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A specific and sensitive antigen capture assay for NS1 protein quantitation in Japanese encephalitis virus infection

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A B S T R A C T

Japanese encephalitis virus (JEV) is a human pathogenic, mosquito-borne flavivirus that is endemic/epidemic in Asia. JEV is rarely detected or isolated from blood or cerebrospinal fluid (CSF), and detection of IgM is generally diagnostic of the infection. The flavivirus nonstructural glycoprotein NS1 is released transiently during flavivirus replication. The aim of this study was to set up a quantitative JEV NS1 antigen capture assay. A soluble hexameric form of JEV NS1 protein was produced in a stable *Drosophila* S2 cell clone and purified from supernatant fluids. Two IgG1 monoclonal antibodies (MAbs) with high affinity against two different epitopes of JEV NS1 antigen were used to develop an antigen-capture assay with a limit of detection of 0.2 ng ml^{-1} NS1. Up to 1 μg ml^{-1} JEV NS1 protein was released in supernatants of mammalian cells infected with JEV but <10 ng ml^{-1} was released in sera of virus-infected mice before the onset of encephalitis and death. Moreover, NS1 protein was detected at low levels (<10 ng ml^{-1}) in 23.8% of sera and in 10.5% of CSF of patients diagnosed as IgM-positive for JEV. This quantitative test of NS1 protein is proposed for highly specific diagnosis of acute infection with JEV genotypes I to IV.

1. Introduction

Japanese encephalitis (JEV) belongs to the family *Flaviviridae*, genus *Flavivirus* comprising dengue virus (DENV), West Nile virus (WNV) and Murray Valley encephalitis virus (MVEV). JEV is one of the most important mosquito-borne viral pathogens that cause encephalitis in Asia (Solomon, 2006). Outbreaks of seasonal JEV pose a serious health threat to the susceptible population in the area. Many children remain asymptomatic after JEV infection but might show encephalitis in about 1:300 cases (Burke et al., 1985). After bite from infected mosquito, JEV replicates in the skin and lymphoid organs and is transported by the blood to peripheral organs, before crossing the blood–brain barrier to infect neurons and trigger infiltration of inflammatory cells (Huang and Wong, 1963; Myint et al., 2007). Virus is rarely isolated from the blood or cerebrospinal fluid (CSF), although it has been detected by reverse transcription-polymerase chain reaction (RT-PCR) (Saxena et al., 2009; Swami et al., 2008; Wang et al., 2007b), and the presence of IgM antibodies, which appear at the onset of the encephalitis symptoms is generally presumptive of diagnosis (Burke et al., 1985; Solomon et al., 1998; Vaughn and Hoke, 1992). However, previous infection with other flaviviruses that co-circulate, might alter the specificity of the immune response to JEV antigens (A-Nuegoonpipat et al., 2008; Dejnirattisai et al., 2010).

The flaviviral genome is a single-stranded, positive-sense RNA that includes two untranslated regions at its 5′ and 3′ ends and one unique translated region that encodes a polyprotein C-prM-E-NS1-NS2A/2B-NS3-NS4A/4B-NS5. The polyprotein is cleaved by host furin or viral protease NS3 at different sites, to generate three structural proteins and seven non-structural proteins (Chambers

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et al., 1990). The flavivirus non-structural protein 1 (NS1) of 48 kDa is a secreted glycoprotein that contains two or three conserved N-linked carbohydrates (Mason, 1989; Chambers et al., 1990) and six conserved disulfide bridges (Wallis et al., 2004). The NS1 protein has a complex maturation process that leads to at least one dimeric form remaining inside the rough endoplasmic reticulum for regulatory activity during viral RNA replication (Muylaert et al., 1997; Lindenbach and Rice, 1999), a cell-membrane-associated form with a lipid raft (Mason, 1989; Noisakran et al., 2008), and an extracellular soluble hexameric form associated to lipids (Flamand et al., 1999; Gutsche et al., 2011). The secretion level of DENV NS1 in patient serum can be as high as 50 μg ml^{-1}, which appears concomitantly with viral particle secretion, and earlier than IgM. It can still be detected a few days after the onset of the IgM response (Alcon et al., 2002). Therefore, NS1 capture from sera of patients is now in use as an early diagnosis of DENV infection (Young et al., 2000; Alcon et al., 2002; Zainah et al., 2009). *In vivo* studies in hamsters or mice infected with WNV have shown a concentration of NS1 of 1–10 μg ml^{-1} or 10–30 ng ml^{-1}, respectively, and a secretion time course concomitant with that of viremia (Macdonald et al., 2005; Chung and Diamond, 2008). Nevertheless, the role of NS1 protein in the pathophysiology and severe clinical features of flaviviral disease is not well understood.

An early and specific diagnosis of the disease is highly desired for surveillance of JEV in non-diagnosed febrile illness in Southeast Asia (Hills et al., 2010). In the present study, a JEV-specific and quantitative NS1 antigen-capture ELISA has been developed using a flavivirus-specific and a JEV-specific monoclonal antibody (MAb), respectively. This assay allowed the analysis of NS1 secretion in JEV-infected cell culture, the follow-up of sequential appearance of virus NS1 in sera of JEV-infected mice, and the detection of NS1 protein in JEV-infected patients.

2. Materials and methods

2.1. Cells and viruses

Baby hamster kidney (BHK-21) and mouse neuroblastoma (Neuro2a) cell lines were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) that contained 3% fetal bovine serum (FBS) and 100 U penicillin and 100 μg streptomycin, at 37 °C in 5% CO_2. JEV Nakayama strain RNA was extracted from Vero cell supernatant. The JEV SA14 strain was a generous gift from Professor Yu Yongxin (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) and virus stock was produced in BHK-21 cells infected at a multiplicity of infection (MOI) of 0.1 pfu/cell. JEV Nakayama (genotype III), WNV (strain IS1999), MVEV, DEN-2 V, Usutu virus, tick-borne encephalitis virus, yellow fever 17D virus, and Chikungunya virus were grown in Vero cells in the Arbovirus Reference center in Institut Pasteur, Paris. JEV 2371 (genotype I), Sarawak CNS138/11 (genotype II), P3 (genotype III), 6468 (genotype IV), and Muar (genotype V) were grown in Vero cells in Dr Solomon's lab in Liverpool University. The *Drosophila* S2 cells were purchased from Invitrogen and cultured in Schneider's *Drosophila* medium (Invitrogen) with 10% FBS, 50 U penicillin and 50 μg streptomycin, and incubated at 28 °C.

2.2. Plasmid construction

To construct the expression plasmids NS1 and its fragments, full-length NS1 that contained amino acids 1–352 ($NS1_{1-352}$), NS1 N-terminus that contained amino acids 1–143 ($NS1_{1-143}$), and NS1 C-terminus that contained amino acids 224–352 ($NS1_{224-352}$), were amplified from RNA of JEV Nakayama strain with specific primers, including restriction sites at their 5′ extremities for cloning: $NS1_{1-352}$ and $NS1_{1-143}$ forward 5′-ATA**GGATCC**GACACTGGATGTGCCA-3′, $NS1_{1-352}$ reverse 5′-TAT**GCGGCCGC**AAAGCATCAACCTGTGA-3′, $NS1_{1-143}$ reverse 5′-TT**GCGGCCGC**ACAGGGCATTCCTTTGTCTC-3′, $NS1_{224-352}$ forward 5′-TTT**AGATCT**ACTTGGCCAGAGACACACAC-3′, $NS1_{224-352}$ reverse 3′-TTT**GCGGCCGC**TTAGCATCAACCTGTGATC-3′. All PCR products were digested with *Bam*HI/*Bgl*II and *Not*I and inserted into pMT/BiP/V5-His A vector (Invitrogen) to generate pMT-$NS1_{1-352}$, pMT-$NS1_{1-143}$, and pMT-$NS1_{224-352}$ expression plasmids. All genes were under the control of inducible metallothionein promoter, and in-frame with BiP secretion signal peptides of *Drosophila* cells. The recombinant proteins included at their C terminus the V5 tag for expression detection and six histidine residues for purification.

2.3. Recovery and cloning of stable S2 cells that produce JEV NS1 proteins

The pMT vectors that contained genes for JEV $NS1_{1-352}$, $NS1_{1-143\ and}$ $NS1_{224-352}$ proteins were co-transfected with pCoblast (Invitrogen) into *Drosophila* S2 cell according to manufacturer's instructions. Briefly, 3×10^6 S2 cells were grown in each well of a six-well plate, cultured overnight, and co-transfected with 19 μg recombinant pMT plasmids and 1 μg pCoblast plasmid, using the calcium phosphate transfection kit (Invitrogen), and incubated for 1 day at 28 °C. The cells were washed three times, and incubated in fresh medium that contained 25 μg ml^{-1} blasticidin (Invitrogen) for selection of transfected cells. After 4 weeks of selection, expression of NS1 proteins in cells was assessed by Western blotting using anti-V5 MAb (Invitrogen). The S2-$NS1_{1-352}$ cells were sub-cloned by limiting dilution to generate clones with high NS1 protein expression. For cloning, the naïve S2 cells were diluted to 50,000 cells ml^{-1} in Schneider's *Drosophila* complete medium with 25 μg ml^{-1} blasticidin as feeding layer cells, and S2-NS1 cells were diluted to 25 cells ml^{-1} and 5 cells ml^{-1} into the feeding layer cell medium, and 200 μl of cells/well were plated into 96-well plates. One month later, the cells from S2-NS1-cell-positive wells were amplified successively into 48-, 24- and six-well plates and tested for NS1 protein expression by Western blotting, and analyzed in an LSR II Flow Cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) using a dengue-2 flavivirus cross-reactive anti-NS1 MAb 17A12 (M. Flamand, data not shown).

2.4. Recombinant NS1 protein expression and purification

Cells that showed the highest $NS1_{1-352}$ protein expression levels were cultured in T150 flasks with Express Five medium (Invitrogen) that contained 10% FBS and 50 U penicillin, 50 μg streptomycin, and 10 μg ml^{-1} blasticidin. After 1 week of culture, the cells were centrifuged and resuspended in Express Five medium without FBS, at a concentration of 3×10^6–5×10^6 cells ml^{-1} and cultured in a Wave Bioreactor™ (GE Amersham, Uppsala, Sweden). One week later, $CdCl_2$ (Sigma–Aldrich, St. Louis, MO, USA) was added to the medium at a concentration of 5 μM to induce recombinant protein expression. After 3 days of induction, the cell supernatant was harvested, filtrated and concentrated, and buffer was exchanged for binding buffer (25 mM Tris–HCl, pH 7.5, 300 mM NaCl, 2 mM imidazole, and 10% glycerol) with the QuixStand Benchtop system (GE Amersham). The resultant fluid that contained NS1 protein was loaded onto a chelating sepharose column (GE Amersham) that was activated with nickel ions. The column was washed with five column volumes of washing buffer 1 (25 mM Tris–HCl, pH 7.5, 300 mM NaCl, 15 mM imidazole, and 10% glycerol); 10 column volumes of washing buffer 2 (25 mM Tris–HCl pH 7.5, 300 mM NaCl, 45 mM imidazole, and 10% glycerol); and the recombinant protein was eluted with 10 column volumes of elution buffer (25 mM Tris–HCl, pH 7.5, 300 mM NaCl, 500 mM imidazole, and 10% glycerol) (Chung et al., 2008). After