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Naturally mutated envelope protein domain I of Chinese B dengue virus attenuated human dendritic cell maturation

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ABSTRACT

Dengue virus (DENV) can infect human dendritic cells (DCs), and cause a spectrum of clinical symptoms. Envelope protein of DENV contains three distinct domains, including domain I (DI), domain II (DII) and domain III (DIII), and plays important roles in receptor binding and induction of protective antibodies. Previously, a new DENV-2 type virus (named B strain) with eight gene mutations in DI of the envelope protein was isolated from a dengue hemorrhagic fever patient. BALB/c mice infected with DENV B strain showed more prolonged viremia than mice infected with the New Guinea C (NGC) strain. However, the mechanism of prolonged viremia was not determined. In this study, DI proteins derived from B and NGC strains of DENV were expressed in Rosetta (DE3) host bacteria and purified by affinity chromatography after refolding. A flow cytometry-based binding assay and confocal microscopy indicated that both proteins could bind to human DCs induced from peripheral blood mononuclear cells (PBMCs), but DI of the B strain had a lower affinity than DI of the NGC strain, and viable B virus also show less binding efficiency with DCs. In addition, DI of the NGC strain, but not the B strain, induced IL-12 secretion and phenotypic maturation of DCs, such as up-regulated expression of CD80, CD83, CD86 and HLA-DR. NGC strain could induce more virus specific IgM/IgG. These results suggest that the naturally mutated envelope protein DI of the Chinese B strain of DENV cannot induce DC maturation as high efficiency as that of NGC strain, which may be the partial reason that DENV B strain escapes immune recognition and induce prolonged viremia. The mutated B strain envelope protein is not a good candidate for subunit vaccine target.

1. Introduction

Dengue virus infection is a worldwide health problem. An estimated 50 million to 100 million people suffer from DENV infections, and DENV causes 25,000 deaths annually. Moreover, approximately 2.5 billion people are at risk for DENV infection due to the expanding habitat of the mosquito and increasing international travel [1,2]. Infection with DENV generates flu-like symptoms that can progress to dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which is characterized by vascular leakage and thrombocytopenia [2,3]. Although the development of a DENV vaccine is important, it may be difficult because every serotype can cause severe DHF/DSS during secondary heterotypic infection, this phenomenon is known as the antibody-dependent enhancement (ADE) [4].

DENV is a member of the *flaviviridae* family and contains four serotypes. The genome of DENV is composed of an 11 kb positive-stranded RNA that encodes three structural proteins, including the capsid, the premembrane/membrane (prM/M) and the envelope (E) protein, and seven nonstructural proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [5]. The envelope proteins are composed of three domains (DI, DII and DIII) and are localized on the outer surface of the mature virions. The E protein exists as a dimer on the native virion and forms a trimer under low pH conditions to allow fusion of the viral envelope with the membranes of target cells [6]. The E protein mediates viral entry and induces a protective immune response [7,8]. Induction of protective levels of neutralizing antibodies is the major goal of immunization, and neutralizing antibodies directed against the E protein have been shown to be the primary mediators of protection against DENV infection [9–12]. In our previous study, a new DENV-2 type dengue virus strain, named DENV B, was isolated from a DHF patient in the Guizhou province [13]. Compared with the DENV NGC strain, DENV B was found to have eight gene mutations (four amino acids mutations) in DI of the E protein [14]. The 158 amino acid motif of D1 located at the N-terminal region of the E protein in the mutant dengue virus has been shown to induce lower levels of antibodies [15]. BALB/c mice infected with the DENV B strain showed prolonged

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viremia compared with mice infected with the NGC strain. In addition, the B strain had a lower ability to induce IgM than the NGC strain during initial infection [16]. It may be that the precise amino acid residues of the DENV B strain have evolved to escape immunological pressure and thus cannot induce protective immune responses.

Dendritic cells (DCs) are the first target of DENV infection. During a blood meal, an infected mosquito injects the virus into the skin, where DCs reside and take up the viral particles [4,17]. Previous studies have indicated that monocytes, but not T or B cells, are the principal target cells of DENV infection in human PBMCs [18]. Recent research has further found that the DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) molecule mediates viral entry, thereby supporting the involvement of DCs in DENV infection [19–21]. In response to pathogenic stimulation, such as with dengue viruses and their components or products, immature DCs undergo a process of maturation and become activated to allow for antigen processing and presentation [22].

In this study, the mechanism of prolonged viremia by the DENV B strain was partly explained. DI of the E proteins from both viral strains was constructed, expressed, refolded and purified, and binding assays indicated that DI of the NGC strain had higher binding affinity to DCs than DI of the B strain. Furthermore, DI of the NGC strain, but not the B strain, induced phenotypic maturation of and secretion of IL-12 by DCs. Taken together, our results suggest that the naturally mutated envelope protein DI of the Chinese B strain of DENV escapes immune detection partly because of its weak binding to DCs and subsequent inability to induce DC maturation.

2. Materials and methods

2.1. Animals and reagents

Male BALB/c mice (8 weeks old, weighing 20–24 g) were obtained from the Shanghai Experimental Center, Chinese Science Academy (Shanghai, China), and maintained at an animal facility under pathogen free conditions. The handling of mice and experimental procedures was conducted in accordance with the experimental animal guidelines.

The pET-28a(+) vector was purchased from Novagen (Darmstadt, Germany). Human recombinant GM-CSF and IL-4 were purchased from PeproTech (PeproTech Inc., NJ). FITC-conjugated anti-human CD80 and HLA-DR, APC-conjugated anti-human CD83, PE-Cy5-conjugated anti-human CD86, and PE-conjugated anti-human CD11c were purchased from BD Biosciences (BD Pharmingen, CA). FITC-conjugated lineage cocktail 1 (lin 1; CD3, CD14, CD16, CD19, CD20, CD56) was bought from BD Biosciences (BD Immunocytometry Systems, CA). A human IL-12p70 ELISA kit was purchased from Bender MedSystems (Burlingame, CA).

2.2. Construction of the recombinant plasmid

The 937 to 1413 bp gene fragment from the envelope protein of DENV was amplified and sub-cloned into the Nde1 and Xho1 sites of the pET-28a(+) vector. The fragment was amplified by polymerase chain reaction (PCR) from either a virus isolated from a DHF patient (the DENV-2 B strain) or the cDNA library of the DENV-2 New Guinea-C (NGC) strain with the primers, sense 5′-CGCCATATGTAT GCTTGCATAG-3′, antisense 5′-CGCCCTCGAGTTAGCCAGTTTTTCCTGTG-3′, and sense 5′-CGCCATATGATGCGTTGCATAGGAATATCAAATAGAGA C-3′, antisense 5′-CGCCCTCGAGTTAGCCATGTTTTCCTGTGTCATTT-3′, respectively. The following PCR conditions were used: 30 cycles at 94 °C for 30 s, 56 °C for 90 s and 72 °C for 90 s. The plasmids were confirmed by sequencing. There were eight gene mutations (four amino acids mutations) in DI of the E gene derived from the DENV-2 B strain [Genbank ID: AY179733] compared with DI of the DENV-2 NGC strain, which is consistent with the previous report [14]. Alignment of the derived amino acid sequences was shown in Supplementary Fig. 1. The recombinant pET-28a(+) vectors were chemically transformed into the competent *E. coli* Rosetta (DE3) bacteria.

2.3. Purification of recombinant DENV B-E and NGC-E proteins

Expression of the E protein was performed in pET-28a(+)-B-E or pET-28a(+)-NGC-E transformed *E. coli*. Rosetta cells follow the induction with 1 mM IPTG for 4–5 h at 37 °C. The cell pellet was disrupted with a high pressure homogenizer. The inclusion body was collected and washed with wash buffer (300 mM NaCl, 50 mM Tris, 2 M urea, pH 8.4). The inclusion body was resolved in 8 M urea, and the denatured protein was refolded in refolding buffer (300 mM NaCl, 50 mM Tris, pH 8.4) at a final concentration of 1 mg/ml. The refolded protein was collected by the affinity chromatography with a Ni^{2+} column. After dialysis, the purified B-E and NGC-E protein concentration was measured using the Bradford method (Bio-rad protein assay), and identified by SDS-PAGE and western blotting.

2.4. Generation of mouse polyclonal anti-serum

The B-E and NGC-E proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Millipore, MA). The membrane containing protein was cut and ground. Eight-week-old male BALB/c mice were immunized by subcutaneous injection of B-E or NGC-E (35 μg/mouse) and then boosted (18 μg/mouse) once a week for two weeks. At 4 weeks post-immunization, the animals were sacrificed and total serum was collected.

2.5. Western blot

The recombinant DENV B-E and NGC-E protein samples were separated by 12% SDS-PAGE and transferred to PVDF membranes. After being blocked in Tris-buffered saline with 5% (w/v) nonfat dry milk, membranes were incubated with a polyclonal anti-DENV-2 E antibody (1:500) for 1 h at 37 °C. After being washed, a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG was applied to the membranes (Boster, Wuhan) for 1 h at 37 °C. The proteins were detected by the enhanced chemiluminescence (ECL) system (Pierce, IL) using X-ray film (Kodak).

2.6. Virus stocks and DV infection

NGC and B strain (serotype 2), which were initially isolated from a dengue hemorrhagic fever patient, were used for infection. The preparation of virus stocks was described previously [16]. Briefly, C6/36 Aedes albopictus cells obtained from the American Type Culture Collection (ATCC No: CRL-1660) were cultured in Minimal Essential Media with Earl's salts (E-MEM) containing 10% fetal bovine serum (FBS, Gibco/Invitrogen) at 28 °C in a humidified atmosphere of 5% CO_2. DENV stocks were grown in C6/36 monolayer cells. After seven days, media was harvested and clarified by centrifugation. The virus containing supernatant was supplemented with 20% FBS and stored at −80 °C.

For infection, 1×10^5 DCs were exposed to DV for 1 h at 37 °C at 0.1 m.o.i. in FBS-free RPMI 1640 supplemented with 0.2% BSA, pH 7.5. Cells were subsequently washed with complete RPMI 1640 to remove excess virus.

2.7. Induction of DCs from PBMCs

PBMCs were isolated from normal donors by density gradient centrifugation. Monocytes were then isolated by the plastic adherence by the incubation of PBMCs in tissue culture dishes (BD Falcon, Franklin Lakes, NJ, USA) for 1 h at 37 °C in a 5% CO_2 incubator. The plates were washed thrice with RPMI1640 to remove nonadherent T cells. To generate immature DCs, the monocytes were cultured in 6-well plates in RPMI-1640 medium containing 10% FBS, 500 U/ml GM-CSF