Generation and Characterization of a Monoclonal Antibody Against prM Protein of West Nile Virus

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West Nile virus (WNV), which is an emerging pathogenic flavivirus with increasing distribution worldwide, is the cause of major human and animal health concerns. The pre-membrane (prM) protein of WNV is cleaved during maturation by the furin protease into the structural protein M and a pr-segment. In this study we generated and characterized a monoclonal antibody (MAb) against the WNV prM protein. Western blot analysis showed that the MAb reacted with WNV prM specifically. Immunohistochemistry assays demonstrated that the MAb recognized native prM protein in transfected BHK-21 cells. Preliminary studies were performed to identify the epitope recognized by the MAb using a set of synthesized overlapping peptides spanning the whole length of the prM protein. The MAb reported here may provide a valuable tool for the further exploration of the biological properties and functions of the prM protein and may also be developed for potential clinical applications.

Introduction

WEST NILE VIRUS (WNV) IS A ZOONOTIC mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae* and is widely prevalent in Africa, Southern Europe, Russia, the Middle East, India, Australia, and North America.^(1,2) Since its first large outbreak in Romania in 1996,⁽³⁾ WNV has become a major public health and veterinary concern. In 2012, the US experienced a WNV outbreak, with 5387 cases of WNV disease reported, including 243 deaths.⁽⁴⁾

WNV was a single-stranded (positive sense) RNA virus with a genome of approximately 11 kb that encodes three structural proteins (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).^(5,6) The prM protein comprises 167 amino acids, with a molecular weight of 20 to 26 kDa. The prM protein, which acts as a chaperone that assists in the maturation of the E protein, interacts with E as a prM/E heterodimer, which is important for the formation of immature virions.^(7,8) The pr peptide is contained within the amino terminal region of the prM protein and is cleaved from prM by the protease furin in the trans-Golgi apparatus during release of the virus from the cell. The proteolytic cleavage of prM to generate the M protein results in the formation of a fusion competent particle.^(9,10) However, the functional role of prM in replication, virus infectivity, and induction of neutralizing activity⁽¹¹⁾ is not fully understood.

In this study we generated a hybridoma cell line secreting a monoclonal antibody (MAb) specific for the pr region of the WNV prM protein. We subsequently characterized the MAb

produced, which may be a useful tool for further investigations of the characteristics and functions of the prM protein, and as a potential candidate for clinical applications in the diagnosis and therapy of WNV.

Materials and Methods

Cell lines, virus, and other reagents

Baby hamster kidney (BHK-21) cells and SP2/0 myeloma cells were cultured in RPMI-1640 medium (Hyclone, Beijing, China) supplemented with 10% fetal calf serum (PAA, Somerset, United Kingdom) and antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin). All cells were maintained in a humidified 5% CO₂ atmosphere at 37°C. Inactivated WNV (strain Chin-01) cell cultures and rabbit anti-WNV sera were kindly provided by Dr. Cheng-Feng Qin (Beijing Institute of Microbiology and Epidemiology, Beijing, China).

Expression and purification of prM protein

The recombinant plasmids were constructed and confirmed in our laboratory prior to transformation of *E. coli* ER2523 (NEB Express, Ipswich, MA) cells for protein expression. Recombinant protein was expressed according to a previously described procedure.⁽¹²⁾ Briefly, overnight cultures of the transformed cells were diluted 1:100 in 50 mL Luria-Bertani (LB) broth containing 100 µg/mL ampicillin at 37°C. When the OD₆₀₀ reached 0.6, isopropyl-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO) was added to the broth at a

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final concentration of 0.3 mM to induce protein expression and the cells were incubated at 16°C overnight. Subsequently, bacterial cells were removed from the growth medium by centrifugation at 5000 g for 15 min and lysed by sonication in cold phosphate-buffered saline (PBS, pH 7.4). Predominant expression of the recombinant prM protein in the maltose-binding protein (MBP) fusion protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) and Western blotting. The fusion protein was affinity-purified for use as an immunogen with an amylose resin column.

Generation, selection, and purification of monoclonal antibodies against prM

The purified recombinant protein was used as an immunogen in mice. Hybridomas secreting anti-prM antibodies were generated according to a previously described method.⁽¹³⁾ Female BALB/c mice (aged 5 weeks) were immunized subcutaneously with recombinant protein emulsified with Freund's complete adjuvant (Sigma, St. Louis, MO). Two booster injections were given at 2-week intervals with the same immunogen emulsified in an equal volume of Freund's incomplete adjuvant (Sigma). A final intraperitoneal booster immunization of soluble prM protein without adjuvant was administered 3 days before cell fusion. Three days after the final administration, the mice were euthanized humanely; spleen cells were harvested and subsequently fused with SP2/0 myeloma cells (5:1 ratio) using polyethylene glycol (PEG 4000, Sigma). The hybridoma cells were seeded into 96-well plates and selected in HAT medium (RPMI-1640 medium containing 10% fetal bovine serum, 100 mg/mL streptomycin, 100 IU/mL penicillin, 100 mM hypoxanthine, 16 mM thymidine, and 400 mM aminopterin). On days 3, 6, and 9, half of this medium was removed and replaced with fresh medium containing HAT. After HAT/HT medium selection, culture supernatants of surviving clones were screened for antibody reactivity and specificity by indirect enzyme-linked immunosorbent assay (ELISA). Positive clones were subcloned three times by the limiting dilution method. Selected clones were cultured in the peritoneal cavities of atollin-primed BALB/c mice to obtain ascitic fluid. MAb isotypes were determined with a mouse MAb isotyping kit according to the manufacturer's instructions (Hycult Biotechnology, Uden, The Netherlands).

Enzyme-linked immunosorbent assay

Ninety-six well microtiter plates were coated with purified fusion protein in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight and blocked with 5% skimmed milk for 2 h at 37°C. After washing three times with PBST (PBS plus 0.5% Tween-20), 100 µL of hybridoma supernatant containing MAbs were added to each well and incubated at 37°C for 1 h, followed by three washes with PBST. The bound MAbs were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Zhongshan Biotechnology, Beijing, China). After further incubation at 37°C for 1 h and thorough washing with PBST, 100 µL of 3,3'-5,5'-tetramethylbenzidine (TMB) was added as a substrate for HRP. The reaction was stopped after 10 min with 2 M H₂SO₄, and the absorbance was measured at 450 nm by an ELISA plate reader (Bio-Rad, Hercules, CA). Mannose-binding protein (MBP) was used as negative control.

Western blot analysis

Virus or prokaryotic expressed fusion proteins were subjected to electrophoresis on 12% SDS-PAGE after reduction with dithiothreitol (DTT) at 100°C for 5 min. The samples were then transferred to a nitrocellulose membrane. Non-specific antibody binding sites were blocked by immersion of the membrane in 5% skimmed milk in PBS overnight at 4°C. The membranes were incubated with MAbs as the primary antibody, and then washed five times with PBST and treated with IRDye 700-conjugated goat anti-mouse secondary antibodies (Licor Biosciences, Lincoln, NE). Blots were visualized by scanning the membranes with a Licor Odyssey infrared image system (Licor Biosciences).

Immunohistochemistry assays

The prM-E gene was cloned into the pCAGGS⁽¹⁴⁾ vector to generate expressing plasmid pCAGGS-WNV-ME and then transfected into BHK21 cells. BHK21 cells expressing prM/E proteins were cultured as monolayers in 24-well cell culture plates. Cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Then, cells were washed with PBS and permeabilized with 1% Triton X-100 at 4°C for 10 min, after which cells were blocked with 4% BSA in PBS at 37°C for 30 min. MAbs were added to the cells as the primary antibody and incubated at 37°C for 1 h. After washing with gentle shaking in PBS, cells were incubated with HRP-labeled goat anti-mouse IgG (Sigma) diluted 1:2000 in PBS at room temperature for 1 h. After washing three times as described previously, the enzyme reaction was developed with AEC (3-amino-9-ethylcarbazole). Finally, the cells were observed by microscopy (IMT2 Olympus, Tokyo, Japan).

Epitope mapping

To map the WNV prM protein epitope recognized by the MAb, a set of 20 partially overlapping short peptides (16 amino acids in length, with the exception of the last one,

TABLE 1. SEQUENCE OF OVERLAPPING PEPTIDES DERIVED FROM WNV prM PROTEIN

No. of peptide	Peptide sequence
prM-1	¹ VTLSNFQGVMMTVNA ¹⁶
prM-2	⁸ KVMMTVNATDVTDVIT ²⁴
prM-3	¹⁷ TDVTDVITIPTAAGKN ³²
prM-4	²⁵ IPTAAGKNLCIVRAMD ⁴⁰
prM-5	³³ LCIVRAMDVGYMCDT ⁴⁸
prM-6	⁴¹ VGYMCDTITYECPVL ⁵⁶
prM-7	⁴⁹ ITYECPVLSAGNDPED ⁶⁴
prM-8	⁵⁷ SAGNDPEDIDCWCTKS ⁷²
prM-9	⁶⁵ IDCWCTKSAVYVRYGR ⁸⁰
prM-10	⁷³ AVYVRYGRCTKTRHSR ⁸⁸
prM-11	⁸¹ CTKTRHSRRSRSLTV ⁹⁶
prM-12	⁸⁹ RSRRSLTVQTHGESTL ¹⁰⁴
prM-13	⁹⁷ QTHGESTLANKKGAWM ¹¹²
prM-14	¹⁰⁵ ANKKGAWMDSTKATRY ¹²⁰
prM-15	¹¹³ DSTKATRYLVKTESWI ¹²⁸
prM-16	¹²¹ LVKTESWILRNPGYAL ¹³⁶
prM-17	¹²⁹ LRNPGYALVAAVIGWM ¹⁴⁴
prM-18	¹³⁷ VAAVIGWMLGSNTMQR ¹⁵²
prM-19	¹⁴⁵ LGSNTMQRVVFFVLLL ¹⁶⁰
prM-20	¹⁵³ VVFFVLLLLLVAPAYS ¹⁶⁷

FIG. 1. Expression, purification, and Western blot analysis of WNV prM protein. The expressed MBP fused prM protein was analyzed by SDS-PAGE (**A**, **D**) and Western blot with rabbit anti-WNV sera (**E**) and MBP-specific MAb (**B**, **F**). In Western blots (**B**, **F**), the MBP-prM protein was probed with a commercial MAb against MBP. In Western blot (**E**), the MBP-prM and MBP control were probed with rabbit anti-WNV sera. M, protein marker; lanes 1 and 5, uninduced bacterial cell lysates; lanes 2, 6, 16, 18, and 20, induced bacterial cell lysates contain MBP-prM; lane 3, induced bacterial culture supernatant contain MBP-prM; lane 4, induced bacterial precipitate. The MBP-prM fusion protein was purified and analyzed by SDS-PAGE (**C**). Lane 7, unpurified supernatant; lanes 8–15, fractions of purified MBP-prM protein; lanes 17, 19, and 20, induced bacterial cell lysates contain MBP protein.

which was 15 amino acids) spanning the entire length of the prM protein were designed (Table 1) as described previously.⁽¹⁵⁾ All the fragments were fused with GST and expressed in the pGEX-6p-1 vector. The epitope peptides were screened by indirect ELISA using synthesized peptides as the coating antigen using the method described previously.

Results

Expression of recombinant prM protein

To detect the expression of WNV prM in *Escherichia coli*, the bacterial lysates were analyzed by SDS-PAGE and Western blotting. Results indicated that the recombinant protein was successfully expressed after IPTG induction and was predominantly expressed in a soluble form (Fig. 1A, D). Western blot analysis showed that the recombinant MBP-prM protein was recognized by a commercially available MAb against MBP (Fig. 1B, F). SDS-PAGE results showed that the fusion protein was successfully purified using an amylose resin column (Fig. 1C). The recombinant protein MBP-prM could be specifically recognized by rabbit anti-WNV sera but MBP protein was not (Fig. 1E).

Preparation of prM protein-specific MAb

Female BALB/c mice (aged 5 weeks) were immunized with the fusion protein as described in the Materials and

FIG. 2. Isotyping of monoclonal antibody 10F7. The isotype of MAb 10F7 was identified with mouse MAb isotyping kit. Developed dark point shows positive signal.

FIG. 3. Titration of MAb 10F7 by ELISA. Culture supernatants and ascites of MAb 10F7 were titrated by ELISA. Cell culture medium was used as negative control. Dilution titers of MAb 10F7 in culture supernatants and ascites were 2048 and over 409,600, respectively.

Methods section. After the third immunization, the mouse with the highest antibody titer to the immunogen was selected and a booster immunization was administered prior to cell fusion. After screening with ELISA, one positive clone was obtained. After three rounds of subcloning, one strain showing strong and specific reactivity to the prM protein was selected and designated as 10F7. Isotype investigations revealed that the MAb produced by this clone was identified as the IgG2a subtype, and the light chains were κ type (Fig. 2).

Biological activity of MAb 10F7

MAb dilution titers were evaluated by indirect ELISA. The antibody titer of the cell supernatant was 2048, while the titer of ascites fluid exceeded 409,600 (Fig. 3). The specificity and reactivity of the MAbs were further investigated by Western blot analysis using WNV-infected BHK-21 cells and MBP-prM protein expressed by *E. coli* ER2523. Western blot analysis suggested that MAb 10F7 reacted with WNV-infected cell lysates (Fig. 4A) and MBP-prM (Fig. 4C), demonstrating that the MAbs had good specificity and reactivity, and showed no cross-reactivity with JEV. The ability of specific MAbs to recognize the native prM protein in cells was investigated by immunohistochemistry analysis of BHK-21 cells transfected with the pCAGGS vector containing the prM gene. A dark red signal was detected, thus confirming that the MAb recognized the native prM protein (Fig. 5).

Epitope mapping of MAb 10F7

A set of overlapping synthesized peptides was successfully expressed (Fig. 6A) and used as coating antigens in ELISAs for mapping of the epitope recognized by MAb 10F7. Among these 20 peptides, MAb 10F7 reacted strongly with peptide prM-1 (VTLSNFQGKVMMTVNA) only (Fig. 6B). Western blot analysis showed that MAb 10F7 specifically recognized GST-prM-1 but not GST (Fig. 4D).

FIG. 4. Western blot analysis of MAb 10F7. Western blots showed that MAb 10F7 specifically recognized prM protein in lysates of cells infected with WNV (A, lane 1) and MBP-prM protein expressed in *E. coli* (lane 4) and GST-prM-1 (lane 8), while JEV prM specific MAb recognized prM of JEV specifically (B, lane 2). M, protein marker; lane 2, lysates of BHK-21 cells infected with JEV; lane 3, lysates of BHK-21 cells; lane 5, lysates of bacteria expressing MBP; lane 6, uninduced bacterial lysates; lane 7, lysates of bacteria expressing GST.

FIG. 5. Micrographs of immunoperoxidase-stained transfected cells. BHK-21 cells transfected with pCAGGS-WNV-ME (A) or untransfected (B) were immunoperoxidase-stained with MAb 10F7 and developed with AEC. Dark red cytoplasmic staining was the result of specific immunoreactivity with prM protein specific MAb 10F7.

Discussion

Following rapid expansion, WNV is now a major threat to human and animal health worldwide. In many areas, WNV co-exists with other flaviviruses, such as JEV in parts of India, St. Louis encephalitis virus (SLEV) on the American continent, and Murray Valley encephalitis virus (MVEV) in Australia.⁽¹⁶⁾ WNV shares some common features with these

other flaviviruses and shows cross-reactivity with these viruses in serological assays. These cross-reactive responses could confound the interpretation of serological testing. It is reported that only mature flavivirus particles are infectious.⁽¹⁷⁾ The cleavage of the prM protein by furin-like protease during virus release from infected cells is necessary for the maturation of flavivirus particles.⁽¹⁸⁾ However, the mechanism of this reaction and the functional role of prM in

FIG. 6. Peptide expression and epitope mapping. (A) SDS-PAGE analysis of peptides fusion expressed with GST. Lanes 1–20, lysates of bacteria expressing fusion proteins GST-prM-1 to GST-prM-20; lane 21, lysates of bacteria expressing GST. (B) Epitope mapping of MAb 10F7 against WNV prM protein by ELISA. Peptides prM-1 to prM-20 were expressed as GST fusions and screened by indirect ELISA. MBP-prM fusion protein was used as positive control; MBP and GST were used as negative controls, respectively. The values represent mean values of triplicates.

replication and virus infectivity have not yet been characterized in detail. Previous studies^(19,20) indicate that prM has relatively high antigenicity and human sera infected with dengue virus did not show cross-reaction with JEV prM in Western blot analyses and vice versa.⁽²¹⁾ These results indicate that prM and antibodies against prM may have potential use for conducting seroepidemiological studies of flavivirus infections in the regions that have prevalence of more than one flavivirus.

In this study, a prokaryotic expression system was used to generate a recombinant protein for use as an antigen to immunize mice. A WNV-prM protein-specific MAb (10F7) was generated using hybridoma technology. Immunohistochemistry assays showed that the MAb reacted with native prM protein expressed by transfected BHK-21 cells and epitope mapping showed that the MAb generated specifically recognized the pr-segment of WNV prM protein.

In conclusion, the MAb generated in this study may be useful for investigating the properties and functions of prM and the mechanism of the cleavage reaction in flaviviruses. Furthermore, this MAb represents a potential candidate for development of extensive applications in the diagnosis of WNV infections.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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