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Production and characterization of monospecific and bispecific antibodies against dengue virus NS1 protein

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Dengue is a mosquito borne infection, which in recent years has become a major international public health concern. Annually, 100 million dengue virus infections are reported worldwide. The nonstructural protein 1 (NS1) of dengue virus is a useful target for diagnostics of dengue infection since the protein is abundantly circulating in the blood during acute phase of the disease, in both primary and secondary infections. This research paper highlights the development of a panel of Mab and bsMab for dengue NS1 detection. The P148 series of Mabs showed high specificity for recombinant dengue NS1 antigen. These antibodies showed no cross reactivity with recombinant dengue envelope protein and other viral proteins. The hybrid-hybridoma approach to generate the P156.1 and P156.2 bsMabs from the P148 monoclonal antibody method was used during this study. Furthermore, the affinity purification provided good yields of quadromas associated with HRPO in two steps. Direct detection method involved coating of plates with different concentrations of recombinant antigen and detecting with bsMab. Sensitive sandwich assay with Mabs and bsMabs was also done. Detection of nonstructural dengue antigens may be of benefit for early and rapid diagnosis of dengue infection due to their long half-life in the blood.

1. Introduction

Dengue is one of the leading vector borne diseases worldwide, and more than 100 million people are infected every year (Gubler and Meltzer, 1999; Ganguly et al., 2013a). Dengue fever is an important mosquito-borne viral disease of humans. This has been a recurrent phenomenon throughout the tropics in the past decade. During 2002, more than 30 Latin-American countries reported over a million dengue fever (DF) cases with large number of dengue hemorrhagic fever (DHF) (Whitehorn and Farrar, 2010). Increasingly cases of the more severe and potentially lethal DHF and dengue shock syndrome (DSS) are reported with children bearing much of the disease burden (Ganguly et al., 2013b). Dengue virus is endemic in at least 100 countries worldwide and causes more human cases than any other mosquito-borne virus. The mortality rate of DHF in most countries is 5%, primarily among children and young adults. In several Asian countries, this virus is the leading cause of hospitalization and death in children. There have been a large number of dengue epidemics that resulted in enormous economic and human loss in parts of Asia and South America (Whitehorn and Farrar, 2010). Hence, there is an urgent need for diagnostic, prophylactic and therapeutic reagents to manage DHF. Dengue virus has four serotypes, which are antigenically distinct (Calisher et al., 1989; Ganguly et al., 2013c). Among those, the dengue virus nonstructural (NS) 1 protein is a 46–50 kDa glycoprotein expressed in infected mammalian cells. All 7 NS proteins are intracellular proteins with the exception of dengue NS1 protein, which exists as secreted as well as a membrane-associated protein. Both forms are demonstrated to be immunogenic (Young et al., 2000; Flamand et al., 1999; Falconar and Young, 1990). It was also reported that NS1 is one of 7 NS proteins produced during viral replication. It possesses not only group specific but also type specific determinants and has been recognized as an important

Abbreviations: BSA, bovine serum albumin; bsMab, bispecific monoclonal antibodies; DHF, dengue hemorrhagic fever; ECL, enhanced chemiluminescence; ELISA, enzyme linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAM-HRPO, goat anti-mouse horseradish peroxidase; HAT, hypoxanthine, aminopterin, and thymidine; HT, hypoxanthine, aminopterin; HRPO, horseradish peroxidase; IMAC, immobilized metal affinity chromatography; Mab, monoclonal antibody; NS1, nonstructural protein 1; OD, optical density; PEG, polyethylene glycol; PBS, phosphate buffered saline; PSG, streptomycin–penicillin–glutamine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMB, tetramethylbenzidine; TRITC, tetramethylrhodamine isothiocyanate.

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antigen in dengue infection (Young et al., 2000; Falconar and Young, 1990; Henchal et al., 1987). A high circulating level of NS1 was demonstrated in the acute phase of dengue by antigen capture ELISAs (Young et al., 2000; Alcon et al., 2002). The precise function of dengue NS1 protein remains unclear. However, antigen detection of nonstructural dengue antigens may be of benefit for an early stage rapid diagnosis of infection due to its long half-life in the blood. At present there are no vaccines or drugs available to counter this deadly disease and diagnosis of infections particularly in endemic regions are clinical (Farrar et al., 2007). Dengue causes a range of syndromes ranging from asymptomatic infection to a severely debilitating and life threatening hemorrhagic condition (Kalayanarooj et al., 1997). Detection of nonstructural dengue proteins is great importance for an early stage rapid diagnosis of infection as a result of its long half-life in the blood (Das et al., 2009). Morbidity and mortality incidence can be significantly reduced with early and accurate detection. Dengue NS1 antigen screening is one of the important tools for early detection of dengue infection after the onset of fever (Malabadi et al., 2011).

Bispecific-monoclonal antibodies have many applications in the area of immunodetection and have been exploited previously for the targeted drug delivery, vaccine development and gene therapy (Khan et al., 2011, 2012; Malabadi et al., 2012; Sunwoo et al., 2013). In this study, we have produced monoclonal antibodies, and bispecific antibodies against dengue NS1 protein. The monoclonal antibodies were characterized by dengue NS1 recombinant antigen based ELISA, SDS-PAGE and Western blot. The isotypes of the antibodies were also determined, and used subsequently to develop preliminary a specific and sensitive antigen capture ELISA for the detection of dengue NS1. The sensitivity and specificity of a preliminary antigen capture ELISA were also assessed. The development of user-friendly, inexpensive, sensitive and specific assay for dengue NS1 is very essential. Here we define the development and characterization of dengue monoclonal antibody (Mab) and bispecific monoclonal antibody (bsMab).

2. Materials and methods

2.1. Preparation of dengue nonstructural protein (NS1)

A full-length codon optimized NS1 gene was chemically synthesized and purchased from Geneart Inc. (Germany), and was cloned in a bacterial expression vector pBM802 digested with NdeI and EcoRI (procured from New England Biolabs, Mississauga, Canada) followed by gel purification and ligation. The expressed protein approximately 48 kDa was purified from *Escherichia coli* Rosetta (ATCC 87064, *E. coli* DE3) cultures (Purchased from American Type Culture Collections, Manassas, VA, USA) (Das et al., 2009). The gene was cloned in correct reading frame with His_6 tag at the C-terminal end for high-level expression within inclusion bodies of *E. coli* (ATCC 87064, Manassas, VA, USA). Furthermore, the identification of the expressed NS1 gene was confirmed by sequence analysis. Restriction digestion mapping and SDS-PAGE (Bio-Rad, USA) showed that the right size clones (48 kDa) were selected. Immobilized metal-affinity chromatography (IMAC) (Bio-Rad, USA) was used under denaturing conditions to adsorb the His_6-tagged protein to finally elute the pure NS1. The glycosylated NS1 was used to generate anti-NS1 Mabs, and for screening bsMabs.

A single bacterial colony was inoculated in 10 mL Terrific Broth/Tet5 medium and grown overnight at 37 °C shaker. The overnight culture was diluted (1:100) in fresh 1 L TB/Tet5 medium and grown at 37 °C until an $OD_{600\,nm}$ of 0.5–0.6 was reached. NS1 induction was done by adding 0.2% (w/v) arabinose (Sigma, St. Louis, MO, USA) according to optimized parameters and bacterial culture was incubated further for 16 h with vigorous shaking at 30 °C. Culture was harvested by centrifugation at 5000 × *g* for 20 min at 4 °C and the pellet was subject to determine total cell protein (TCP) from induced and un-induced culture. The NS1 protein was analyzed further by SDS-PAGE (Bio-Rad, USA) and also by Western blot probed with anti-His_6 Mab.

The pellet (5 g of bacterial wet pellet) from 1 L bacterial culture was suspended in 50 mL phosphate buffered saline (PBS) (Gibco Inc., USA) and completely lysed by passing through a French Press (20,000 psi). The total cell lysate was clarified by centrifugation at 27,000 × *g* for 30 min at 4 °C and supernatant was collected as total soluble protein. The pellet was resuspended in lysis buffer and then 2% sodium deoxycholate was added. The mixture was incubated at room temperature for 30 min with gentle shaking and centrifuged at 27,000 × *g* for 30 min at 4 °C. The pellet was resuspended in lysis buffer and washed thrice at 27,000 × g for 20 min at 4 °C to completely remove sodium deoxycholate.

2.2. IB solubilization and immobilized metal affinity chromatography (IMAC) purification

The washed pellets, inclusion bodies, were solubilized in denaturing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 8.0) for 1 h at room temperature (RT) with gentle shaking. Solubilized denatured recombinant NS1 proteins from insoluble materials were separated by centrifugation at 27,000 × *g* for 30 min at 4 °C. A Ni–NTA column (Bio-Rad, USA) was prepared by loading the Ni–NTA agarose on a plastic column (Bio-Rad, USA) and equilibrated with 10 bed volumes of the denaturing buffer. Twenty milligrams of solubilized denatured His_6 tagged (purchased from Novagen Inc., Madison, USA) rNS1 protein were loaded on the column and the column was washed with 5–10 bed volumes with washing buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 6.3). After complete wash, bound protein was eluted with elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–Cl, pH 5.9) and subsequently with the same elution buffer at pH 4.5. The eluted fractions were analyzed by SDS-PAGE (Bio-Rad, USA) prior to refolding.

2.3. Refolding

Protein assay was done to quantitate the amount of protein eluted from the Ni–NTA column (Bio-Rad, USA) with a total amount of ~4 mg. Refolding was done in three different concentrations to evaluate the best refolding condition. The eluted protein was adjusted to 100, 75 and 50 μg/mL with refolding Tris–Arginine buffer (Gibco, NY, USA). The refolding was done by dialysis in Tris–Arginine buffer (Gibco, NY, USA) in the presence of 1.0 mM GSH (glutathione, reduced), 0.1 mM GSSG (glutathione, oxidized) (Sigma, St. Louis, MO, USA) for 3 days with two changes at 4 °C. Final dialysis was done in PBS (Gibco, NY, USA) pH 7.4 at 4 °C.

2.4. Western blot analysis

Total cell protein (1 g), inclusion bodies (3 g), IMAC (Bio-Rad, USA) eluted fractions or refolded dengue NS1 protein (10 μL of a 2 mg/mL final concentration of denatured protein per sample well) were electrophoresed on SDS-PAGE (Bio-Rad, USA) using 10% polyacrylamide gel and then electroblotted onto Hybond ECL nitrocellulose membranes (Bio-Rad, USA). The nitrocellulose membrane was blocked with 5% skim milk in PBST (Gibco, NY, USA) (0.1% Tween 20 in PBS, pH 7.3) (Gibco, NY, USA) for overnight at 4 °C. The membrane was washed four times with PBST (Gibco, NY, USA) and incubated with anti-His_6 Mab for 1 h. After washing, the membrane was incubated with HRPO labeled goat anti-mouse IgG (GAM-HRPO) (Sigma, St. Louis, MO, USA) for 1 h. Finally, the membrane was washed with PBS (Gibco, NY, USA) and enhanced