

Monoclonal Antibodies Against NS4B Protein of Japanese Encephalitis Virus

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Japanese encephalitis (JE) is one of the most prevalent global viral encephalitis viruses. The functions of JEV (virus) NS4B protein are still under investigation. In our study, NS4B was expressed in *Escherichia coli* and purified by dialysis. Two clones of monoclonal antibodies (MAbs 1B1 and 1C3) against NS4B protein were generated and their characterizations were investigated. IFA, Western blot, and ELISA results showed that the MAbs were specific against JEV NS4B protein. The epitope of the MAbs was further identified using pairs of synthesized overlapping peptides. These MAbs may provide valuable tools for further exploration of the functions of NS4B and the pathogenesis of Japanese encephalitis virus.

Introduction

JAPANESE ENCEPHALITIS VIRUS (JEV) is a member of the genus *Flavivirus* of the family *Flaviviridae*.⁽¹⁾ It is the cause of the most prevalent viral encephalitis that endangers Asia and the Oceania region.⁽²⁾ It is responsible for 30,000 to 50,000 cases and 10,000 deaths annually in eastern Asia.⁽³⁾ The virus is transmitted to vertebrates by mosquitoes, mainly by *Culex tritaeniorhynchus*.⁽⁴⁾ In human infected cases, children are susceptible to JEV, but less than 1% of humans result in JE.⁽⁵⁾ JE is also an epidemic disease in pigs, which can cause porcine reproductive disorder. The high morbidity and mortality of JE stunt the development of the animal industry. Even though incidences and detriments of JEV were reported before, the pathogenic mechanism of it is still under investigation.

JEV is a single-stranded, positive-sense RNA virus containing 11kb genome. The only open reading frame, which encodes a single polyprotein, is cleaved by viral and cellular proteases to form three structural (C, prM, and E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). It has been reported that non-structural proteins including NS4B of the KUN strain of West Nile virus (WNV) inhibited the IFN- α signal pathway.⁽⁶⁾ The transmembrane region of NS4B of hepatitis C virus (HCV) was recently shown to contain protein-protein interaction motifs, which is crucial for HCV genome replication.⁽⁷⁾ Nevertheless, the functions of NS4B and the protein-protein interactions involved in JEV replication are not yet well defined.

In this study, we generated two clones of MAbs, 1B1 and 1C3, against NS4B of JEV and identified the epitope of the MAbs to investigate whether it could contribute to our understanding of the functions of the NS4B protein. These re-

sults may play an important role in revealing the functions of the protein and be ideal material for clinical applications.⁽⁸⁾

Materials and Methods

Cloning, expression, and purification of recombinant protein NS4B

The NS4B gene fragments were amplified from JEV P3 strain (GenBank: U47032)-infected BHK cells by a one-step RT-PCR. Primer sequences for the target genes are listed in Table 1. The target fragments were then cloned into bacterial expression vector pGEX-KG. The recombinant plasmids pKG-4B and the negative control plasmid pGEX-KG were transformed into competent *Escherichia coli* BL21 cells and were then induced with isopropyl- β -thio-galactopyranoside (IPTG). After centrifugation, the bacteria pellet was suspended and sonicated until a clear lysate was obtained. The target proteins were purified by dialysis and stored at -80°C .⁽⁹⁾

Production of monoclonal antibody

Four adult female SPF BALB/c mice (4~6 weeks of age) were immunized intramuscularly with 50 μL of a 1:2 purified protein pKG-4B (20 μg per mouse) and **Quickantibody adjuvant (Kang Bi Quan Biotech, Beijing, China)** the first time and then boosted with the antigen in Quickantibody adjuvant at 2-week intervals. Before fusion, the mice were boosted with 40 μg of pKG-4B protein again. Three days later, mice splenocytes were harvested and fused with SP2/0 using 50% polyethylene glycol. The fused cells were cultured in HAT medium. Ten days later, the aminopterin was omitted and cells were cultured in HT medium. Hybridoma culture supernatants were screened by ELISA. The positive hybridoma

TABLE 1. PRIMER SEQUENCES

Gene	Primer orientation	Primer sequence (5'-3')
NS4B	Forward	CCGGATCCATGACACTTCACTATGGGTAC
	Reverse	GGCTCGAGTCACCTTTTCAAGGAGGGCTT
NS4B-A	Forward	GAGGATCCACACTTCACTATGGGTAC
	Reverse	CGCTCGAGTTAGACATTAGGGTTGAC
NS4B-B	Forward	TAGGATCCGTGGCAGCGTTTCTCGTC
	Reverse	TACTCGAGCCTTTTCAAGGAGGGC

lines were subcloned three times by limiting dilution method. The stable hybridoma clones (1×10^7 cells/mL) were injected intraperitoneally into BALB/c mice. Three days later, seroperitoneum was collected from the immunized mice. MAbs were purified by an antibody purification kit, according to the manufacturer's instructions.^(10,11)

Identification of MAb subtype

The subtype of the MAbs was identified using a subtype identification kit (Pierce Rapid ELISA Mouse MAb Isotyping Kit, Thermo Scientific, Boston, MA), according to the manufacturer's instructions.

Immunofluorescence assay

BHK-21 cells were cultured in glass-bottom cell culture dishes (NEST) and infected with JEV genotype 3 strain (P3 strain) at one multiplicity of infection (MOI). After post-infection, cells grew 60~70% confluent, were fixed with absolute methanol, and processed for indirect IFA using MAbs 1B1 and 1C3. Then, the secondary antibody, fluorescein isocyanate-conjugated goat anti-mouse IgG, was added. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). Fluorescent images were examined with a confocal fluorescent microscope.

Western blot analysis

MAbs were analyzed by Western blot analysis to determine their specificity. JEV-infected 293T cells were collected and separated by SDS-PAGE, then transferred to a nitrocellulose membrane. The membrane was blocked overnight with 1% bovine serum albumin (BSA) in TBST buffer (0.01 M Tris-HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween-20), then incubated with MAbs 1B1 and 1C3 (1:500 diluted in TBST) at 37°C for 1 h. The binding antibodies were detected by HRP-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL). After washing three times with TBST buffer, the protein bands were developed using an enhanced chemiluminescence system.

Epitope mapping of MAbs

To narrow the scope for epitope mapping, two mutant proteins pKG-4B (1-222 bp) and pKG-4B (193-411 bp) were generated; their gene fragments were amplified from JEV P3 strain (GenBank: U47032)-infected BHK cells by a one-step RT-PCR. Primer sequences for the target genes are listed in Table 1. The target fragments were then cloned into bacterial expression vector pGEX-KG. The recombinant plasmids pKG-4B (1-222 bp) and pKG-4B (193-411 bp) were transformed into competent *E. coli* BL21 cells and were then induced with IPTG. After centrifugation, the bacteria pellet was suspended and sonicated until a clear lysate was obtained. The target proteins were purified by dialysis and identified by SDS-PAGE (Fig. 1). The proteins were coated as antigen in epitope mapping ELISA assay. Based on the results of ELISA, a set of six partially overlapping in length of 15 amino acids (with the exception of the last one, which was 14 amino acids in length) short peptides covering 1-192 bp of the NS4B protein were synthesized. The epitope peptide was screened by ELISA using synthesized peptides as the coating antigen.

Results and Discussion

NS4B expression in Escherichia coli

Recombinant protein pKG-4B was produced in *E. coli* as inclusion bodies. A protein band of 41 kDa was detected in SDS-PAGE and Western blot analysis. The molecular

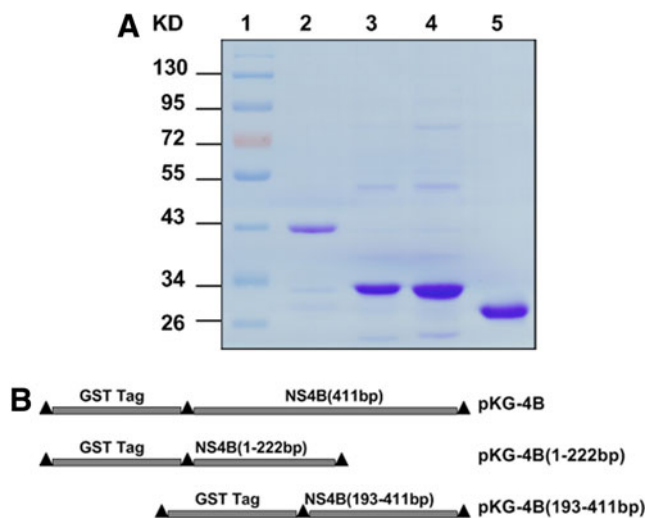


FIG. 1. SDS-PAGE analysis of recombinant proteins pKG-4B, pKG-4B (1-222 bp), and pKG-4B (193-411 bp). (A) Lane 1, protein marker; lane 2, bacilli precipitation of pKG-4B; lane 3, bacilli precipitation of pKG-4B (1-222 bp); lane 4, bacilli precipitation of pKG-4B (193-411 bp); lane 5, bacilli precipitation of pGEX-KG. (B) Construction of recombinant plasmids expressing full length or truncated forms of NS4B.

TABLE 2. DETECTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES

	1B1	1C3
MAb subclass	IgG2b	IgG2b
Light chain	κ	κ

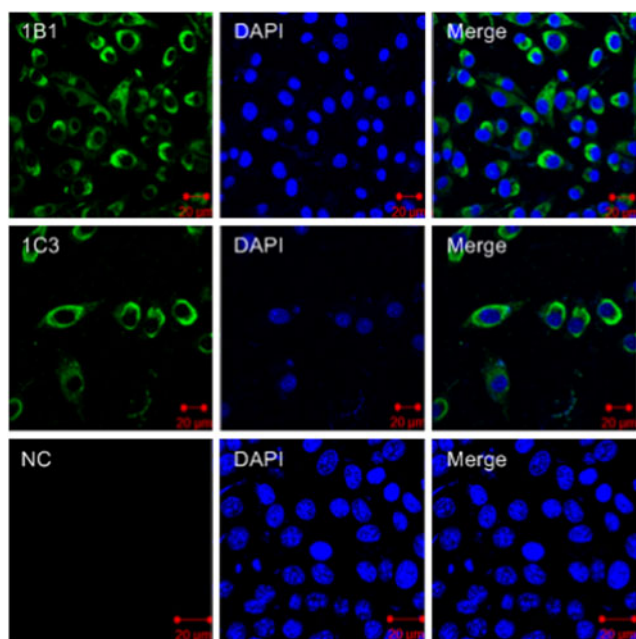


FIG. 2. Immunofluorescence staining (IFA) of JEV P3 strain-infected BHK-21 cells with different MAbs. Fluorescent images were examined with a confocal fluorescent microscope. 1B1 and 1C3 against NS4B, cell control; uninfected BHK-21 cells, control.

weight of pKG-4B protein corresponded to the fusion protein (Fig. 1).

Generation of MAbs against NS4B protein of JEV

Blood samples of mice were analyzed by indirect ELISA to monitor the specific antibody titer during immunization. After the third injection of antigen, the antisera collected from one immunized mouse displayed high binding affinity with coating antigen. Three days after the final booster injection, hybridoma technique was used to produce a JEV NS4B specific monoclonal antibody. Two clones of MAbs (1B1 and 1C3) against NS4B were eventually isolated through hybridoma fusion, and the cell line was injected into the abdomen of BALB/c mice for large-scale antibody production.⁽¹²⁾

Subtype identification of MAbs against NS4B

The subtype of the MAbs was identified by a rapid mouse MAb isotyping kit. The result showed that both MAbs of NS4B (1B1 and 1C3) belonged to the subtype IgG2b; the light chains of these MAbs were kappa (Table 2).

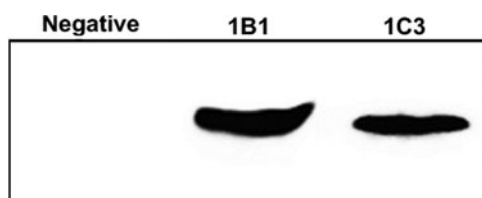


FIG. 3. Specificity of monoclonal antibodies against NS4B protein analyzed by Western blot analysis.

TABLE 3. EPITOPE MAPPING OF MAbs AGAINST JEV NS4B PROTEIN BY ELISA

Mutant proteins	Value of OD630 1B1	Value of OD630 1C3
pKG-4B(1–222 bp)	2.1753	2.0613
pKG-4B(193–411 bp)	0.2943	0.2675
Negative control	0.1107	0.1162

MAbs specifically recognize NS4B of Japanese encephalitis virus

We used two methods to confirm the specificity of the MAbs. On one hand, BHK-21 cells that were infected with JEV P3 strain were analyzed by immunofluorescence assay using MAbs 1B1 and 1C3 as the first antibody. Specific fluorescence signals were observed mainly in the cytoplasm. In contrast, the negative control cells were not detected in any fluorescence staining (Fig. 2). On the other hand, Western blot analysis also showed that MAbs 1B1 and 1C3 specifically reacted with the protein NS4B of JEV, while no reaction was observed with negative control cells (Fig. 3). In conclusion, both clones of MAbs showed high specificity to JEV.

Epitope mapping of MAbs against NS4B

The epitopes of MAbs 1B1 and 1C3 were narrowed by ELISA. Mutant proteins pKG-4B(1–222 bp) and pKG-4B(193–411 bp) were coated as antigen separately. ELISA results indicated that both clones of MAbs reacted with amino acids from 1–64 of NS4B (Table 3). Based on this result, a set of synthesized peptides were generated and used for further epitope mapping. ELISA results showed that MAbs 1B1 and 1C3 strongly reacted with the amino acids 21–35 (RRTAA-GIMKNAVVDG), but not the other five peptides (Table 4). Interestingly, the epitope amino acids GIMKN were consistent with other members of flavivirus, for instance, WNV and DENV. On this basis, we hypothesized that the MAbs would cross-react with NS4B of WNV and DENV. This result may lay the foundation for revealing the structure and functions of NS4B protein.

Conclusion

In this study, a prokaryotic expressed recombinant protein was used as antigen to immunize mice. Hybridoma technique

TABLE 4. EPITOPE SCREENING OF MAbs AGAINST NS4B PROTEIN OF JEV BY ELISA

Synthesized peptide	Value of OD630 1B1	Value of OD630 1C3
TLHYGYMLPGWQAEA	0.1712	0.1594
WQAEALRAAQRRRTAA	0.1530	0.1516
RRTAAGIMKNAVVDG	1.6178	1.8428
AVVDGMVATDVPELE	0.2644	0.1276
VPELERTTPLMQKKV	0.2303	0.1365
MQKKVGQVLLTGVS	0.0519	0.1099
Negative control (no peptide coating)	0.1523	0.1409

was used to produce JEV NS4B-specific monoclonal antibody and two clones, 1B1 and 1C3, were generated. The subtype of the MAbs was IgG2b. In indirect immunofluorescence assay, both clones of the MAbs showed high specificity to JEV and localized in cytoplasm. The specificity of MAbs was also proven by Western blot analysis. We further mapped the epitope of the MAbs by synthesized peptides. We believe that the MAbs would be important tools for illustrating the functions and characteristics of NS4B. These results also lay the foundation for further research of JEV pathogenic mechanisms.

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Author disclosure statement

The authors have no financial interests to disclose.

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