A Monoclonal Antibody Specific for Japanese Encephalitis Virus with High Neutralizing Capability for Inclusion as a Positive Control in Diagnostic Neutralization Tests

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Abstract. Japanese encephalitis virus (JEV) is the most common cause of viral encephalitis in Asia, and it is increasingly a global public health concern because of its recent geographic expansion. Although commercial vaccines are available and used in some endemic countries, JEV continues to cause illness, with more than 60,000 cases reported annually. To develop a reproducible positive control antibody useable in diagnosis of JEV infections, murine hybridomas were developed from mice inoculated with a combination of IXIARO JEV vaccine and JEV domain III of the envelope protein (E-DIII). Monoclonal antibodies (MAbs) were characterized for their ability to neutralize virus in vitro. Monoclonal antibody 17BD3-2 was found to be JEV specific and highly neutralizing, with a plaque reduction neutralization test (PRNT)₉₀ endpoint titer of 1.25 µg/mL. The functional epitopes were mapped using virus neutralization escape variants to amino acid residues S309, K312, and G333 in E-DIII. This MAb may be substituted for human immune sera used as a positive control in PRNT for distribution to public health laboratories worldwide in potential future outbreaks of JEV.

Japanese encephalitis virus (JEV) is a mosquito-borne virus from the genus *Flavivirus* in the family *Flaviviridae* found in southern and eastern Asia. With its increasing geographic distribution and emergence into Pakistan, western Indonesia, and northern Oceania, it has become a growing public health concern, with an estimated three billion people at risk of infection.^{1,2} The WHO estimates 67,900 cases of Japanese encephalitis (JE) are reported annually, making it the most common viral encephalitis in Southeast Asia. Of the cases reported, 25–30% result in death, whereas 50% result in permanent neurologic sequelae.³ Although several JEV vaccines are available, including inactivated whole virus formulations derived from infected mouse brains and cell culture, and live attenuated virus preparations, including chimeric viruses produced in vitro, outbreaks of JEV continue to expand.³

The WHO recommends testing suspected cases of JEV using acute and convalescent samples of cerebral spinal fluid and serum for the JEV-specific IgM antibody by ELISA, followed by confirmatory testing by plaque reduction neutralization test (PRNT).³ Because the suspected cases of JE in the United States come from travelers, JEV-reactive human sera for use in serological assays as positive controls are not readily available, requiring laboratories to rely on reagents produced in laboratory animals such as hyperimmune ascites and immune sera. Here, we describe a highly neutralizing JEV-specific monoclonal antibody (MAb) for use as a positive control in PRNT—substituting for human infection—immune sera. The new reagent can be readily produced and distributed to public health laboratories worldwide in potential future outbreaks.

Three 6-week-old BALB/c mice were inoculated intraperitoneally with 1 µg of inactivated Vero cell culture–derived JEV IXIARO vaccine (Valneva, Livingston, Scotland) on days 0 and 7, followed by a booster inoculation with 50 µg of purified domain III of the JEV SA14-14-2 envelope protein (E-DIII) coupled to maltose-binding protein (MBP) (a kind gift from Dr. Alan Barrett, University of Texas Medical branch,

Galveston, TX) with TiterMax adjuvant (Sigma-Aldrich, St. Louis, MO) on days 14, 28, and 35. Immunization of mice with inactivated virus followed by E-DIII has previously been shown to elicit a highly neutralizing antibody response to West Nile virus (WNV).⁴ Sera collected on days 21, 35, and 39 were assayed by ELISA, described previously, using either purified JEV vaccine strain SA14-14-2 at a concentration of 0.06 µg/well captured with rabbit immune sera diluted 1:1,000 or recombinant E-DIII derived from SA14-14-2 at a concentration of 30 ng/well as the antigen.⁵ On day 21 postinoculation, immunized mice had average reciprocal ELISA endpoint titers to virus and E-DIII of 2.7 log₁₀ and 3.3 log₁₀, respectively. These titers rose to virus on day 35–3.5 log₁₀, but slightly decreased to E-DIII to 3.003 log₁₀. By day 39, an average ELISA endpoint titer of 3.3 log₁₀ to virus remained stable, whereas the average ELISA endpoint titer to E-DIII decreased once again to 2.4 log₁₀ (Figure 1). The lower reactivity in ELISA to E-DIII is not surprising, given that Simmons et al.⁶ found lower antibody titers to E-DIII in ELISA from mice vaccinated with dengue virus type 2 (DENV2) E-DIII coupled to MBP than those vaccinated with E-DIII alone. However, higher neutralizing antibody titers were induced in mice vaccinated with DENV2 E-DIII/MBP than to E-DIII alone, indicating that the carrier protein presents the attached peptide in a native conformation for proper induction of neutralizing antibodies.⁶

On day 39, heterologous fusions of splenocytes harvested from immunized mice and SP2/0-Ag14 myeloma cells were performed.⁷ Hybridomas were grown in high-glucose Dulbecco's minimal essential medium supplemented with L-glutamine, 10% low IgG fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.15% sodium bicarbonate, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 0.1 mM nonessential amino acids. Fifteen hybridoma clones of the 144 clones screened produced an antibody reactive to JEV SA14-14-2 and E-DIII SA14-14-2. Of these, three clones with the highest reactivity to the native antigen were further characterized and tested for their ability to neutralize virus in vitro by PRNT using JEV SA14-14-2 previously described.⁸ Reciprocal endpoint titers were expressed as the concentration of the purified antibody that yielded 90% reduction in the number of plaques (PRNT₉₀). The hybridoma supernatant from clone 17BD3-2 had the highest neutralizing

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FIGURE 1. Reciprocal of geometric mean endpoint titers postimmunization measured in virion ELISA (A) and Japanese encephalitis virus-DIII ELISA (B). Standard error represents results obtained from three immunized mice.

capacity at a dilution of 1:32. Clone 17BD3-6 had a PRNT₉₀ endpoint titer dilution of 1:8, whereas 16AA-1 was unable to neutralize virus even at the lowest dilution of 1:2 (data not shown). Monoclonal antibodies 17BD3-2 and 17BD3-6, which were both found to be an IgG2a isotype, were purified from highly concentrated culture media using Protein A Sepharose (GE Healthcare, Pittsburgh, PA) according to the manufacturer's instructions. Purified MABs were dialyzed against phosphate buffered saline, and the concentration was measured by the Bradford protein assay (Bio-Rad, Hercules, CA).

These MABs were further characterized and compared with previously described anti-JEV MABs J3-14E6 and J3-14H5, which have a limited capability to neutralize virus in vitro.⁹ Viral protein specificity was determined by immunoblot using purified JEV SA14-14-2 and E-DIII SA14-14-2 with or without the reducing agent, dithiothreitol. All MABs reacted specifically with the E protein from purified virus and JEV E-DIII, and all MABs were reactive under reducing and nonreducing conditions, suggesting they recognized linear epitopes on the JEV E-DIII (data not shown). When tested in an ELISA using JEV SA14-14-2 directly coated on the plate at a concentration of 0.25 µg/well, 17BD3-2 had a reciprocal ELISA endpoint titer of 0.002 µg/mL, whereas J3-14E6 and J3-14H5 both had ELISA endpoint titers of 0.006 µg/mL. 17BD3-6 had the highest ELISA endpoint titer of 0.039 µg/mL. When tested in an ELISA with captured virus, 17BD3-2 had the lowest ELISA endpoint titer of 0.002 µg/mL, whereas J3-14H5, J3-14E6, and 17BD3-6 had higher ELISA endpoint titers of 0.391, 0.098, and 0.098 µg/mL, respectively (Table 1).

These clones were also tested for their specificity in a virus capture ELISA, using the DENV2 strain 16681 and the WNV strain NY99 35262 captured with rabbit immune sera homologous to the virus in the assay as described previously for

JEV.⁵ The MAB J3-14H5 reacted with WNV (endpoint titer 0.625 µg/mL) and DENV2 (endpoint titer 0.156 µg/mL) (Table 1). Previously, this MAB was shown to cross-react with WNV, DENV3, and DENV4 by an immunofluorescence assay, whereas J3-14E6 was shown to cross-react with WNV, Murray Valley encephalitis virus, Kunjin virus, and St. Louis encephalitis virus.⁹ When using JEV E-DIII as an antigen, J3-14H5 had the lowest ELISA endpoint titers of 0.019 µg/mL. Monoclonal antibodies 17BD3-6 and 17BD3-2 had ELISA endpoint titers of 0.625 and 5.02 µg/mL, respectively, whereas MAB J3-14E6 did not react with E-DIII by ELISA (Table 1).

In PRNT with JEV SA14-14-2, MAB 17BD3-2 had the highest neutralizing capacity with PRNT₅₀, PRNT₈₀, and PRNT₉₀ endpoint titers of 0.0049, 0.078, and 1.25 µg/mL, respectively. The MAB 17BD3-6 had PRNT₅₀, PRNT₈₀, and PRNT₉₀ endpoint titers of 0.313, 2.5, and 10 µg/mL, and J3-14H5 and J3-14E6 were only able to neutralize 50% of virus in vitro with endpoint titers of 0.313 and 0.078 µg/mL, respectively (Table 1). In addition, MAB 17BD3-2 was tested for its ability to neutralize JEVs of different genotypes. Plaque reduction neutralization test₅₀ endpoint titers of 0.3125, 0.078, and 0.0024 µg/mL were identified for JEV M884 (genotype I), JEV JKT5441 (genotype II), and JEV Nakayama (genotype III), respectively.

The dominant functional epitopes of 17BD3-2 were mapped by selecting for neutralization escape variant viruses.¹⁰ Varying amounts of purified 17BD3-2 (six replicates each of 50, 5, and 0.5 µg) were incubated with approximately 100 plaque forming units of plaque-purified JEV SA14-14-2 overnight at 4°C and then plated on Vero cells in six-well plates. Following incubation, plaques were cored and eluted overnight at 4°C in 0.5 mL of medium. The eluted virus was incubated again with 50 µg of 17BD3-2, followed by adsorption to Vero cells

TABLE 1
In vitro characteristics of JEV-reactive MABs measured by ELISA and PRNT

MAB	ELISA*					PRNT†		
	JEV	JEV E-DIII	Captured JEV	Captured WNV	Captured DENV2	50%	80%	90%
J3-14H5	0.006	0.019	0.391	0.625	0.156	0.313	> 10	> 10
J3-14E6	0.006	> 10	0.098	> 10	> 10	0.078	> 10	> 10
17BD3-2	0.002	5.02	0.002	> 10	> 10	0.0049	0.078	1.25
17BD3-6	0.039	0.625	0.098	> 10	> 10	0.313	2.5	10

DENV = dengue virus; E-DIII = domain III of the envelope protein; JEV = Japanese encephalitis virus; MAB = monoclonal antibody; PRNT = plaque reduction neutralization test; WNV = West Nile virus.

* Reciprocal ELISA endpoint titers expressed as µg/mL of purified MAB using JEV, recombinant JEV E-DIII, and captured JEV, WNV, and DENV2.

† Reciprocal PRNT endpoint titers of MAB expressed as concentration of the purified antibody (µg/mL) able to neutralize 50%, 80%, and 90% of JEV in vitro.

FIGURE 2. Japanese encephalitis virus (JEV) E protein. The image (Protein Data Bank: 3P54) shows a top view toward the viral surface of the JEV E protein. In the inset, the E protein functional and structural domains I, II, and III are shown in red, yellow, and blue, respectively. Epitopes important in monoclonal antibody binding are shown in space-filling representations: S309 (green), K312 (cyan), and G333 (magenta).

containing 25 µg MAb/mL in cell culture medium. The cells were monitored for cytopathic effect, and the supernatant was collected for RNA isolation and cDNA sequencing of the coding region of the structural genes of JEV SA14-14-2 described previously.¹¹ Four neutralization escape variants were isolated and sequenced. All variants were found to have an amino acid change from K to E at residue 312 on the A-strand of E-DIII. In addition, one variant had an amino acid change from S to L at residue 309 on the A-strand of E-DIII, whereas two variants had an amino acid change from G to A at 333 in the BC loop of E-DIII (Figure 2). Similar epitopes of JEV-neutralizing MAbs have been mapped. Fernandez et al.¹² mapped several JEV-neutralizing MAb epitopes to similar regions, including at amino acid residues D332, G333, and P334 in the BC-loop of E-DIII and amino acid residues F308 and F310 in the A-strand of E-DIII. Similar epitopes important in antibody binding and critical for viral fitness have been mapped at the amino acid residue G333 of JEV and on the A-strand and lateral ridge of DIII of DENV2 and WNV.^{13–18}

Reagents used in diagnostic serological assays that do not rely on human or animal material are preferred because these reagents may only be available in limited amounts, and MAbs that can be produced in cell culture are ideal for this application. The MAb 17BD3-2 described here will be a useful positive control in the PRNT and is available from the CDC for distribution to public health laboratories interested in expanding capacity to test for JEV. Humanization and expression of this MAb as a chimeric IgM antibody can be used as a virus-specific and highly reactive positive antibody control in other serological assays, including IgM antibody capture ELISA (MAC-ELISA), which will further increase the public health laboratory capacity for JEV detection and diagnosis.

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REFERENCES

1. Mackenzie JS, Williams DT, 2009. The zoonotic flaviviruses of southern, south-eastern and eastern Asia, and Australasia: the potential for emergent viruses. *Zoonoses Public Health* 56: 338–356.
2. van den Hurk AF, Ritchie SA, Mackenzie JS, 2009. Ecology and geographical expansion of Japanese encephalitis virus. *Annu Rev Entomol* 54: 17–35.
3. WHO, 2015. Japanese encephalitis vaccines: WHO position paper – February 2015. *Wkly Epidemiol Rec* 90: 69–87.
4. Zlatkovic J, Stiasny K, Heinz FX, 2011. Immunodominance and functional activities of antibody responses to inactivated West Nile virus and recombinant subunit vaccines in mice. *J Virol* 85: 1994–2003.
5. Calvert AE, Huang CY, Kinney RM, Roehrig JT, 2006. Non-structural proteins of dengue 2 virus offer limited protection to interferon-deficient mice after dengue 2 virus challenge. *J Gen Virol* 87: 339–346.
6. Simmons M, Nelson WM, Wu SJ, Hayes CG, 1998. Evaluation of the protective efficacy of a recombinant dengue envelope B domain fusion protein against dengue 2 virus infection in mice. *Am J Trop Med Hyg* 58: 655–662.
7. Kohler G, Milstein C, 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.
8. Huang CY, Silengo SJ, Whiteman MC, Kinney RM, 2005. Chimeric dengue 2 PDK-53/West Nile NY99 viruses retain the phenotypic attenuation markers of the candidate PDK-53 vaccine virus and protect mice against lethal challenge with West Nile virus. *J Virol* 79: 7300–7310.
9. Mason PW, Dalrymple JM, Gentry MK, McCown JM, Hoke CH, Burke DS, Fournier MJ, Mason TL, 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. *J Gen Virol* 70: 2037–2049.
10. Kinney RM, Chang GJ, Tsuchiya KR, Sneider JM, Roehrig JT, Woodward TM, Trent DW, 1993. Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein. *J Virol* 67: 1269–1277.
11. Calvert AE, Dixon KL, Delorey MJ, Blair CD, Roehrig JT, 2014. Development of a small animal peripheral challenge model of Japanese encephalitis virus using interferon deficient AG129 mice and the SA14-14-2 vaccine virus strain. *Vaccine* 32: 258–264.
12. Fernandez E et al., 2018. Mouse and human monoclonal antibodies protect against infection by multiple genotypes of Japanese encephalitis virus. *MBio* 9: e00008–e00018.

13. Oliphant T et al., 2005. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. *Nat Med* 11: 522–530.
14. Roehrig JT et al., 2013. Mutation of the dengue virus type 2 envelope protein heparan sulfate binding sites or the domain III lateral ridge blocks replication in Vero cells prior to membrane fusion. *Virology* 441: 114–125.
15. Brien JD, Austin SK, Sukupolvi-Petty S, O'Brien KM, Johnson S, Fremont DH, Diamond MS, 2010. Genotype-specific neutralization and protection by antibodies against dengue virus type 3. *J Virol* 84: 10630–10643.
16. Nybakken GE, Oliphant T, Johnson S, Burke S, Diamond MS, Fremont DH, 2005. Structural basis of West Nile virus neutralization by a therapeutic antibody. *Nature* 437: 764–769.
17. Sanchez MD, Pierson TC, McAllister D, Hanna SL, Puffer BA, Valentine LE, Murtadha MM, Hoxie JA, Doms RW, 2005. Characterization of neutralizing antibodies to West Nile virus. *Virology* 336: 70–82.
18. Cecilia D, Gould EA, 1991. Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. *Virology* 181: 70–77.