

Report

Monoclonal antibody to dengue capsid protein

Its application in dengue studies

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Abbreviations: DF, dengue fever; DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; C, capsid; Den, dengue; Mab, monoclonal antibodies; RNA, ribonucleic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; SMB, suckling mouse brain; FITC, fluorescein isothiocyanate; E, envelope; pi, post infection; ic, intracranial route; MEM, minimal essential medium; HCV, hepatitis C virus; OD, optical density; JE, Japanese encephalitis; BHK, baby hamster kidney; RT-PCR, reverse transcriptase-polymerase chain reaction; MARV, marburg virus

Key words: monoclonal antibodies, capsid protein, dengue virus, diagnosis, immunoassays

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are considered the most important arthropod-borne viral diseases in terms of morbidity and mortality. The emergency and severity of dengue (Den) infections increase the necessity of an early, quick and effective dengue laboratory diagnostic. Viral isolation is considered a gold standard for diagnosis of dengue infection using monoclonal antibodies (mAbs) as a tool for determining serotype specificity. Alternatives have been used to improve sensitivity and time to dengue diagnosis. Based on the early expression of dengue C protein in the life cycle, we focused our study on the application of an anti-dengue 2 virus capsid protein mAb in dengue diagnosis. The kinetic expression of dengue-2 capsid in mosquito cells and its immuno-localization in experimentally infected suckling albin Swiss (OF-1) mice brain tissues was established. The results demonstrate the possible utility of this mAb in early dengue diagnosis versus traditional isolation. In addition, a preliminary study of an enzyme immunoassay method using 8H8 mAb for specific detection of dengue C protein antigen was performed, making possible recombinant C protein quantification. The results suggest that detection of dengue capsid protein could be useful in the diagnosis of early dengue infection.

Introduction

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are considered the most important arthropod-borne viral diseases in terms of morbidity and mortality.¹ It is estimated that more than 100 million people are infected with dengue virus every year.² This situation enhances the need for an early, fast and efficient dengue laboratory diagnostic.

Dengue diagnosis based on antibody identification has become the most practical approach due to a variety of commercial and "in-house" immunoassays for dengue diagnosis that have been developed.³⁻⁶ The IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) is the assay of preference for the serological diagnosis of acute dengue virus infection, and is a very useful tool for sero-epidemiological dengue surveillance.^{7,8} The detection of both anti-dengue IgM and IgG by ELISA permits classification of the dengue infection type (primary or secondary), thereby contributing to a better understanding of the pathogenesis of DHF.^{8,9} On the other hand, molecular biology techniques detecting specific sequences of nucleic acid as well as viral proteins are valuable for diagnosis. However, the sample and RNA must be processed carefully to avoid RNA degradation.⁹

Although the process is time-consuming, isolation and viral identification from infectious samples are considered the gold standard of dengue virus infection diagnosis. Viral isolation allows follow-up virological study and biological characterizations of the agent.⁹⁻¹¹ In order to reduce the time to diagnosis, a variety of modifications, novel methods and tools have been introduced.¹¹⁻¹⁴

Monoclonal antibodies (mAbs) are efficient analytic tools for detection, screening and characterization of biomolecules, and have wide application as diagnostics and immunotherapies. mAbs have been successfully used to identify serotype specificity of dengue viruses in conventional diagnosis as viral isolation. Dengue envelope (E) and non structural 1 (NS1) proteins have been targets

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Figure 1. Immunofluorescence staining with 8H8 mAb of dengue infected cells with Den-2 A15 strain. C6/36-HT cells were infected with Den-2 A15. At 6 (a), 8 (b), 12 (c), 48 (d) and 72 (e) h p.i, the infected cells were reacted 8H8 mAb followed with an FITC-conjugated goat anti-mouse antibody and then examined under a fluorescence microscope. Cytoplasmatic and nuclear staining of infected cells are indicated as C and N, respectively. C6/36-HT cells infected with Den-2A15 reacted with anti-Den-2 polyclonal antibody at 72 h p.i was used as a positive control (f) and non-infected C6/36-HT cells were used as negative control (g).

of these mAbs in different assays.¹⁵⁻¹⁷ Dengue capsid (C) protein has been used for therapeutic and vaccinology purposes, but not in diagnostic research. Studies of this protein have established its intracellular localization in different cellular lines, and its early expression in BHK-21 cell lines has been reported.¹⁸⁻²⁰ In our studies, we evaluated the usefulness of an anti-C mAb in the establishment of the kinetic expression of Den-2 capsid protein in *Aedes albopictus* cells, demonstrated the efficacy of this anti-C mAb in the early identification of dengue virus from viremic sera in conventional diagnosis, and determined its utility in the immuno-localization of Den-2 capsid protein in suckling mice brain by immunohistochemistry assay. Additionally, 8H8 anti-C mAb was used in an antigen-capture Enzyme-Linked Immunosorbent Assay (ELISA) test to quantify a recombinant C protein, suggesting a potential future application in the early diagnosis of dengue infection.

Results

Kinetic expression of the dengue capsid protein. C protein was detected in infected cells during the entire study interval from 6 h to 96 h after infection. At 6 h and 8 h pi a slight positive staining was observed in the cytoplasm of infected cells (Fig. 1A and B). After 12 h pi the signal became more intense at the cytoplasm. At 48 h and 72 h pi the signal appeared as spots in the cytoplasm and in the nuclei (Fig. 1D and E). The highest intensity was observed at 72 h pi (Fig. 1E). No immunofluorescence was observed in non-infected C6/36-HT cells (Fig.

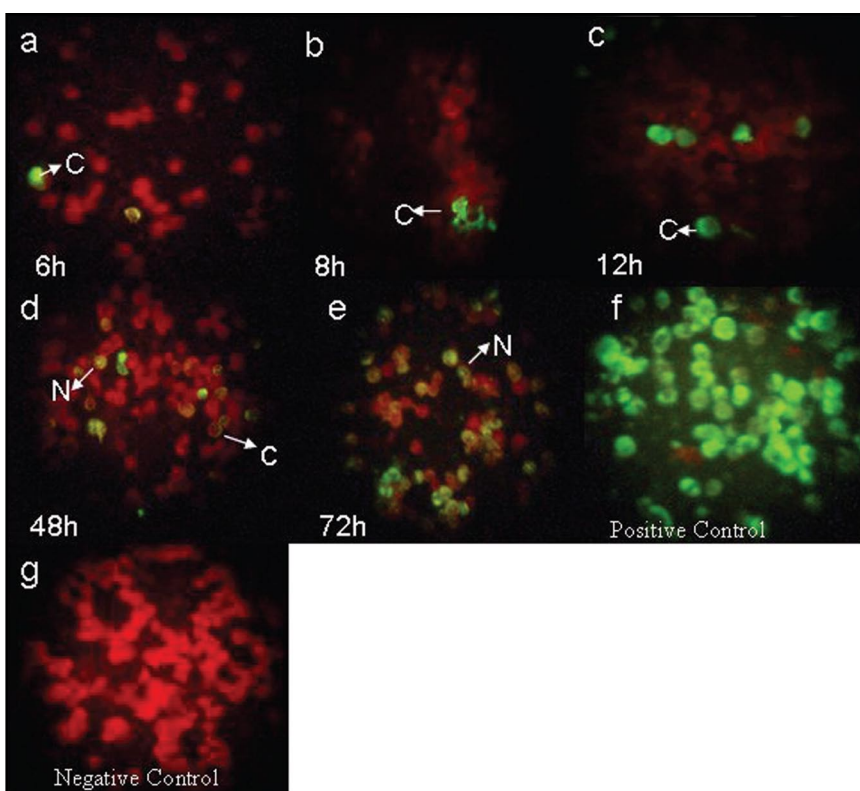
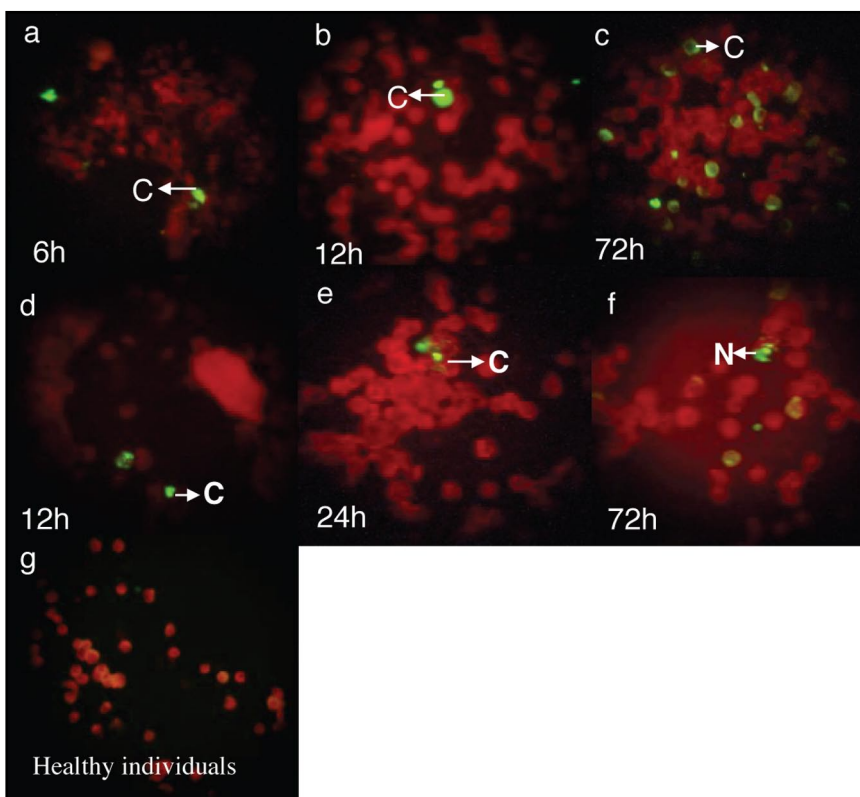


Figure 2. Immunofluorescence staining with 8H8 mAb of dengue infected cells. C6/36-HT cells were infected with samples 57 (a-c) and 59 (d-f) from dengue acute patients. At 6 (a), 12 (b), 72 (c) h pi, for sample 57 and 12 (d), 24 (e), 72 (f) h pi for sample 59, the infected cells were reacted 8H8 mAb followed with an FITC-conjugated goat anti-mouse antibody and then examined under a fluorescence microscope. Cytoplasmatic and nuclear staining of infected cells is indicated as C and N, respectively. Samples from healthy individuals as negative control (g).



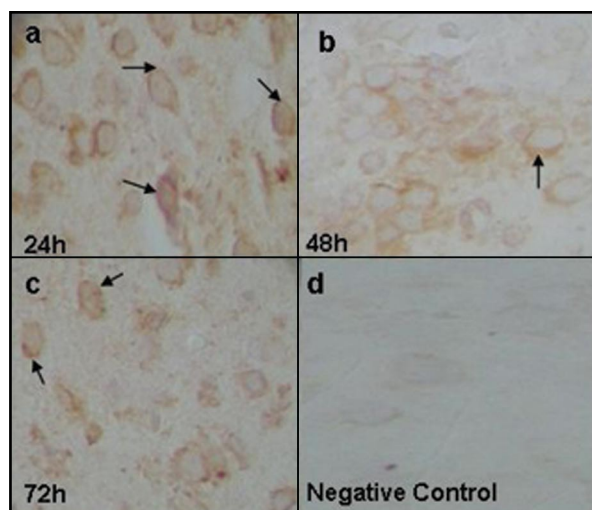


Figure 3. Staining of Dengue capsid protein in suckling mice brain at 24 (a), 48 (b) and 72 h (c) pi. Uninfected suckling mouse brain as negative control (d).

1G). Anti-E H3-6 mAb was used as control assay and it was able to detect the expression of E protein of the Den-2 strain from 24 h pi.

In addition, the kinetic expression of the C protein of Den-2 isolates in C6/36-HT cells from two sera collected from dengue patients was also studied (Fig. 2). In the two cases, an increase in the intensity of the immunofluorescence was observed over time during infection. The C protein of the 57 and 59 isolates began to express at the same time (6 h p.i.) as the Dengue-2 A15 strain (Fig. 1).

Expression of dengue capsid protein in suckling mice brain. Capsid protein expression was demonstrated in the brain of suckling OF-1 mice from 24 h until 72 h pi. C protein antigen was immunolocalized in the cytoplasm (Fig. 3). Nevertheless, no histopathological findings were observed in the hematoxylin-eosin stained tissue sections.

Detection of dengue C protein antigens by ELISA. Based on the utility of the anti-capsid 8H8 mAb in the early detection of the C protein by immunofluorescence assay, an ELISA for the detection and quantification of the Den-2 recombinant C protein

was standardized. The antigen-capture assay using 8H8 mAb might allow viral identification after short incubation times under the recombinant protein up to 1/1,024 dilution which corresponded to 1.67 $\mu\text{g}/\text{mL}$ (Fig. 4).

Discussion

mAbs have previously been used to locate intracellular viral antigens.²⁰ The C protein of Kunjin and Japanese encephalitis viruses (JE) has been detected in the cytoplasm, the nucleus and nucleolus of infected mammalian and mosquito cells.²¹ In addition, mAbs have been used to demonstrate the probable significance of the nuclear localization of the West Nile virus C protein in replication and pathogenesis.²² Dengue C protein has also been detected in the cytoplasm, nucleus^{19,23} and in the nucleolus of infected cells.¹⁸ Nevertheless, the kinetic expression of dengue C protein has been studied only in BHK-21 cells.²⁰

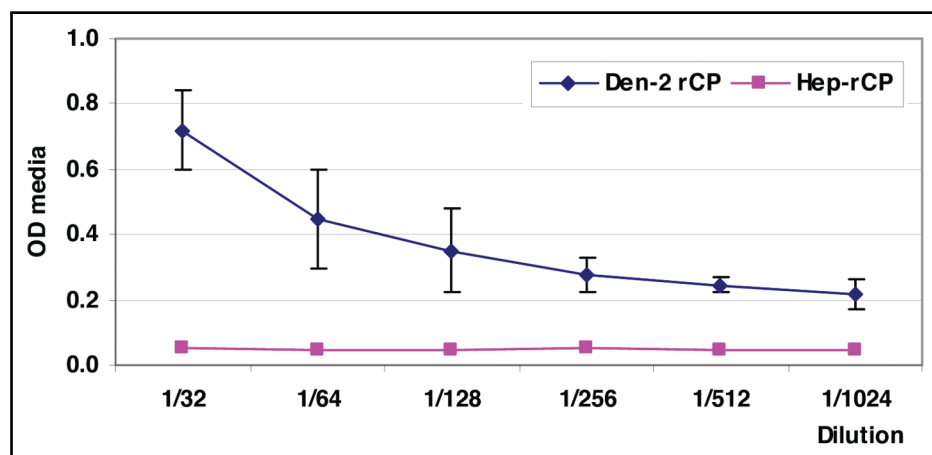
To our knowledge, the present study is the first report of kinetic expression of the Den-2 C protein in C6/36-HT cells, leading to detection of the protein at a very early stage (6 h pi). We observed similar findings when C6/36-HT cells were infected with acute sera from dengue patients. Dengue diagnosis by traditional procedures (viral isolation) requires at least 6–10 days²⁴ and viral identification is done using anti-E mAbs.^{10,11,25} Therefore, the use of anti-capsid 8H8 mAb can allow the viral identification in minor incubation time under epidemiological conditions where serotype 2 is circulating. The specificity and sensitivity of the 8H8 mAb was demonstrated, since the acute sera from dengue patients were positive by isolation and RT-PCR.

mAbs are also considered valuable tools for dengue diagnosis from fatalities through use of immunochemistry studies.^{26–29} Many researchers use this method to determine pathological changes in infected tissues related to the presence of Den antigen.^{30–32} Although we did not find histopathological changes in the mice brain infected tissues, 8H8 mAb was able to detect C protein from the same paraffin-embedded sections. This suggests that 8H8 mAb could also be helpful for the diagnosis of dengue in fatal cases occurring under similar epidemiological conditions.

High titers of viremia are present in patients at the early stage of dengue infection, suggesting that the detection of viral antigens is appropriate for early diagnosis.^{33,34} The antigen capture ELISA test has demonstrated utility in the detection of viral antigens from blood.³⁵ This assay has been used recently for the detection of non-

structural protein 1 (NS1) protein dengue virus.^{16,17,36} C protein has also been used as a target in the antigen capture ELISA for some viruses, such as Rift Valley Virus,³⁷ Hepatitis B virus,³⁸ Hepatitis C virus,³⁹ Marburg⁴⁰ and Ebola⁴¹ using monoclonal antibodies. However, it has not been used for this purpose in dengue diagnosis.

Figure 4. Reactivity of 8H8 mAb in the antigen-capture enzyme-linked immunosorbent assay (ELISA) format. The ability to capture the Dengue-2 recombinant protein (Den-2-rCP) of the monoclonal antibody was evaluated at various concentrations of Den-2-rCP.



Saijo et al. developed an antigen capture ELISA to detect recombinant nucleoprotein of Marburg virus (MARV). The test could detect 40 ng/mL and the authentic MARV nucleoprotein at a level equivalent from 3×10^3 PFU/mL to 1.2×10^4 PFU/mL.⁴² The use of polyclonal antibody against nucleoprotein of MARV as detector contributed to an increase the sensitivity of the assay.

The preliminary results of our study indicate that 8H8 mAb could successfully quantify a C recombinant dengue protein. In order to improve the sensitivity of capture ELISA assay the application of polyclonal antibody or another dengue anti-capsid mAb should be applied since the use of mAbs offer higher specificity, but less sensitivity, to the assay.^{43,44}

Possible improvements in detection of C dengue protein in the ELISA assay requires a more extensive study, including use of a detergent cocktail in pre-treatment of samples, a kinetic study of clinical samples, and increases in the sensitivity of the test.⁴⁵

The applicability of 8H8 mAb in dengue diagnosis is limited because its specificity to C protein of Den-2 serotype. Nevertheless, it provides the possibility of earlier identification of this serotype in viral isolation performed during dengue diagnosis. Our results suggest that generation of monoclonal antibodies against consensus regions of C dengue protein will be a useful tool for early dengue laboratory diagnosis.

Materials and Methods

Cells. *Aedes albopictus* cell line (clone C6/36-HT) was grown at 33°C in MEM medium containing 2 mmol/L glutamine and 10% of heat-inactivated fetal bovine serum (FBS). These cells were maintained in the same medium with 2% of FBS.

Viruses. Den-2 A15 Cuban strain⁴⁶ with a history of four passages in suckling mouse brain (4pSMB) and seven passages in C6/36-HT cells (7pC6/36) with a titer of 2.05×10^5 PFU/mL was employed.⁴⁷

Two dengue acute sera from patients confirmed as Den-2 by isolation in C6/36-HT cell culture and RT-PCR were included in the study. These sera were identified as number 57 and 59.

Monoclonal antibody (mAb). Anti E dengue protein H3/6 mAb was produced by Hermida et al. and is able to recognize the 4 dengue serotype, it is an IgG₂ immunoglobulin subclass capable to neutralize in vitro.⁴⁸ This mAb was used as a control for detecting of viral presence in immunofluorescence assay.

Anti-capsid 8H8 mAb was generated by Pupo et al. It was characterized as IgG₁ immunoglobulin subclass, specific to Den-2 serotype as stated by immunofluorescence and immunoassay. It was unable to inhibit the hemagglutination and neutralize the virus.⁴⁹

Both hybridoma cells producing 8H8 and H3/6 mAbs were maintained in RPMI 1640 medium supplemented with 10% FBS, non essential amino acids, and antibiotics (streptomycin and penicillin). A suspension of cells (2×10^6) was injected intraperitoneally in ten Pristane primed mice, collected ascetic fluids were clarified by centrifugation at 1,500 rpm for 10 min at 4°C.

8H8 mAb purification was achieved by immunochromatographic methods using protein A conjugated to Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden). Purified antibodies were conjugated with horseradish peroxidase enzyme (Sigma) following the periodate method.

Immunofluorescence staining. To determine C dengue protein expression, C6/36-HT cells were inoculated with Den-2A15 (4pSMB, 7pC6/36) (m.o.i 0.01) following the shell vial method described elsewhere.⁵⁰ The cells were harvested every 12 h from 6 h to 96 h post-infection (pi) to be fixed on slides with cold acetone for 20 min. Fixed cells were covered with the appropriate antibodies and examined under a fluorescence microscope (Leitz Wetzlar Germany). The capsid protein expressed in infected cells was detected by immunofluorescence staining using 8H8 mAb at 1:50 dilution in phosphate buffered saline (PBS) as the primary antibody and FITC-conjugate goat anti-mouse IgG (Sigma) diluted 1:40 in PBS as the secondary antibody.

Similarly, acute sera collected from two cases of Den-2 infections were used for C6/36-HT inoculation. These patients had been classified as dengue infection clinically and by RT-PCR assay in the laboratory. Serum from the blood of a healthy donor was included as a negative control.

Envelope (E) protein was also detected using the anti-E monoclonal antibody H3-6.⁴⁸

Immunohistochemistry study. Suckling OF-1 mice were inoculated by intracranial route (ic) with 0.02 ml of a 1:10 dilution in MEM medium of Den-2A15 strain. Brain tissues samples were obtained during three days at 24 h interval and paraffin embedded in a routine fashion for histopathology studies described elsewhere.⁵¹

Hematoxylin and eosin staining was performed for histopathology studies. The C protein antigen presence was assayed by indirect immunoperoxidase method using the 8H8 mAb as primary antibody and anti-mouse rabbit immunoglobulins conjugated with horseradish peroxidase (Dako Cytomation) as a secondary antibody. The reaction was detected as a golden brown stain when 3,3'-diaminobenzidine (Sigma) was used as the chromogen. Uninfected suckling mouse brains collected at the same time as the infected tissues samples were employed as negative control.²⁸

ELISA for detection of dengue capsid protein. An ELISA was performed using 8H8 mAb for the capture and the revelation of C antigens. Microtiter plates (Nuncs) were coated with 100 µL per well containing 5 µg/well of 8H8 mAb in carbonate-bicarbonate buffer pH 9.6 (coating buffer) and incubated overnight at 4°C. Afterward, the wells were washed, blocked by 2% skimmed milk solution and dried. Serial dilutions of a Den-2 recombinant capsid protein⁵² (100 µL) were then added. Plates were incubated for 2 h at 37°C and washed three times. A 100 µL of a 1/3,000 peroxidase-conjugated 8H8 mAb dilution was added to each well and incubated for 1 h at 37°C followed by three washes; 100 µL of the substrate solution (*o*-phenyldiamine and H₂O₂ in 0.1 M citrate buffer, pH 5.0) were added and incubated for 30 min at room temperature. The stop reagent, 12% H₂SO₄, was added and the plates were read at 492 nm. Serial dilutions of a recombinant hepatitis C virus (HCV) capsid antigen (from UMELISA HCV kit, Immunoassay Center, Cuba) were used as negative control. Samples were considered positive when optical density (OD) was twice OD of negative control. The assay was performed five times for duplication, and the graphic was achieved using OD arithmetic mean and standard deviation.

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