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Monoclonal antibodies against dengue NS2B and NS3 proteins for the study of protein interactions in the flaviviral replication complex

Nicole J. Moreland [a], Moon Y.F. Tay [a], Elfin Lim [a], Abhay P.S. Rathore [a], Angeline P.C. Lim [b], Brendon J. Hanson [b], Subhash G. Vasudevan [a,*]

[a] Program in Emerging Infectious Diseases, DUKE-NUS Graduate Medical School, Singapore
[b] Defence Medical and Environmental Research Institute, DSO National Laboratories, Singapore

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The replication of dengue virus (DENV) RNA requires at least two viral non-structural (NS) proteins, NS3 and NS5. To facilitate the study of the DENV replication complex, human monoclonal IgG that are specific for NS proteins have been generated and characterised. The anti-NS3 IgG, 3F8, binds a conserved epitope (aa526-531) in the NS3 helicase domain, and cross-reacts with NS3 from all four DENV serotypes and the related yellow fever virus. The anti-NS2B IgG, 3F10, binds aa49-66 of NS2B (CF18), which forms part of the 47 aa hydrophilic cofactor region required for NS3 protease activity. The specificity of the IgG for their respective non-structural proteins has been demonstrated by immunofluorescence of cells infected with DENV and Western blotting. 3F8 is able to co-immunoprecipitate NS3 and NS5 from BHK-21 cells infected with DENV2, and 3F10 is able to detect an interaction between recombinant $NS2B_{CF18}$NS3 full-length protein and the NS5 RNA-dependent RNA polymerase (RdRp) domain in an ELISA-based binding assay. The assay is specific and highly reproducible, with a clear binding curve seen when RdRp is incubated with increasing amounts of full-length NS3, but not the NS3 protease domain. The NS3 helicase domain competes with NS3 full-length for NS5 RdRp binding, with a K_d of 2.5 μM. Since NS3 and NS5 are required for DENV replication, this fascile assay could be used to screen for non-nucleoside, allosteric inhibitors that disrupt the interaction between the two proteins.

1. Introduction

Dengue virus (DENV) is a positive-sense, single stranded RNA virus with four antigenically related serotypes (DENV1-4). The 11-kb genome encodes a single polyprotein that is proteolytically cleaved into three structural proteins (capsid, premembrane and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers et al., 1990). Polyprotein processing is conducted by host signalase and the two-component viral protease NS2B/NS3 (Yusof et al., 2000). The NS3 protein (69 kDa) has protease activity localized within the N-terminal 169 residues and requires the hydrophilic co-factor region of NS2B (aa49-95) to cleave the polyprotein (Leung et al., 2001; Li et al., 2005; Yusof et al., 2000). The remainder of NS3 forms the helicase domain which comprises nucleotide binding, nucleotide triphosphate (NTPase) and RNA binding motifs (Luo et al., 2008; Xu et al., 2005).

The replication of DENV RNA requires at least two viral proteins – NS3, and the largest of the DENV non-structural proteins, NS5. The amino terminal region of NS5 harbors a methyltransferase (MTase) domain involved in RNA cap formation, while the carboxyl terminal region comprises an RNA-dependent RNA polymerase (RdRp) domain (Ackermann and Padmanabhan, 2001; Tan et al., 1996). NS3 and NS5 have been shown to form a complex in DENV2 infected monkey kidney (CV-1) cells (Kapoor et al., 1995), and yeast-two hybrid studies have mapped the interaction between the proteins to the C-terminal region of NS3 (aa303-618) and the N-terminal region of the NS5 RdRp domain (aa320-368) (Johansson et al., 2001). A recent functional analysis of NS5 identified residue K330 as being critical for interaction with the NS3 helicase domain (Zou et al., 2011). It has been suggested that the cavity occupied by K330 could be a target for rational drug design (Zou et al., 2011), but there are no fascile assays available to screen for compounds that can disrupt the NS3–NS5 interaction.

Antibodies are valuable tools for investigating protein–protein interactions such as that between NS3 with NS2B or NS5, and there is a current lack of epitope mapped, monoclonal antibodies for flaviviral research. In a previous study a panel of NS2B/NS3 specific antibody fragments (Fab) were identified from a naïve human Fab library using phage display technology (Moreland et al., 2010). In this study, their conversion from Fab to IgG and the development of selected antibodies as DENV research reagents is presented.

* Corresponding author at: DUKE-NUS Graduate Medical School, 8 College Road, Singapore 169857. Tel.: +65 6516 6718; fax: +65 6221 2529.
E-mail address: subhash.vasudevan@duke-nus.edu.sg (S.G. Vasudevan).

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Table 1
Characteristics of the NS2B/NS3 specific antibodies.

Name	Type/host/isotype	DENV epitope	Cross-reactivity	Concentration (nmol/L)			
				Immunoblot	ELISA	IFA[a]	IHC[b]
3F8	mAb/human/IgG1	NS3 aa526-531	DENV1-4, other flaviviral NS3 with RGExRK motif	0.2	0.1	3	3
3F10	mAb/human/IgG1	NS2B aa49-66	DENV2, 3 and 4	0.25	1	6	ND[c]

[a] Immunofluorescence.
[b] Immunohistochemistry.
[c] Not done.

The anti-NS3 helicase IgG, 3F8, detects NS3 from all four DENV serotypes and the related yellow fever virus, while 3F10 IgG binds the hydrophilic co-factor region of NS2B. The antibodies are specific for their respective non-structural proteins and have enabled further investigations into the NS3–NS5 interaction in infected cells, and with recombinant proteins *in vitro*. A sensitive enzyme-linked immunosorbant assay (ELISA) that reliably detects an NS3–NS5 interaction is described. The assay incorporates 3F10 as a detection reagent and could be used to screen for compounds that disrupt the NS3–NS5 interaction as part of a DENV drug-discovery effort.

2. Materials and methods

2.1. Preparation and analysis of Fab and IgG1

Phagemids were transformed into *E. coli* Top10 F′ cells (Invitrogen, Carlsbad, USA) for expression and periplasmic extraction of Fab as previously described (Moreland et al., 2010). Selected Fab were converted to human IgG1 by cloning the variable light and heavy chain sequences into an IgG1 vector containing human constant coding sequences via ApaL1 and BsmB1 sites as previously described (Lim et al., 2008). IgG were produced by transient transfection of human embryonic kidney (HEK293T) cells with lipofectamine 2000 (Invitrogen, Carlsbad, USA). Transfected cells were maintained in 10% ultra-low IgG FCS (Invitrogen, Carlsbad, USA) in Dulbeco's modified eagle's medium (DMEM) for 5–6 days. The medium were collected and replaced on day three. Collected medium was subjected to affinity chromatography on a recombinant Protein G column (GE Healthcare, Waukesha, USA) and eluted IgG were dialysed into PBS for storage.

For ELISA, Maxisorb Immunoplates (Nunc, Rochester, USA) were coated with the relevant NS3 antigen (0.25 μM) in PBS pH 7.5 and blocked with 5% skim milk in PBS-T (0.1% Tween-20). Blocked wells were incubated with purified Fab or IgG at room temperature for 1 h. Plates were washed with PBS-T and incubated with an anti-human IgG HRP conjugate (MyBiosource, San Diego, USA) for detection. For Western blot, infected cells were lysed and total protein concentration was determined by the BCA Assay (Pierce, Pittsburgh, USA). Equivalent amounts of protein lysate were separated on 12% SDS-PAGE gels, and Western blotted with the various anti-NS2B/NS3 IgG followed by an anti-human IgG HRP conjugate.

2.2. Immunostaining and microscopy

BHK-21 cells grown on cover slips at a density of 1×10^5 were infected with DENV2 (GenBank accession EU081177.1) at an MOI of 0.3. Cells were fixed 24 h post-infection with 80% acetone at 4 °C for 15 min, and washed three times with PBS. Cells were blocked with 5% BSA in PBS overnight at 4 °C followed by an overnight incubation with primary antibody (3F8, 3 nmol/L or 3F10, 6 nmol/L) at 4 °C. Cells were washed three times with PBS and incubated with goat anti-human AF594 (Invitrogen, Carlsbad, USA) for 2 h at 4 °C. Labeled cells were washed three times with PBS, mounted with ProLong Gold containing DAPI (Invitrogen, Carlsbad, USA) and imaged using an inverted fluorescence microscope (Olympus IX71, Center Valley, USA) at 40× magnification. Image analysis was performed with ImageJ software (Collins, 2007).

2.3. Mouse infections and immunohistochemistry

Type I and type II interferon receptor deficient 129/Sv (AG129) mice were bred at the Duke-NUS Graduate Medical School animal facility. All experimental procedures were performed according to Institutional Guidelines. Mouse adapted DENV2 virus (S221) at 5×10^4 pfu was administered by the intra-peritoneal route. Mice were euthanized on day three post-infection and organs were harvested for staining. Intestinal samples were processed using a tissue processor (Leica TP 1020, Hesse, Germany), fixed in 4% para-formaldehyde for 24 h, and subject to an ascending series of ethanol prior to being cleared with xylene and embedded in paraffin. Paraffin embedded small intestine samples were sectioned at a thickness of 4 μm using a microtome (Leica RM 2135, Hesse, Germany). The sections were mounted on SuperFrost Plus slides (Thermo Fischer Scientific, Waltham, USA) and air-dried for storage.

On the day of staining slides were heated at 60 °C for 1 h, immersed in xylene for 10 min and rehydrated in an ethanol series. Slides were treated with antigen retrieval buffer (100 mM sodium citrate pH 6.0) and blocked with PBS supplemented with 5% BSA and 0.1% Triton X-100 for 2 h. The primary antibody, 3F8, was diluted to 3 nmol/L in PBS supplemented with 5% BSA and used to probe for DENV2 NS3 in tissues. After rinsing with PBS, slides were incubated with goat anti-human AF594 (Invitrogen, Carlsbad, USA) for 2 h at 4 °C. Slides were then rinsed again with PBS and mounted with ProLong Gold containing DAPI (Invitrogen, Carlsbad, USA). Immunofluorescence images were captured and analysed using an inverted fluorescence microscope (Olympus IX71, Center Valley, USA) and ImageJ software (Collins, 2007).

2.4. NS3–NS5 immunoprecipitation and ELISA

For immunoprecipitation from BHK-21 cells 3F8 Fab (240 pmol) was incubated overnight with 100 μl of lysate from uninfected or DENV2 (GenBank accession EU081177.1) infected cells and anti-*c-myc* resin (Pierce, Rockford, USA) that was previously blocked with BSA (5 mg/mL). DENV2 infected lysate incubated with anti-*c-myc* resin in the absence of 3F8 was included as a no antibody control. Samples were subject to 12% SDS-PAGE, and Western blotted with anti-NS3 and anti-NS5 polyclonal antibodies followed by anti-rabbit-HRP conjugates for ECL detection.

The NS5 RdRp domain, $NS2B_{CF18}NS3$ full-length, $NS2B_{CF47}NS3$pro and NS3 helicase proteins from DENV3 (GenBank accession AY662691) required for the ELISA were expressed and purified as previously published (Li et al., 2005; Moreland et al., 2010; Yap et al., 2007). Maxisorb Immunoplates were coated with