Production of a Monoclonal Antibody Against Non-Structural Protein 3 of Dengue-2 Virus by Intrasplenic Injection

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Dengue fever and dengue hemorrhagic fever/dengue shock syndrome are highly infectious diseases caused by dengue virus (DV). DV non-structural protein 3 (NS3) is known to possess ATPase, helicase, and protease activity that is a constitutive part of the replication complex of DV. In this study, we discuss the cloning, expression, and purification of the DV-2 NS3 protein to immunize mice by intrasplenic injection and then to generate a monoclonal antibody (MAb). One MAb, named 4F5, was obtained and it was specific to NS3 of DV-2. Immunofluorescence show that 4F5 recognizes the native protein in infected ECV304 cells. Likewise, C6/36-infected lysates were used in Western blot analysis, and we observed the specific characteristic band that defines NS3. We conclude that MAb 4F5 may be a useful tool, not only to study the replicative process of DV, but also to generate specific diagnostic tools for DV infection.

Introduction

Dengue virus (DV) infection is an acute mosquito-transmitted viral disease characterized by a mild febrile illness known as dengue fever (DF). DV affects more than 100 million people worldwide every year. Some infections result in more severe dengue hemorrhagic fever and/or dengue shock syndrome (DHF/DSS), which can threaten the patient’s life, primarily through increased vascular permeability and shock. Epidemiological studies indicate that DHF/DSS is more commonly seen during secondary infection with a different serotype of DV from that which caused the primary infection. There are four serotypes of DV (DV-1 to DV-4), whose genome contains a single open-reading frame (ORF) of ~11 Kb encoding a polyprotein precursor that is proteolytically cleaved into three structural proteins (capsid [C], premembrane [prM], and envelope [E]) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5).

DV-2 NS3 is a multifunctional protein of ~69 kDa involved in polyprotein processing, RNA replication, and capping of the viral genomic RNA. NS3 serine protease domain is contained within the N-terminal 180 amino acids and requires the protein NS2B for protease activity. The conserved hydrophilic domain of NS2B alone is sufficient for activation of the protease domain of NS3. The C-terminal region of NS3 contains RNA helicases/NTPases. It is demonstrated that both DV-2 NS3 and NS5 are capable of converting the replicative form RNA (RF) to replicative intermediate (RI). The NS3 acts as a helicase unwinding the double-stranded RF to a partially single-stranded RI. RNA helicase activity of NS3 has also been demonstrated in Japanese encephalitis virus, Langat virus, hepatitis C virus, and pestivirus proteins.

DV-2 NS3 is also an important target for human T cells and able to elicit specific antibodies. The NS3 protein appears to be a dominant target for DV-specific CD4+ and CD8+ T cells, and most DV NS3-specific T cells are serotype cross-reactive. The abundance of T cell epitopes on the flavivirus NS3 protein is not well explained, although multiple human T cell epitopes on the NS3 protein have been identified.

The NS3 protein is also able to elicit specific antibodies. It is reported that monoclonal antibodies (MAb) to NS3 of DV-1 are able to increase the survival time of mice challenged with a lethal dose of DV-1, although the mechanism remains to be defined. In DV-2 infections, the presence of NS3 antibodies in acute-phase samples from primary and secondary cases was reported. It suggests the possibility of implementing ancillary diagnostic assays with higher sensitivity for NS3 antigen detection in some DF and DHF/DSS cases.

To further investigate the role of NS3 protein in pathogenesis of DV infections and to develop a new diagnostic assay with NS3 antigen detection, in this study we report the cloning, expression, and purification of DV-2 NS3 protein. BALB/c mice were immunized with the NS3 protein by intrasplenic injection and then one MAb named 4F5 was...
generated through hybridoma technique. The results showed that MAb 4F5 was specific to NS3 protein of DV-2, suggesting that it might be a useful tool not only to study the replicative process of DV but also to generate specific diagnostic tools for DV infection.

Materials and Methods

Cell lines, virus, and other reagents

*Aedes albopictus* mosquito cells (C6/36) (European Collection of Cell Culture, Salisbury, United Kingdom) were grown at 28°C in Dulbecco’s modified Eagle’s minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY). ECV304 cells (European Collection of Cell Culture) were cultured at 37°C in DMEM and used for immunofluorescence staining. DV-2 (strain TR1751) isolated from a patient with DF was kindly provided by Dr. A. Oya (National Institute of Infectious Disease, Japan) and was propagated in C6/36 cells. *Escherichia coli* JM109 and T4 ligase were purchased from Takara Ltd. (Dalian, China). Restriction enzymes were purchased from MBI (Vilnius, Lithuania). The plasmid pQE-31 and Ni²⁺-nitrilotriacetic acid (NTA)–agarose resin was purchased from Qiagen (Chatworth, CA).

Expression of DV NS3 protein

Viral RNA was isolated from DV-infected C6/36 cells usingBiozol reagent (Bioflux, Tokyo, Japan) and reverse transcribed to cDNA using primer DEN (−): CTCCCGCT CATCAAGAATAA. With the primers of P1 (+), cggtatcgc GCTGGAGTATTGTGGGACG, and P2 (−), ccaaggtcttATT CTTCCGGCTGCGA, the coding gene of protein NS3 was amplified by 14,000 cycles from a restriction enzyme site, BamHI (G*GATCC), was added to the 5'-terminal of P1 (+); HindIII (A*AGCTT) and stop codon (CTA) were added to the reverse primer P2 (−). The BamHI and HindIII were designed to ligate PCR fragments into the expression vector pQE-31. A BamHI/HindIII enzyme fragment that contained NS3 sequence was cloned into pQE-31 vector and named pQE-NS3. Using this strategy, the DV NS3 gene was fused in-frame with pQE-31 plasmid with the 6×His purification tag, naming pQE31-NS3.

*E. coli* JM109 with pQE31-NS3 expression vectors was inoculated in LB medium (containing 100 μg/mL ampicillin) (Amresco, Solon, Ohio) and allowed to grow at 37°C until the absorbance of the culture reached 0.6 at 600 nm; then the cells were induced with 1 mM IPTG (Amresco) and harvested 10 h after induction by centrifugation at 7000g for 20 min at 4°C. The induced-expression cells were disrupted by sonication in an ice-water bath for 40 min. The inclusion bodies were isolated by 12% SDS-PAGE and then transferred onto a PVDF membrane and probed with mouse anti-His antibody followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Dako, Carpinteria, CA).

Immunization through intrasplenic injection

For intrasplenic injection of DV NS3 protein, 8-week-old female BALB/c mice (Laboratory Animal Center, Academy of Military Medical Sciences, Beijing, China) were anesthetized with an intraperitoneal injection of pentobarbitone (45 mg/kg), followed by an intramuscular injection of ketamine hydrochloride (50 mg/kg). An area (2×2 cm) in the left mid-scapular line at the lower costal margin was shaved and swabbed with a 70% ethanol solution. A small incision was made through the skin, the muscles were separated by blunt dissection, and the underlying spleen was visualized through the intact peritoneal membrane. A 30-gauge needle (Top Injection Needle, Tokyo, Japan) was inserted parallel to the spleen, and two injections of DV NS3 protein (each containing 200 μg in 20 μL of physiologic saline) were delivered into the spleen. The overlying skin edges were approximated, and the incision was closed in a single step with 4-0 silk.

Preparation of NS3 protein-specific MAb

Three weeks after immunization, the specific antibody titer of immunized mice sera was detected by indirect ELISA and indirect immunofluorescence staining assay (described below). Then the spleen cells from the best immunized mice were fused with SP2/0 myeloma cells, and hybridomas were generated by the method described earlier. When cells became 80% confluent, the supernatants were subjected to test antibody titer by indirect ELISA. Positive hybridomas were selected and cloned 3~5 times; then monoclonal hybridoma was obtained. The MAb was harvested from hybridoma grown in 1640 medium without FBS. MAb was isotyped by Mouse MAb Isotyping Kit (Sigma, St. Louis, MO). The hybridoma was intraperitoneally injected in BALB/c mice (pretreated with pristane) for production of ascites of MAb. The ascites were collected for the following examinations.

Indirect ELISA

96-well plates (BD Biosciences, San Jose, CA) were coated with 2 μg/well of purified NS3 or bovine serum albumin (BSA) overnight at 4°C, and then blocked with 1% BSA/PBS for 1 h at 37°C. The supernatant of hybridoma was added as primary antibody respectively and incubated overnight at 4°C. Mouse anti-DV2 sera and normal mouse sera (Nms) were used as positive and negative controls, respectively. After washing three times with PBS containing 0.05% Tween-20 (PBS-T), the plates were incubated with HRP-conjugated goat anti-mouse immunoglobulin (IgG) (Dako) for 1 h at 37°C, followed by extensive washing and coloration with o-phenylenediamine (OPD, Amresco). The absorbencies were determined at 492 nm with Sunrise Absorbance Reader (Tecan, Salzburg, Austria).

Indirect immunofluorescence staining assay

A specific MAb was used to identify the native protein on DV-2-infected ECV304 cells. Briefly, ECV304 cells were grown on coverslips and infected with DV-2 for 48 h; then the cells were fixed with 4% paraformaldehyde in PBS (pH 7.2) and blocked with 1% BSA in PBS. A specific MAb to NS3 was added, and its reactivity was made evident by the recognition with FITC-conjugated goat anti-mouse IgG (Sigma). After the appropriate wash, preparations were mounted with...
Vecta-Shield and evaluated by fluorescence microscopy (Olympus BX51, Tokyo, Japan). Anti-DV2 sera and NMS were used as positive and negative controls, respectively.

**Electrophoresis and Western blot analysis**

At 72h after infection, C6/36 cells infected with DV-2 or mock infected were washed with non-supplemented medium; then the cells were lysed with RIPA buffer (100 mM Tris-HCl [pH 8.3], 2% Triton X-100, 150 mM NaCl, 0.6 M KCl, 5 mM EDTA, 1% Aprotinin, 3 mM PMSF, 1 μg/mL leupeptin, and 5 μg/mL soybean trypsin inhibitor). The recombinant NS3, DV-infected C6/36, and uninfected cell lysates were analyzed by SDS-PAGE and transferred onto nitrocellulose membrane (Millipore). Hybridoma supernatant was incubated with the nitrocellulose membrane, followed by washing in PBS-T, and then incubated with HRP-conjugated goat anti-mouse IgG for 1 h. Finally, substrate was added.

**Results**

**Expression and purification of NS3 protein**

In order to produce DV-2 NS3 protein, viral RNA was isolated from DV-2-infected C6/36 cells and reverse transcribed to cDNA using primer DEN. The gene of NS3 protein (about 1854 bp) was amplified by PCR with primers P1 (+) and P2 (−) from cDNA and was cloned into pQE-31 vector to give the recombinant pQE-NS3. The DNA sequencing showed that the gene sequences of interest were authentic. The transformant, E. coli JM109, with pQE-NS3 was induced by 1 mM IPTG. A band of ∼70 kDa proteins appeared, but not in normal JM109 lysates. The inclusion bodies were completely dissolved in 8 M urea and the fusion proteins could be captured by Ni^{2+}-NTA affinity chromatography. The specific characteristic bands were confirmed by Western blot using anti-His monoclonal antibody (Fig. 1).

**Immunization by intrasplenic injection and preparation of NS3-specific MAb**

Three weeks after immunization with purified NS3 protein by intrasplenic injection, the sera was collected from an immunized mouse by cutting the tail. Immunostaining showed that intense fluorescing clusters were noted in the perinuclear of infected ECV304 cells and diffuse fluorescence with some speckling was seen in the cytoplasm (Fig. 2A). Infected cells incubated with the pre-vaccinated mouse serum (Fig. 2B) and uninfected cells incubated with the mouse anti-recombinant NS3 protein PAbs (Fig. 2C) showed no specific fluorescence. Immunostaining analysis indicated that the mice sera were specific for native NS3 protein using DV-2-infected ECV304 cells.

The mouse whose serum showed the highest antibody titer and strong immuno-fluorescence in infected ECV304 cells was used to obtain the splenocytes that were fused with SP2/0 cells; the resulting hybridomas were initially screened for the secretion of anti-His-NS3. Some MABs produced by the hybridomas may react with the His-tag, or with the combination of His and NS3. To determine whether these MABs recognized NS3 exclusively, the same supernatants were tested simultaneously with wells coated with His-tag and with His-NS3. From the 10 clones obtained, only four were specific for NS3 (data not shown). The positive hybridoma that gave the highest response to NS3 protein (MAb 4F5) was cloned. The isotype of this antibody was found to be IgG2a by Mouse MAb Isotyping Kit. Finally, the hybridoma was inoculated intraperitoneally into pristine-primed BALB/c mice to produce MAb ascitic fluid.

**MAb 4F5 recognizes DV-infected ECV304 cells by immunofluorescence**

The MAb 4F5 was tested by immunofluorescence staining for its reactivity to DV-infected and mock-infected ECV304 cells. Strong fluorescence intensity was observed in the cytoplasm of infected cells by anti-recombinant NS3 protein PAbs; (B) infected cells incubated with the pre-vaccinated mouse serum; (C) uninfected cells incubated with the mouse anti-recombinant NS3 protein PAbs.
plasm of the cells (Fig. 3B). The distribution of specific reaction was similar with that seen in a section stained with mouse anti-DV2 sera (Fig. 3A). No evident cross-reactivity with other components of the uninfected host cell was observed (Fig. 3C).

**MAb 4F5 recognizes the band of NS3 in immunoblotting**

Immunoblotting was performed to further characterize the denatured protein. Lysates of infected and mock-infected C6/36 cells were harvested 7 days after infection and separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with the MAb 4F5. The presence of a 70 kDa protein was observed in the infected cell lysate (lane 1), in contrast with that observed in the mock-infected cells, where no bands were observed (lane 2, Fig. 4).

**Discussion**

There is currently no effective vaccine or antiviral drug to protect against dengue. Passive or active immunizations directed against a variety of DV proteins are currently being investigated as a potential therapeutic approach in the field of DV infectious disease. As a multifunctional protein, NS3 plays an important role in the replication of dengue viruses. The N-terminal region of NS3 interacts with NS2B and functions as a two-component serine protease involved in processing the viral polyprotein precursor. The C-terminal region of NS3 contains RNA helicases/NTPases. Moreover, the NS3 protein is also able to elicit specific antibodies. NS3 looks like a potential antivirus target.

To further elucidate the roles of NS3 in the pathogenesis of DV-mediated diseases, we report on the cloning, expression, and purification of the DV-2 NS3 protein under denaturing conditions. Mice were immunized with the NS3 protein by intrasplenic injection and MAb 4F5 was generated. Western blot analysis showed that 4F5 against the NS3 protein of DV-2 was specific. Using immunostaining, a strong positive response was observed in perinuclear of infected ECV304 cells, when probed with our anti-NS3 antibody.

The conventional immunization process is subcutaneous intraperitoneal and intramuscular injections of target proteins, which requires more immunizations. Intraspnic injection with DV NS3 fusion protein resulted in immune response, which needs only one immunization. The response was much faster than that in animals receiving a conventional immunization of DV NS3 in IFA. The time was cut down to 3 weeks and the operations were reduced to one step. Immunization through intraspnic injection was more efficient than the classical means of immunization.18

In this study, we demonstrate that intraspnic immunization of DV NS3 fusion protein leads to a rapid immune response. The purified NS3 protein had strong immunogenicity, and its MAb could recognize the native and denatured proteins. The MAb might be a useful tool, not only to study the replicative process of DV but also to generate ancillary diagnostic and therapeutic tools for some DV infections.

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