Cross-reactivity of human monoclonal antibodies generated with peripheral blood lymphocytes from dengue patients with *Japanese encephalitis virus*

**Background:** Hybridomas that produce human monoclonal antibodies (HuMAbs) against *Dengue virus* (DV) had been prepared previously using peripheral blood lymphocytes from patients with DV during the acute and convalescent phases of a secondary infection. Anti-DV envelope glycoprotein (E) 99 clones, anti-DV premembrane protein (prM) 8 clones, and anti-DV nonstructural protein 1 (NS1) 4 clones were derived from four acute-phase patients, and anti-DV E 2 clones, anti-DV prM 2 clones, and anti-DV NS1 8 clones were derived from five convalescent-phase patients.

**Methods and results:** In the present study, we examined whether these clones cross-reacted with *Japanese encephalitis virus* (JEV), which belongs to the same virus family. Forty-six of the above-described 99 (46/99) anti-E, 0/8 anti-prM, and 2/4 anti-NS1 HuMAbs from acute-phase, and 0/2 anti-E, 0/2 anti-prM, and 5/8 anti-NS1 HuMAbs from convalescent-phase showed neutralizing activity against JEV. Thus, most of the anti-E and anti-NS1 (but not the anti-prM) antibodies cross-reacted with JEV and neutralized this virus. Interestingly, 3/46 anti-E HuMAbs derived from acute-phase patients and 3/5 anti-NS1 HuMAbs from convalescent-phase patients showed particularly high neutralizing activity against JEV. Consequently, the HuMAbs showing neutralization against JEV mostly consisted of two populations: one was HuMAbs recognizing DV E and showing neutralization activity against all four DV serotypes (complex-type) and the other was HuMAbs recognizing DV NS1 and showing subcomplex-type cross-reaction with DV.

**Conclusion:** Anti-DV E from acute phase (46/99) and anti-DV NS1 (7/12) indicate neutralizing activity against JEV. In particular, three of 46 anti-DV E clones from acute phase and three of five anti-NS1 clones from convalescent phase showed strong neutralizing activity against JEV.

**Keywords:** Dengue virus, Japanese encephalitis virus, viral neutralization, human monoclonal antibody, envelope, nonstructural protein 1

**Introduction**

*Dengue virus* (DV) encodes capsid protein (C), premembrane protein (prM), and envelope glycoprotein (E), in addition to seven nonstructural proteins (NS). There are four antigenically distinct serotypes (DV1–DV4), which share major antigens with each other and with other mosquito-borne and tick-borne flaviviruses, including *Japanese encephalitis virus* (JEV). DV and JEV are closely related, belonging to the same virus family, Flaviviridae. Both viruses are cocirculating in areas of Southeast Asia, including Thailand. Indeed, vaccination rates against JEV in Thailand are high, at 84% in 1998 and 98% in 2008. The immune response to a primary DV infection generates anti-DV neutralizing antibodies, which then protect against subsequent infection by the same serotype.
Materials and methods

Cell lines and viruses

Previously, 121 hybridomas were derived from dengue patients during the acute phase of a secondary DV infection and 15 were derived from patients during the convalescent phase. For the present study, Vero cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and maintained in a 5% CO₂ incubator at 37°C. The mosquito-derived cell line, C6/36, was cultured at 28°C in Leibovitz’s L-15 medium supplemented with 10% minimum essential medium and 0.3% tryptose phosphate broth. JEV (Nakayama strain) was cultured in C6/36 cells and the culture supernatants were used as viral stocks. Infectivity titers were estimated in Vero cells according to the number of focus-forming units, as previously described.

Immunofluorescence assay

Vero cells were plated in 96-well plates at a density of 2.5 × 10⁴ per well and either mock-infected or infected with JEV. After incubation for 16 hours, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline and permeabilized with 1% Triton X-100 in phosphate-buffered saline. Undiluted hybridoma culture fluid was used as a source of HuMAbs. Bound antibodies were visualized using Alexa Fluor 488-conjugated anti-human or anti-mouse IgG secondary antibody (1:1,000, Invitrogen, Carlsbad, CA, USA).

Viral neutralization assay

The viral neutralization assay was performed using undiluted hybridoma culture fluid as previously described. The fluid, or Dulbecco’s Modified Eagle’s Medium with 15% fetal bovine serum (as a negative control), was added with 100 focus-forming units of JEV (25 μL). After incubating for one hour, the mixture was used to infect Vero cells in a 96-well microplate. After inoculation at 37°C for 2 hours, 100 μL of minimum essential medium containing 3% fetal bovine serum was added to the wells. After a further overnight incubation at 37°C, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline and permeabilized with 1% Triton X-100 in phosphate-buffered saline. The plates were then stained with murine anti-E 4G2 monoclonal antibody at 4°C overnight for the immunofluorescence assay, as above. Bound antibodies were visualized using an Alexa Fluor 488-conjugated anti-mouse IgG (1:1,000; Invitrogen). All assays were performed in duplicate and the results were expressed as the mean. The viral neutralization assay

However, severe dengue infections often occur in patients who are secondarily infected with a different DV serotype. The reason for this may be that the second virus uses pre-existing anti-DV antibodies (raised during the primary infection) to gain entry to macrophages expressing Fc receptors, a process called antibody-dependent enhancement. Interestingly, most DV infections are asymptomatic, even in individuals who are secondarily infected with a heterotypic DV. However, in symptomatic cases, it can cause a wide spectrum, ranging from a mild illness, such as dengue fever, to severe illnesses, such as dengue hemorrhagic fever and dengue shock syndrome.

There have been several trials examining the clinical implications of prior exposure to JEV, or vaccination against JEV, which may increase the severity of subsequent DV infections. The results showed that neutralizing antibodies against JEV have both protective and detrimental effects upon subsequent DV infection.

Examination of the humoral immune status of DV-infected individuals, including dengue patients in the acute and convalescent phases of the secondary infection with heterotypic DV, may provide valuable information that will inform the development of anti-dengue vaccines. Previous reports showed that antibodies raised during primary infections were more type-specific, whereas those raised during secondary infections were more heterogeneous and wide-ranging in their ability to cross-react with heterotypes. Several groups have reported successful generation of hybridomas that produce anti-DV human monoclonal antibodies (HuMAbs), and all used peripheral blood mononuclear cells isolated from patients during the convalescent phases of primary and secondary infections. However, there are no reports of hybridomas being generated using peripheral blood mononuclear cells derived from the acute phase of a secondary DV infection. Information on the anti-DV antibodies derived from patients during the acute phase after secondary infection could be useful for understanding the mechanism(s) underlying dengue immunopathogenicity.

Recently, we reported the preparation of several hybridomas that secrete anti-DV HuMAbs by using peripheral blood mononuclear cells from dengue patients at the acute and convalescent phases of secondary infection with DV.

The aim of the present study was to investigate whether these dengue patient-derived HuMAbs showed neutralizing activity against JEV. The results showed that two populations of HuMAbs, anti-E from acute-phase patients and anti-NS1 from convalescent-phase patients, showed neutralizing activity against JEV at high rates.

Viral neutralization assay

The viral neutralization assay was performed using undiluted hybridoma culture fluid as previously described. The fluid, or Dulbecco’s Modified Eagle’s Medium with 15% fetal bovine serum (as a negative control), was added with 100 focus-forming units of JEV (25 μL). After incubating for one hour, the mixture was used to infect Vero cells in a 96-well microplate. After inoculation at 37°C for 2 hours, 100 μL of minimum essential medium containing 3% fetal bovine serum was added to the wells. After a further overnight incubation at 37°C, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline and permeabilized with 1% Triton X-100 in phosphate-buffered saline. The plates were then stained with murine anti-E 4G2 monoclonal antibody at 4°C overnight for the immunofluorescence assay, as above. Bound antibodies were visualized using an Alexa Fluor 488-conjugated anti-mouse IgG (1:1,000; Invitrogen). All assays were performed in duplicate and the results were expressed as the mean. The viral neutralization assay
activity of HuMAbs generated by the hybridoma clones was expressed as follows: $-\. <50\%; +\. 50\% to <80\%; or ++. \geq80\% reduction of focus-forming units compared with the negative control.

Western blot analysis
The JEV-infected Vero cells were suspended in loading buffer containing 2-mercaptoethanol, electrophoresed in 10\% sodium dodecyl sulfate polyacrylamide gels, then blotted onto polyvinylidene fluoride membranes. The blots were then incubated with undiluted hybridoma culture fluid at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for a further 2 hours at room temperature. The peroxidase reaction was visualized using ECL Plus Western Blotting Detection System (GE Healthcare UK Ltd, Little Chalfont, UK).

Results
Patient demographics
In total, peripheral blood mononuclear cell samples from nine secondarily infected dengue patients were used for anti-DV HuMAbs.\[26\] The peripheral blood mononuclear cells from D23, D32, and D33 (who had dengue fever), and D30 (who had dengue hemorrhagic fever) were obtained 6–8 days after the onset of fever (acute phase), and those from D25, D26, and D28 (who had dengue fever), and D22 and D27 (who had dengue hemorrhagic fever) were obtained between 12 and 15 days after the onset of fever (convalescent phase). The four acute-phase patients were all infected with DV2.

Cross-reactivity of HuMABs with JEV
A previous study examined the serologic activity of the HuMAbs against all four serotypes using immunofluorescence and viral neutralization assays\[26\]. The present study went on to examine possible cross-reactions with JEV-infected cells by immunofluorescence. The results are summarized in Table 1. The results from a DV2-derived viral protein expression system showed that 99, eight, and four of the 121 HuMAbs derived from four acute-phase patients recognized DV E, prM, and NS1, respectively, whereas two, two, and eight of 15 HuMAbs derived from five convalescent-phase patients recognized DV E, prM, and NS1, respectively.\[26\] As summarized in Table 2, immunofluorescence analysis showed that 93 of the above-described 99 (93/99) anti-E clones, 1/8 anti-prM clones, and 1/4 anti-NS1 clones (anti-NS1 D30-2B1g5 also showed a weak reaction with E) derived from acute-phase patients were cross-reactive with JEV, whereas 2/2 anti-E clones and 0/8 anti-prM clones were cross-reactive with JEV, and 1/8 anti-NS1 clones were cross-reactive with JEV (anti-NS1 D25-2B11G11 also showed a weak reaction with E and prM).

### Table 1 Patient disease status and the human monoclonal antibodies obtained in this study

| Dengue patient’s clinical phase for HuMAb preparation | Reaction with DV2 protein | Total HuMAb clone number | Patient (clone number) | Dengue patient-derived HuMAb clone number | IF with JEV | VN against JEV[a] |
|------------------------------------------------------|--------------------------|-------------------------|------------------------|------------------------------------------|-----------|------------------|
|                                                      | E                        | 99                      | D23 (56)               | -                                       | 5         | 51               |
|                                                      |                          |                         | D30 (23)               | +                                       | 0         | 23               |
|                                                      |                          |                         | D32 (5)                | +                                       | 1         | 4                |
|                                                      |                          |                         | D33 (15)               | ++                                      | 0         | 15               |
|                                                      | prM                      | 8                       | D23 (8)                | +                                       | 8         | 0                |
|                                                      |                          |                         | D23 (3)                | -                                       | 3         | 2                |
|                                                      |                          |                         | D30 (1)                | ++                                      | 0         | 1\[b\]          |
|                                                      | NS1                      | 4                       | D22 (1)                | +                                       | 0         | 1                |
|                                                      |                          |                         | D26 (1)                | +                                       | 0         | 1                |
|                                                      |                          |                         | D22 (1)                | -                                       | 1         | 0                |
|                                                      |                          |                         | D27 (1)                | ++                                      | 1         | 0                |
|                                                      | prM                      | 2                       | D25 (5)                | +                                       | 4\[c\]   | 1                |
|                                                      |                          |                         | D26 (1)                | +                                       | 1         | 0                |
|                                                      |                          |                         | D27 (1)                | +                                       | 1         | 0                |
|                                                      | NS1                      | 8                       | D28 (1)                | +                                       | 1         | 0                |

Notes: $-\. <50\%; +\. 50\% to <80\%; and ++. \geq80\% FFU reduction compared with the negative control; \[1\]HuMAb D30-2B1G5 also showed a weak reaction with DV2 E; \[2\]HuMAb D25-4D4F10 also shows a weak reaction with DV2 prM; \[3\]HuMAb D25-2B11G11 also shows a weak reaction with DV2 E and prM.

Abbreviations: DV, Dengue virus; E, envelope glycoprotein; HuMAb, human monoclonal antibody; prM, premembrane protein; NS1, nonstructural protein 1; IF, immunofluorescence; VN, viral neutralization; FFU, focus-forming units; JEV, Japanese encephalitis virus.
The results of the viral neutralization assay revealed that 46/99 anti-E, 0/8 anti-prM, and 2/4 anti-NS1 derived from acute-phase showed neutralizing activity against JEV, whereas 0/2 anti-E, 0/2 anti-prM, and 5/8 anti-NS1 derived from convalescent-phase showed neutralizing activity against JEV (Table 3). Interestingly, three anti-E HuMAbs derived from acute-phase and three anti-NS1 HuMAbs derived from convalescent-phase showed very high viral neutralization activity against JEV, as indicated by ++ (Table 1) and clone numbers on the right side in parentheses (Table 3). There were no viral neutralization-positive clones among HuMAbs recognizing prM, irrespective of their origins (acute or convalescent phase). The characteristics of the individual HuMAbs are summarized in Table S1. Of the three clones that reacted with DV NS1 but also showed a weak cross-reaction with E and/or prM in the immunofluorescence with 293T cells transfected with individual DV2 viral genes,26 D30-2B1G5 and D25-2B11G11, but not D25-4D4F10, showed neutralization against JEV.

Most of the HuMAb clones showing a positive reaction with JEV in immunofluorescence recognized DV E and showed a complex-type cross-reaction with all four serotypes (91/96 clones [95%], Table 2). Similarly, viral neutralization assay showed that 46/99 (46%) anti-E and 2/4 (50%) anti-NS1 HuMAbs from acute-phase showed neutralizing activity against JEV, as did 5/8 (63%) anti-NS1 HuMAbs from convalescent-phase (Table 3). Interestingly, 38/46 of the above anti-E (83%) were HuMAbs showing neutralization against all four serotypes (complex-type, Table 3). On the other hand, five clones (one from acute-phase and four from convalescent-phase) of anti-NS1 HuMAbs were positive for viral neutralization activity against JEV, without viral neutralization activity against all four DV (<50% against all four serotypes); whereas two clones (one from acute-phase and one from convalescent-phase) of anti-NS1 HuMAbs were positive for viral neutralization activity against JEV, with subcomplex-type viral neutralization activity against DV2, DV3, and DV4.

Figure 1 shows the staining of Vero cells infected with serotypes DV1 to DV4 and Vero cells infected with JEV by several HuMAb clones (anti-E, anti-prM, or anti-NS1, Figure 1A) and the percent reduction of viral replication in Vero cells incubated with the same HuMAb clones (Figure 1B).

In this study, hybridoma culture fluids were prepared under the same conditions. These culture fluids from individual hybridoma cells are also positive against DV by immunofluorescence. In addition, the same lots of individual culture fluids were used throughout the experiments in this study, such as immunofluorescence (against both DV and JEV), viral neutralization (against both DV and JEV), and western blotting test. The IgG concentration of the HuMAbs was not adjusted in this study. Therefore, there are some risks that some antibody may have low immunofluorescence, viral neutralization, or western blot signal, because it may be produced from hybridoma at a low concentration/amount.

**Cross-reactivity of HuMAbs with JEV as assessed by western blotting**

The cross-reactivity of HuMAbs with JEV antigens was also examined by western blotting. The results are summarized in Table 4. More than half of the anti-DV E HuMAbs were also reactive with JEV E on western blots. Also, most of the anti-DV NS1 HuMAbs were reactive with JEV NS1. In contrast, all of the anti-DV prM HuMAbs were not reactive with JEV prM.
Table 3 Cross-reactivity of Japanese encephalitis virus with human monoclonal antibodies derived from dengue patients as assessed by the viral neutralization assay

| DV serotype | HuMAbs positive against DV in the VN assay (positive against JEV in the VN assay) |
|-------------|---------------------------------------------------------------------------------|
|             | Derived from acute-phase patients | Derived from convalescent-phase patients |
|             | E | prM | NS1 | E | prM | NS1 |
| DV1 | DV2 | DV3 | DV4 | - | - | - | - | 5 (2/0)<sup>a</sup> | 5 (0/0) | 3 (1/0) | 1 (0/0) | 1 (0/0) | 6 (1/3) |
| + | - | + | - | - | 3 (1/0) | 1 (0/0) | 2 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) |
| - | - | - | + | - | 1 (0/0) | 2 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) |
| - | + | - | - | - | 1 (0/0) | 2 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) |
| + | + | + | + | - | 1 (0/0) | 2 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) |
| - | + | + | + | + | 1 (0/0) | 2 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) |
| + | + | + | + | + | 1 (0/0) | 2 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) |
| - | ++ | ++ | ++ | ++ | 1 (0/0) | 2 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) | 1 (0/0) |

Notes: <sup>a</sup>50%; <sup>b</sup>80% FFU reduction of DV1–DV4 replication compared with the negative control; <sup>c</sup>90% FFU reduction of DV1–DV4 replication compared with the positive control; <sup>d</sup>number of HuMAb clones reactive with JEV in the VN assay is shown in parentheses; number of clones showing ≥50 and <80% FFU reduction of JEV replication compared with the negative control/number of clones showing ≥80% FFU reduction of JEV replication compared with the negative control; <sup>e</sup>90% reduction of the replication of any DV serotype; <sup>f</sup>HuMAb D30-2B1G5 is also weakly reactive with DV2 E; <sup>g</sup>HuMAb D25-4D4F10 is also weakly reactive with DV2 E and prM; <sup>h</sup>HuMAb D25-2B11G11 is also weakly reactive with DV2 E and prM.

Abbreviations: DV, Dengue virus; E, envelope glycoprotein; huMAb, human monoclonal antibody; prM, premembrane protein; NS1, nonstructural protein 1; IF, immunofluorescence; VN, viral neutralization; FFU, focus-forming units; JEV, Japanese encephalitis virus.

JEV prM. Interestingly, three of the anti-DV E clones and two of the anti-DV NS1 clones that showed high neutralizing activity (+++) against JEV showed no apparent reaction with JEV on the western blots.

Discussion

Overall, hybridomas previously generated from peripheral blood mononuclear cells isolated from dengue patients secondarily infected with DV<sup>26,27</sup> were producers of HuMAbs cross-reactive with JEV-infected cells by immunofluorescence and showed high rates of neutralization against JEV by viral neutralization assay. The clones that cross-reacted with JEV and showed neutralizing activity against JEV were mainly classified into two groups: one contained clones that recognized DV E and were derived from acute-phase, and the other contained clones that recognized DV NS1 and were mostly derived from convalescent-phase. In contrast, none of the anti-DV prM clones showed neutralizing activity against JEV.

Of all the viruses that belong to the family Flaviviridae, JEV shows the closest antigenic relationship with West Nile virus, Murray Valley encephalitis virus, and St Louis encephalitis virus; JEV is much less antigenically related to DV<sup>8,31</sup>. However, the present study showed that most of the anti-DV E antibodies from hybridomas generated from peripheral blood mononuclear cells from DV patients during the acute phase showed complex-type cross-reactions and neutralizing activity against all four DV serotypes; indeed, not only do these show strong viral neutralization activity against DV2 (which was replicating in these patients), they are also strongly cross-reactive with other DV serotypes<sup>26</sup> and with JEV. These results showed there are common antigenic sites between DV E and JEV E, although a low antigenic relationship exists between DV and JEV. This supports previous reports of flavivirus
Figure 1 Staining of Vero cells infected with serotypes DV1 to DV4 and of Vero cells infected with Japanese encephalitis virus using several human monoclonal antibody clones (anti-E, anti-prM, or anti-NS1). (A) Anti-DV E (D23-1C2D2 and D23-1E12E8), anti-DV prM (D23-1D5G10), and anti-DV NS1 (D27-1E8A4 and D28-2B11F9) clones were incubated with Vero cells infected with Dengue virus serotypes 1-4 or with Vero cells infected with Japanese encephalitis virus. (B) Percent reduction of viral replication in Vero cells incubated with the same human monoclonal antibody clones.

Abbreviations: DV, Dengue virus; E, envelope glycoprotein; prM, premembrane protein; NS1, nonstructural protein 1.

Table 4 Reactivity of dengue patient-derived human monoclonal antibodies with Japanese encephalitis virus-infected cell lysate on western blots

| DV protein used as a target for dengue patient-derived HuMAbsa | Reactivity with JEV-infected cells on western blots | Dengue patient-derived HuMAb clone numbers showing a positive activity against JEV in the VN assayb |
|-------------------------------------------------------------|--------------------------------------------------|---------------------------------------------------------------|
|                                                            | −       | +      | ++        |
| E                                                          | 22      | 17     | 3         |
| prM                                                        | 33      | 26     | 0         |
| NS1                                                        | 4       | 3      | 2         |

Notes: aViral proteins targeted by the HuMAbs were identified using DV2 E, prM, and NS1 proteinsb, c, d, <50%; +, ≥50% and <80%; and ++, ≥80% FFU reduction of JEV replication compared with that observed for the negative control.

Abbreviations: DV, Dengue virus; E, envelope glycoprotein; HuMAb, human monoclonal antibody; prM, premembrane protein; NS1, nonstructural protein 1; VN, viral neutralization; JEV, Japanese encephalitis virus.
envelope glycoprotein cross-reactive epitopes analyzed by mouse monoclonal antibodies indicating that DV and JEV envelope proteins have sequence similarity and also share structure similarity in causing the cross-activity effects.\(^{12,33}\) In addition, anti-DV NS1 antibodies showing neutralizing activity against DV are 3/4 clones from acute-phase and 2/8 clones from convalescent-phase,\(^{26}\) and 2/4 clones from acute-phase and 5/8 clones from convalescent-phase also showed neutralizing activity against JEV in the present study. There has been no report about cross-reactivity in flaviviruses. However, the NS1 gene shares a high degree of homology.\(^{34}\) The results of the present study also showed that anti-prM HuMAbs (eight derived from acute-phase patients and two from convalescent-phase patients) had no neutralizing activity against JEV. This supports the findings of a previous study showing that anti-prM antibodies in sera from DV-infected or JEV-infected patients during the convalescent phase did not react with either DV or JEV on western blots.\(^{35}\) The present study showed that most of the anti-DV E and anti-DV NS1 HuMAbs derived from dengue patients during the acute phase of a secondary infection (around one week after the onset of illness) neutralized JEV, but none of the anti-DV prM HuMAbs derived from acute-phase and convalescent-phase patients neutralized JEV; this is despite the finding that anti-prM antibody responses are amplified after a secondary infection.\(^{36}\) A previous report described the generation of HuMAbs by transforming B cells derived from dengue patients (who were secondarily infected with DV) during the convalescent phase (15–24 days after defervescence). The results showed that 64% of anti-E, 7% of anti-NS1, and 3% of anti-prM antibodies were cross-reactive with JEV on western blots.\(^{34}\)

The E protein of flaviviruses is the principal antigen responsible for eliciting neutralizing antibody responses; however, neutralizing antibodies specific for the prM and NS1 proteins have also been detected.\(^{37–40}\) The anti-DV NS1 HuMAbs used in the present study were mostly derived from dengue patients during the convalescent phase of a secondary DV infection.\(^{26}\) Several anti-NS1 HuMAbs showed strong neutralizing activity against JEV, although those antibodies showed no apparent reactions with JEV-infected cells on immunofluorescence analysis (Table 1). A previous paper also reported several cases that were negative by immunofluorescence (anti-JEV IgG IIFT, Euroimmune, Lübeck, Germany), but positive by plaque reduction neutralization test.\(^{41}\) The present study examined three anti-NS1 clones that reacted with DV NS1, but also showed a weak cross-reaction with DV E and/or prM. Two of the three showed neutralizing activity against JEV. A previous study reported that all convalescent serum samples obtained from Japanese encephalitis patients contained anti-JEV NS1 IgG antibodies. Of these, 65% and 40% contained JEV NS1-specific IgM and IgA antibodies, respectively; also, these IgM and IgA antibodies did not cross-react with JEV.\(^{42}\) The anti-NS1 clones used in the present study (four clones obtained from acute-phase patients and eight from convalescent-phase patients) were all IgG antibodies.\(^{26}\)

No information regarding the JEV infection or vaccination status of patients from whom the HuMAbs were derived was available; therefore, the possibility that the HuMAbs might have (at least in part) originated from memory immune cells that were initially primed with JEV antigens after a natural JEV infection or an anti-JEV vaccination cannot be ruled out. However, the acute phase of the disease in patients secondarily infected with heterotypic DV may be due to IgG antibodies induced by the secondary infection. Thus, it may be possible that some of the HuMAbs used in the present study could be derived from immune cells that had been primed after exposure to JEV.

**Acknowledgments**

The authors thank P Singhasivanon for valuable discussions, and P Sawanpanyalert and J Boon-Long for coordinating the project. This study was partly supported by Japan Science and Technology Agency/Japan International Cooperation Agency (SATREPS, 08080924), the Founding Research Center for Emerging and Re-emerging Infectious Diseases Program, which was launched through a project commissioned by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Thailand Research Fund through the Royal Golden Jubilee PhD Program and Mahidol University (Grant PHD/0260/2550, to CP).

**Disclosure**

The authors report no conflicts of interest in this work.

**References**

1. Kuhn RJ, Zhang W, Rossmann MG, et al. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell*. 2002;108(5):717–725.
2. Innis BL, Thirawuth V, Hemachudha C. Identification of continuous epitopes of the envelope glycoprotein of dengue type 2 virus. *Am J Trop Med Hyg*. 1989;40(6):676–687.
3. Calisher CH, Karabatsos N, Dalrymple JM, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol*. 1989;70 Pt 1:37–43.
4. Makino Y, Tadano M, Saito M, et al. Studies on serological cross-reaction in sequential flavivirus infections. *Microbiol Immunol*. 1994;38(12):951–955.
15. Harris E, Videa E, Pérez L, et al. Clinical, epidemiologic, and virologic aspects of West Nile fever in Cuba. J Clin Virol 2010;47(1):24–26.

16. Sabin AB. Research on dengue during World War II. Am J Trop Med Hyg 1952;1(1):30–50.

17. Halstead SB, Chow J, Marchette NJ. Immunological enhancement of dengue virus replication. Nat New Biol 1973;243(122):24–26.

18. Hoke CH, Nisalak A, Sangawhipa N, et al. Protection against Japanese encephalitis virus neutralizing antibodies and increased symptomatic admissions for dengue to estimate the frequency of third or fourth dengue infection. Int J Infect Dis 2010;14(10):e888–e892.

19. Gibbons RV, Kalanaraoj S, Jarman RG, et al. Analysis of repeat hospital admissions for dengue to estimate the frequency of third or fourth dengue infections resulting in admissions and dengue hemorrhagic fever, and serotype sequences. Am J Trop Med Hyg 2007;77(5):910–913.

20. Anderson KB, Gibbons RV, Thomas SJ, et al. Pre-existing Japanese encephalitis virus neutralizing antibodies and increased symptomatic dengue illness in a school-based cohort in Thailand. PLoS Negl Trop Dis 2011;5(10):e1311.

21. Wahala WM, Kraus AA, Haymore LB, Accavitti-Loper MA, de Silva AM. Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody. Virology 2009;392(1):103–113.

22. Beltramello M, Williams KL, Simmons CP, et al. The human immune response to dengue virus is dominated by highly cross-reactive antibody -endowed with neutralizing and enhancing activity. Cell Host Microbe 2010;8(3):271–283.

23. Schieffelin JS, Costin JM, Nicholson CO, et al. Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived from a naturally infected patient. Virol J 2010;7:28.

24. De Alwis R, Beltanemello M, Messer WB, et al. In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection. PLoS Negl Trop Dis 2011;5(6):e1188.
### Table S1 Cross-reactivity of the individual dengue patient-derived human monoclonal antibodies with four dengue virus serotypes and with *Japanese encephalitis virus*

| Patient (acute or convalescent) | Hybridoma | IF reactivity | VN activity | Target protein determined by IF performed on cells expressing DV2 protein | Western blotting with JEV-infected cell lysates |
|-------------------------------|-----------|--------------|-------------|---------------------------------------------------------------------|-----------------------------------------------|
|                               | DV1       | DV2          | DV3         | DV4        | DV1<sup>a</sup> | DV2<sup>a</sup> | DV3<sup>a</sup> | DV4<sup>a</sup> | JEV<sup>b</sup> | prM | NS1 | E |
| D22 (convalescent)            | D22-1B7G2 | +            | +           | +          | +          | +          | +          | +          | +          | +   | +   | + |
|                               | D22-2G4E3 | +            | +           | +          | +          | +          | +          | +          | +          | +   | -   | - |
| D23 (acute)                   | D23-1A10H7| +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1A2B6 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1A5D7 | +            | +           | +          | +          | +          | +          | +          | +          | +   | -   | - |
|                               | D23-1A8H5 | +            | -           | +          | +          | -          | -          | -          | -          | +   | -   | - |
|                               | D23-1B10C7| +            | +           | +          | +          | -          | +          | +          | +          | +   | -   | - |
|                               | D23-1B1E5 | +            | +           | +          | +          | ++         | +          | ++         | ++         | +   | -   | - |
|                               | D23-1B3B9 | +            | +           | +          | +          | ++         | ++         | ++         | +          | +   | -   | - |
|                               | D23-1B8F7 | +            | +           | +          | +          | +          | -          | +          | +          | +   | -   | - |
|                               | D23-1C1G4 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1C2D2 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1C6F6 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1D4E2 | +            | +           | +          | +          | -          | +          | +          | +          | +   | -   | - |
|                               | D23-1D5G10| +            | +           | +          | +          | -          | -          | -          | -          | -   | +   | - |
|                               | D23-1E12E8| +            | +           | +          | +          | -          | ++         | +          | +          | +   | -   | - |
|                               | D23-1E3B1 | +            | +           | +          | +          | +          | ++         | +          | +          | +   | -   | - |
|                               | D23-1G1H8 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1G5C8 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1G7C2 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1H1C2 | +            | +           | +          | +          | -          | -          | -          | -          | -   | -   | - |
|                               | D23-1H5A11| +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-1A11B7| +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-2A5B5 | +            | +           | +          | +          | -          | -          | -          | -          | -   | +   | - |
|                               | D23-2A8D2 | +            | +           | +          | +          | -          | -          | -          | -          | +   | -   | - |
|                               | D23-2A8G5 | +            | +           | +          | +          | -          | -          | -          | -          | +   | NS1 | - |
|                               | D23-2C11G6| +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-2C3D8 | +            | +           | +          | +          | -          | -          | -          | -          | -   | -   | - |
|                               | D23-2D1C6 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-2E1D7 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-2F2B1 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-2G4E7 | +            | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-3A10G12| +           | +           | +          | +          | ++         | ++         | ++         | ++         | +   | -   | - |
|                               | D23-3A2F9 | +            | +           | +          | +          | -          | -          | -          | -          | -   | prM | - |

(Continued)
**Table S1 (Continued)**

| Patient (acute or convalescent) | Hybridoma | IF reactivity | VN activity | Target protein determined by IF performed on cells expressing DV2 protein | Western blotting with JEV-infected cell lysates |
|--------------------------------|-----------|---------------|-------------|--------------------------------------------------------------------------|-----------------------------------------------|
|                                | DV1       | DV2           | DV3         | DV4 | JEV | DV1^a | DV2^a | DV3^a | DV4^a | JEV^a |                                                |                                               |
| D23-3B2G9                      | +         | +             | +           | +   | ++  | +     | -     | -     | +     | E     |                                                | +                                             |
| D23-3C5A9                      | +         | +             | +           | -   | -   | -     | -     | -     | -     | prM   |                                                | -                                             |
| D23-3E6D7                      | +         | +             | -           | +   | ++  | +     | ++    | +     | +     | E     |                                                | +                                             |
| D23-4A11A2                     | +         | +             | +           | +   | ++  | +     | +     | +     | +     | E     |                                                | -                                             |
| D23-4A6F9                      | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | -                                             |
| D23-4B12C5                     | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-4B9H3                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-4C5D7                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-4D10E9                     | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | +                                             |
| D23-4D6F9                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | +                                             |
| D23-4D9G5                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | +                                             |
| D23-4E9A10                     | +         | +             | +           | +   | ++  | +     | +     | +     | +     | E     |                                                | -                                             |
| D23-4F10H7                     | +         | +             | +           | -   | -   | -     | -     | -     | -     | E     |                                                | -                                             |
| D23-4F5E1                      | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | +                                             |
| D23-4H12C8                     | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | +                                             |
| D23-5A2C10                     | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5A4E8                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5A7F6                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5A8B1                      | +         | +             | +           | -   | -   | -     | -     | +     | -     | prM   |                                                | -                                             |
| D23-5B5E1                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | +                                             |
| D23-5B9C9                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5C1G7                      | +         | +             | +           | +   | ++  | +     | +     | +     | +     | E     |                                                | -                                             |
| D23-5C3B2                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5C7G1                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5D6D1                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | nsI   |                                                | -                                             |
| D23-5E3G3                      | -         | +             | +           | -   | -   | +     | +     | +     | +     | E     |                                                | -                                             |
| D23-5E6B1                      | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | -                                             |
| D23-5E8B8                      | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | -                                             |
| D23-5F3B8                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5F4B3                      | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5G10G8                     | +         | +             | +           | +   | ++  | +     | +     | -     | -     | E     |                                                | -                                             |
| D23-5G2D2                      | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | +                                             |
| D23-5G8E3                      | +         | +             | +           | +   | ++  | ++    | +     | +     | +     | E     |                                                | +                                             |
| D25 (convalescent)             |           |               |             |     |     |       |       |       |       |       |                                                |                                               |
| D25-2B11C3                     | +         | +             | +           | -   | -   | -     | -     | -     | -     | NSI   |                                                | -                                             |
| D25-2B11G11                    | +         | +             | +           | +   | ++  | +     | +     | +     | +     | NSI, prM |                                                | -                                             |
| D25-4D3D2                      | +         | +             | -           | -   | -   | -     | -     | -     | -     | NSI   |                                                | -                                             |
| D25-4D4C3                      | +         | +             | -           | -   | -   | -     | -     | -     | -     | NSI   |                                                | -                                             |
| D25-4D4F10                     | +         | +             | +           | -   | +   | +     | +     | +     | +     | prM, NSI |                                                | +                                             |
| NS1 | E | pM | NS1 | E | NS1 | E | NS1 | E | NS1 | E | NS1 | E | NS1 | E | NS1 | E | NS1 | E | NS1 | E | NS1 | E | NS1 | E |
|-----|---|----|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|-----|---|
| +   | + |    | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + |
| +   | + |    | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + |
| +   | + |    | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + |
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| +   | + |    | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + |
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| +   | + |    | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + | +   | + |
Table S1 (Continued)

| Patient (acute or convalescent) | Hybridoma    | IF reactivity | VN activity | Target protein determined by IF performed on cells expressing DV2 protein | Western blotting with JEV-infected cell lysates |
|---------------------------------|--------------|---------------|-------------|--------------------------------------------------------------------------|-----------------------------------------------|
|                                 |              | DV1  | DV2  | DV3  | DV4  | JEV  | DV1<sup>a</sup> | DV2<sup>a</sup> | DV3<sup>a</sup> | DV4<sup>a</sup> | JEV<sup>a</sup> |                                                         |
|                                 | D33-3A4A6    | +    | +    | +    | +    | +    | -    | -    | -    | -    | -    | E                                                          | +                                                      |
|                                 | D33-3A5C7    | +    | +    | +    | +    | +    | -    | -    | +    | -    | -    | E                                                          | +                                                      |
|                                 | D33-3A7B4    | +    | +    | +    | +    | +    | +    | -    | +    | -    | -    | E                                                          | +                                                      |
|                                 | D33-3D12D4   | +    | +    | +    | +    | +    | +    | -    | -    | -    | -    | E                                                          | +                                                      |
|                                 | D33-3D8A9    | +    | +    | +    | +    | +    | +    | -    | +    | -    | -    | E                                                          | +                                                      |
|                                 | D33-3E4H10   | +    | +    | +    | +    | +    | +    | -    | +    | -    | -    | E                                                          | +                                                      |
|                                 | D33-3G12C3   | +    | +    | +    | +    | +    | +    | -    | +    | -    | -    | E                                                          | +                                                      |
|                                 | D33-4C10C10  | +    | +    | +    | +    | +    | +    | -    | +    | -    | -    | E                                                          | +                                                      |
|                                 | D33-5D12B11  | +    | +    | +    | +    | +    | +    | -    | +    | -    | -    | E                                                          | +                                                      |

**Notes:** <sup>a</sup>, <50%, +, 50%-90%, and ++, >90% FFU reduction compared with the negative control; <sup>b</sup>, <50%, +, 50%-90%, and ++, >80% FFU reduction compared with the negative control; <sup>c</sup>, in addition to NS1, the E protein was also weakly reactive with this HuMAb; <sup>d</sup>, in addition to NS1, E, and prM proteins were also weakly reactive with this HuMAb; <sup>e</sup>, in addition to NS1, the prM protein was also weakly reactive with this HuMAb.

**Abbreviations:** DV, Dengue virus; E, envelope glycoprotein; HuMAb, human monoclonal antibody; prM, premembrane protein; NS1, nonstructural protein 1; IF, immunofluorescence; VN, viral neutralization; FFU, focus-forming units; JEV, Japanese encephalitis virus.
Cross-reactivity of HuMAbs between Dengue virus and Japanese encephalitis virus