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Dengue virus envelope domain III protein based on a tetravalent antigen secreted from insect cells: Potential use for serological diagnosis

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ABSTRACT

In the present study, we developed a tetravalent protein by connecting the receptor-binding envelope domain III (EDIII) of the four dengue virus serotypes in the order of D1–D3–D4–D2. Using a baculovirus expression system, the protein was secreted into the supernatant of infected *sf*9 cells in a stable form with preserved native conformation. Using immobilized affinity chromatography, the recombinant EDIII (rEDIII) protein was purified with a yield of 300 μg per 10^6 cells. The purity and reactivity of the protein were determined via SDS-PAGE and Western blot respectively. A MAC-ELISA method based on the secreted rEDIII protein was subsequently established and evaluated using a panel of pre-characterized dengue IgM-positive and -negative human sera. We obtained a specificity of 100% and sensitivity of 93% using this method. Our data collectively suggest that the secreted tetravalent rEDIII protein has potential utility in the diagnosis of dengue virus infections.

1. Introduction

The four distinct serotypes of dengue viruses (DENV1, 2, 3, 4) are mosquito-borne flaviviruses belonging to the Flaviviridae family, which additionally includes yellow fever virus, Japanese encephalitis virus and West Nile virus (Gubler et al., 2007). DENV infection causes a wide spectrum of clinical symptoms ranging from inapparent or mild dengue fever (DF) to severe and fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Approximately 2.5 billion individuals living in over a hundred tropical and sub-tropical countries, representing 40% of the world's population, are at risk of DENV infections with 400 million cases of DENV infections worldwide estimated every year (Guzmán and Kouri, 2002; Bhatt et al., 2013).

No effective drugs or vaccines are available for the clinical treatment of DENV as yet, which poses a public health threat to nearly half of the global population (Swaminathan and Khanna, 2009). Infection with any one DENV serotype provides lifelong immunity against that particular serotype, but cross-protection against the remaining three serotypes is transient (Innis, 1997). Epidemiologic and laboratory data suggest that cross-reactive antibodies produced during a primary infection may predispose an individual to potentially fatal DHF and DSS during a subsequent infection through antibody-dependent enhancement (ADE) mechanism (Halstead et al., 2005). Diagnosis of dengue virus infection on the basis of clinical symptoms is not reliable, and should be confirmed with laboratory studies, since more than half of the infected individuals are either asymptomatic or have a mild undifferentiated fever (Burke et al., 1988; Endy et al., 2002). Hence, to facilitate timely clinical treatment and etiologic investigation and disease control, rapid detection and differentiation of dengue virus infection in the acute phase of illness is an urgent medical requirement.

Definitive laboratory diagnosis of dengue infection relies on the identification of the virus, viral genome RNA, viral antigens or virus-induced antibodies. Viral isolation, the current gold standard for diagnosis, is time-consuming and requires sophisticated laboratory procedures. Despite the high degree of sensitivity, molecular diagnosis based on real-time reverse transcription PCR is not prevalent due to its wide variability, which requires specialized equipment and experienced personnel, and short duration of viremia. Thus, in most cases, feasible diagnosis specifically depends on the identification of dengue viral antigen or antibodies. Serological-based assays are most commonly employed and despite the many efforts

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to develop a single assay to confirm dengue infection, that goal has not been reached owing to the complicated pathogenesis and multiple infections in dengue endemic areas. Recently, numerous commercial dengue serological diagnostic kits measuring the levels of anti-dengue IgM have become available, some of which are recommended by WHO. Among these, an IgM capture test based on a mixture of recombinant DENV 1–4 antigens (80% N-terminal portion of the envelope protein) from Panbio Diagnostics has shown high sensitivity and specificity. The IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) is a valuable tool for rapid diagnosis of acute viral infection, since the IgM antibody appears early in infection, increases rapidly during the disease course, and is usually less cross-reactive than the IgG antibody (Kuby, 1997).

As DENV co-circulates with other flaviviruses that often display common antigenic similarities, efforts to eliminate the cross-reactivity have begun to focus on the utility of DENV envelope protein domain III (EDIII), which contains both serotype-specific as well as DENV complex-specific epitopes (Ludolfs et al., 2002; Hapugoda et al., 2007; Guzman et al., 2010). Owing to the similarity between the amino acid sequences and predicted structures of the E protein of flaviviruses, construction of hybrid EDIII proteins is feasible and EDIIIs of all four DENV serotypes can be used to detect anti-DENV antibodies.

In this study, we designed a tetravalent protein by combining the EDIIIs of the four DENV serotypes using flexible peptide linkers. The recombinant rEDIII protein was expressed successfully in *Spodoptera frugiperda* (*sf9*) lepidopteron cells and secreted into the supernatant of cell culture. The protein was subjected to affinity chromatography on the Ni-NTA column under non-denaturing conditions to retained functional activities relevant for DENV diagnosis. The rEDIII protein was further labeled with horseradish peroxidase (HRP) and a panel of pre-characterized human DENV antibody-positive and -negative sera ($n = 249$), used for evaluation of rEDIII protein based MAC-ELISA. The method reported in this study led to the successful development of a DENV diagnostic reagent based on a single antigen secreted from eukaryotic cells.

2. Materials and methods

2.1. Plasmid constructs

Each domain III antigen contains 104 amino acid residues spanning DENV envelope aa298-400, corresponding to DENV 1–4 serotypes. A linker sequence, namely GGCGGTGGCGGTAGCGGCGGTGGCGGT, was added to the 5′ end of the primers. Oligonucleotide primers used here included (i) the domain III gene of DEN1 with the forward primer J1: 5′–3′ ATGTCATATGTAATGTGCAC and the reverse primer J2: 5′–3′ CATTGCATAGCTCATACCGCCACCGCCGCTACCGCCACCGCCTTTC, (ii) the domain III gene of DEN3 with the forward primer J3: 5′–3′ ATGAGCTATGCAATG and the reverse primer J4: 5′–3′ CATCGTGTATGACATACCGCCACCGCCGCTACCGCCACCGCCCTTC, (iii) the domain III gene of DEN4 with the forward prime J5: 5′–3′ ATGTCATACACGATG and the reverse primer J6: 5′–3′ CATAGAATATGACATACCGCCACCGCCGCTACCGCCACCGCCCTTG, and (iv) the domain III gene of DEN2 with the forward primer J7: 5′–3′ ATGTCATATTCTATG and the reverse primer J8: 5′–3′ CCGCTCGAGTTAATGGTGATGGTGATGGTGTTGGCCAATAGAGCT. Each PCR product was 312 bp and named D1, D3, D4, and D2, respectively, in which D1, D3, D4 had linker sequence and some overlap sequence. In the second round of amplification, D1, D3 were mixed to primers J1, and J4 to construct fragments D1–D3 and D4, D2 were mixed to primers J5, and J8 to amplify fragments D4–D2 respectively. In the third round of amplification, D1–D3, D4–D2, primers J1 and J8 were mixed to construct the full-length tandem domain III gene (Fig. 1). The final recommended PCR product was purified using a Qiaquick Gel extraction kit (Qiagen, Germany), and verified using restriction enzyme digestion and sequencing. To obtain secreted recombinant protein, pAcSecG2T, pAcGP67B and pAcUW51 vectors were selected as the expression plasmids. The pAcSecG2T vector contained one signal sequence and G2T tag sequence. The recombinant plasmid generated using this vector as the template was designated pAcSecG2T-rEDIII. The pAcGP67B vector also contained one signal peptide, but no tag sequence. Subsequently, then a 6× His tag sequence was added to the 3′ end of the tandem rEDIII gene fragment to obtain the pAcGP67B-rEDIII-His construct. The pAcUW51 vector had neither signal peptide nor tag sequence. A JEV signal peptide and 6× His tag sequence were added to the 5′ and 3′ ends of the tandem rEDIII gene fragment, respectively, to generate the pAcUW51-rEDIII-His plasmid. An additional two plasmids containing no 6× His tag sequence were used to examine the influence of the tag on protein secretion.

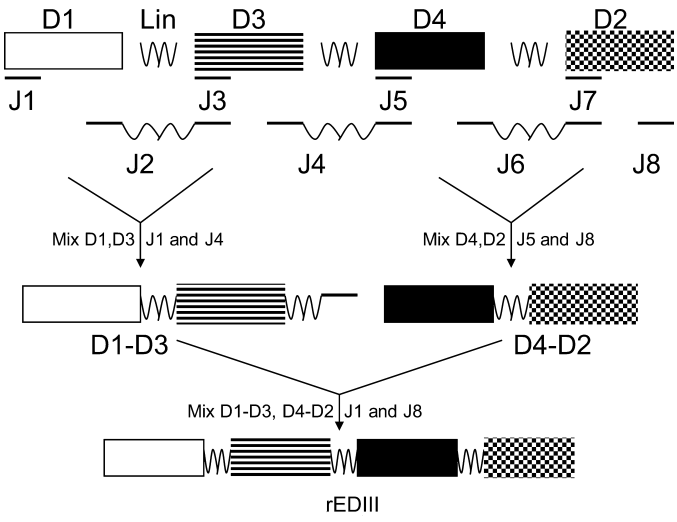

Fig. 1. Diagram showing the construction of the tandem domain III of the dengue virus (DENV) envelope protein. First, domain III genes of the four serotypes of DENV (D1, D3, D4, and D2) were amplified with primers J1/J2, J3/J4, J5/J6, and J7/J8 respectively. Next, two fragments (D1–D3 and D4–D2) were amplified via overlapping PCR. Finally, the tetramer (rEDIII) containing D1, D3, D4, and D2 was generated using another round of overlapping PCR.

2.2. Preparation of recombinant baculoviruses

PAcSecG2T-rEDIII, pAcGP67B-rEDIII, pAcGP67B-rEDIII-His, pAcUW51-rEDIII, pAcUW51-rEDIII-His recombinant plasmids and linearized AcPak6 DNA (Invitrogen, USA) were employed to co-transfect *sf9* cells according to the manufacturer's instructions. Recombinant baculoviruses were propagated by infecting *sf9* cells. Expression of rEDIII protein was validated via immunofluorescence. Mouse anti-DENV1 E protein sera were tested against virus-infected and uninfected *sf9* cells and an FITC-labeled secondary antibody diluted in PBS containing 0.01% Evans blue was employed for detection. The target protein in the supernatant culture was detected using SDS-PAGE.

2.3. Production and purification of recombinant protein

Recombinant rEDIII protein could only be secreted by pAcGP67B vector, and the 6× His tag had no effect on the secretion. Accordingly, the pAcGP67B-rEDIII-His plasmid was selected for protein generation. After large-scale cell culture, 1000 ml medium was harvested and centrifuged at 1000 rpm for 15 min. The supernatant fluid was clarified and subjected to chromatography on a 5 ml