[DESCRIPTION]

[Title of Invention]
DENGUE-VIRUS SEROTYPE NEUTRALIZING ANTIBODIES

[Technical Field]
[0001]
The present invention relates to materials and methods for the treatment of dengue viral infections. The present invention relates to an anti dengue virus (DENV) monoclonal antibody or an antigen-binding fragment thereof, the monoclonal antibody or the antigen-binding fragment thereof comprising a neutralization activity against serotypes of DENV-1, DENV-2, DENV-3 and DENV-4, wherein the monoclonal antibody comprises a human monoclonal antibody or a humanized monoclonal antibody.

[0002]
RELATED APPLICATIONS
The present application claims the benefit of U.S. Provisional Patent Application Nos. 61/532,605, filed September 9, 2011, and 61/532,671, filed September 9, 2011, both of which are incorporated herein by reference in their entireties.

[0003]
GOVERNMENT FUNDING
The subject matter described herein was supported, at least in part, by the Japan Science and Technology Agency (JST)/Japan International Cooperation Agency (JICA) as part of the Science and Technology Research Partnership for Sustainable Development (SATREPS) and the program of the Founding Research Center for Emerging and Reemerging Infectious Diseases, which was launched through a project commissioned by the Ministry of Education, Cultures, Sports, Science, and Technology of Japan.

[Background Art]
[0004]
There are many infectious viral diseases lacking prophylactic vaccine and/or appropriate anti-viral drugs for the conventional therapies. Mosquito-borne dengue virus (DENV) infection occurs in tropical and subtropical regions around the world. An estimated 50 million cases of dengue infection occur worldwide each year. There

are four antigenically distinct dengue-virus serotypes (DENV-1 to DENV-4) sharing major antigens within the group, and with other mosquito and tick-borne flaviviruses. Innis et al., Am. J. Trop. Med. Hyg. 40:676-687 (1989); Calisher et al., J. Gen. Virol. 70: 37-43 (1989). The global spread of four dengue-virus serotypes (DENV-1 to DENV-4) has made dengue virus a major and growing public-health concern. Generally, pre-existing neutralizing antibodies from primary DENV infection play a significant role in providing protective neutralizing antibodies against infection with the same serotype. However, these prior antibodies mediate a non-protective response to subsequent heterotypic DENV infections and are hypothesized to enhance the course of disease, via antibody-dependent enhancement (ADE). DENV infections may be asymptomatic, even in secondary infections.

[0005]

When humans have multiple infections with the same virus, such as influenza virus and DENV, pre-existing memory cells producing specific antibodies could play a significant role in quickly providing neutralizing antibodies to protect against the current virus infection. In DENV, pre-existing neutralizing antibodies raised by the primary infection are protective against infection with the same DENV serotype. Severe dengue cases may mostly occur among patients secondarily infected with different DENV serotypes. This may be due to antibody-dependent enhancement (ADE), where part of the pre-existing anti-DENV antibodies raised by the primary DENV infection, by which the current infecting virus can be amplified in Fc receptor-positive macrophages. Sangkawibha et al., Am. J. Epidemiol. 120: 653-669 (1984); Halstead et al., J. Exp. Med. 146:201-217 (1977); Diamond et al, Immunol. Rev. 225:212-225 (2008). Most DENV infections may be asymptomatic, even among individuals secondarily infected with heterotypic DENV. Some of these cases may show a wide spectrum of clinical symptoms, from a mild dengue fever to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Harris et al., Am. J. Trop. Med. Hyg. 63:5-11(2000).

[0006]

No specific antiviral drug or vaccine has been approved for clinical use against DENV. Therefore, therapies against this disease are urgently needed. Passive immune therapy may be one approach to treating dengue, if effective neutralizing monoclonal antibodies (MAbs) to all serotypes of this virus can be developed, without ADE activity with any DENV serotypes. Several prophylactic and therapeutic trials have been

conducted using murine MAbs (Sukupolvi-Petty et al., J. Virol., 84:9227-9239 (2010).), and chimpanzee-derived humanized IgG1 antibody (Goncalvez et al., J. Virol. 78:12910-12918 (2004).). To obtain a useful candidate for an anti-DENV human MAb (HuMAb), a protocol for obtaining hybridoma clones efficiently is needed.

[0007]
HuMAbs are increasingly used in the treatment of cancer, and more recently, infectious diseases (Reichert et al., Nat. Biotechnol. 23:1073-1078 (2005)), such as RS virus (Frogel et al., J. Manag. Care Pharm. 16:46-58 (2010); Mansbach et al., Pediatr Emerg Care 23: 362-367 (2007)). HuMAbs can be produced by the immortalization of B cells with Epstein-Barr virus (EBV) (Kozbor, J. Immunol., 127:1275-1280 (1981); Steinitz et al., J. Immunol., 141: 3516-3522 (1988); Lanzavecchia et al., Curr. Opin. Biotechnol., 18:523-528 (2007)) or by fusion of B cells with an appropriate partner to produce hybridomas (Kozbor et al. Proc. Natl. Acad. Sci. USA, 79:6651-6655 (1982); Karpas et al., Proc. Natl. Acad. Sci. USA, 98:1799-1804(1998)).

[0008]
Such methods are very inefficient. Alternative strategies have been developed, including humanization of murine MAbs through protein engineering (Jones et al., Nature, 321:522-525(1986)), selection of antibodies from phage-display libraries of human antibody fragments (McCafferty et al., Nature 348:552-554 (1990)), and immunization of transgenic mice carrying human immunoglobulin loci, followed by the production of monoclonal antibodies using hybridoma technology (Green et al., J. Immunol. Methods, 231:11-23 (1999)). Nevertheless, the number of therapeutic antibodies against infectious agents remains limited (Lanzavecchia et al., Curr. Opin. Biotechnol., 18:523-528 (2007)).

[0009]
Many studies successfully developed HuMAbs using patients' memory B cells, against SARS-coronavirus (Traggiai et al, Nat Med 10: 871-875 (2004).), highly pathogenic avian influenza virus H5N1 (Simmons et al., PLoS Med 4:e178 (2007)), the seasonal influenza viruses H1N1 and H3N2 (Frank et al., Viral Immunol., 2:31-36 (1989)), and DENV (Schieffelin et al., Virol. J. 7:28 (2010), Dejnirattisai et al., Science 328:745-748 (2010); Beltramello et al., Cell. Host. Microbe 8:271-283 (2010), de Alwis et al., PLoS NTG 5:e1188 (2011)), via transformation with EBV. In addition, several studies have successfully prepared specific HuMAbs using PBMCs around 6-7 days' post-vaccination,

such as influenza virus (Wrammert et al., Nature 453:667-671 (2008).) or booster vaccinations (Lanzavecchia et al., Curr. Opin. Biotechnol., 18:523-528 (2007)). Several HuMAbs were prepared against seasonal influenza A viruses, H1N1 and H3N2, by fusion of PBMC from influenza-vaccinated volunteers with newly developed murine-human chimera fusion partner cells, named SPYMEG (Kubota-Koketsu et al., Biochem. Biophys. Res. Commun., 387:180-185 (2009)).

[0010]
Preparing HuMAbs through the immortalization of patient-derived B cells with EBV method, Dejnirattisai et al. (Science 328:745-748 (2010)) prepared anti-E and anti-prM HuMAbs using B cells from DENV-infected 7 patients on 15-24 days after defervescence. They also observed that 89% of anti-E HuMAbs were complex-type. However, they prepared more anti-prM than anti-E HuMAbs. The anti-prM HuMAbs were also highly cross-reactive with all 4 serotypes of DENV (94%) and potently promoted ADE.

[0011]
Also using the immortalization method, Bettramello et al. (Cell. Host. Microbe, 8:271-283 (2010)) used B cells from three primarily infected patients after 200 days to 8 or more years after infection and from two secondarily infected patients at 212 to 510 days after infection. Because the domain III of the E protein is the main target of neutralizing anti-flavivirus in mice (Oliphant et al., "J. Virol. 81:11828-11839 (2007).), they also performed a large screen to gain insights into a domain specificity and cross-reactivity of E domain III-specific antibodies isolated from 2 patients, one with a primary infection (donor 13) and the other with a secondary infection (donor 12). From donor 13, 18 of 152 HuMAbs were reactive with domain III, which consisted of 13 specific-type, 1 subcomplex-type, and 4 complex-type antibodies. From donor 12, 138 of 313 HuMAbs were reactive with domain III, which consisted of 44 specific-type, 31 subcomplex-type, and 63 complex-type antibodies.

[0012]
de Alwis et al. (PLoS NTG 5:e1188 (2011)) showed the preparation of anti-DENV HuMAbs using memory B-cells from 2 patients who had been confirmed to be infected with DENV 1, 8 years ago. Most of the HuMAbs obtained were weakly neutralizing and not bound to the E protein. Thus, the efficiency to prepare complex-type neutralized HuMAbs was significantly higher in secondary infection cases, as shown in Beltramello et al. (Cell. Host. Microbe 8:271-283 (2010)).

[0013]
Wrammert et al. (Nature 453:667-671 (2008)) found that rapid and robust influenza-specific IgG+ antibody-secreting plasma cells at peak levels approximately 7 days after vaccination. Those cells accounted for up to 6% of the peripheral blood B cells. However, influenza-specific IgG+ memory B cells at peak levels 14-21 days after vaccination, which accounted for average of 1% of all B cells. Generally, reports show a difference in the B cell phenotype between acute- and convalescent-phase patients of infectious diseases (Leyendeckers et al., Eur. J. Immunol. 29:1406-1417 (1999)). Schieffelin et al. (Virol. J. 7:28 (2010)) obtained complex-type human MAbs have been previously obtained.

[0014]
There remains a need for new therapies against Dengue fever.

[Citation List]
[Non Patent Literature]
[0015]
[NPL 1] Sukupolvi-Petty et al., J. Virol., 84:9227-9239 (2010)

[Summary of Invention]
[Technical Problem]
[0016]
Passive immune therapy as a treatment of dengue is provided by preparation of human monoclonal antibody (HuMAb) that shows strong neutralization to all serotypes (complex-type) . The present invention provides for the efficient preparation of hybridomas producing HuMAbs against DENV, using peripheral blood mononuclear cells (PBMCs) from patients with secondary infections. Hybridoma clones were efficiently prepared that produce robust HuMAb using the PBMCs from infant patients at acute phase of infection (around 1 week after the onset of illness).

[0017]
The efficiency of preparing hybridoma clones producing robust HuMAb using PBMCs from patients in the acute (around 1 week after onset of illness) and convalescent phases (around 2 weeks after onset of illness) was compared. Surprisingly, significantly higher efficiencies were obtained from patients in the acute phase of

infection (121 HuMAbs from 4 patients) than those in the convalescent phase (15 HuMAbs from 5 patients). Most HuMAbs from acute-phase infections were complex-type by immunofluorescence: 90.1% (109/121) from acute versus 46.7% (7/15) from convalescent phases. 57.9% (70/121) from the acute phase, and only 6.7% (1/15) from the convalescent phase showed significant complex-type neutralization activity.

[0018]
Unexpectedly, HuMAbs that neutralize all 4 serotypes of DENV were obtained efficiently when PBMCs from acute-phase patients were used, with 57.9% (70/121) from acute and only 6.7% (1/15) from convalescent phase. They showed 50% or more reduction in the proliferation of all 4 DENV serotypes. Collectively, the secondary virus infection can play a significant role as a boost stimulation of the memory cells, to transiently increase the number of antibody-secreting plasma cells in patients in the early phase of infection. These HuMAbs provide for the understanding of protective and pathogenic roles in patients, and enable the design of prophylactic and/or therapeutic antibodies against dengue.

[0019]
Many infectious viral diseases lack prophylactic vaccines and/or appropriate anti-viral drugs for conventional therapy. The 4 DENV serotypes are partially cross-reactive with each other; it is believed that this is related to the mechanism that induces severe cases of dengue in secondarily infected patients, antibody-dependent enhancement (ADE). In the present invention, PBMCs were sampled from patients with dengue infection, to prepare hybridomas producing neutralizing HuMAbs against all DENV serotypes. When the efficiency of obtaining neutralizing HuMAbs with PBMCs from patients in the acute and convalescent phases was compared, it was found that efficiency was much greater in the acute phase, although there was a slight tendency towards higher neutralizing antibody titers in plasma from the convalescent than acute phase. The HuMAbs described herein are useful for characterizing the molecular mechanisms to induce pathogenicity, including ADE in dengue infection, and for the identification of promising candidates for antibody therapeutics.

[0020]
It is therefore a feature of the present invention to provide materials and methods for treating dengue infections.

[0021]
Another feature of the present invention is to provide human monoclonal antibodies against all serotypes of dengue virus.

[0022]
A further feature of the present invention is to use human monoclonal antibodies to neutralize all dengue-virus serotypes using patients' peripheral blood lymphocytes.

[0023]
Additional features and advantages of the present invention will be set forth in part in the description that follows, and in part will be apparent from the description, or may be learned by practice of the present invention. The objectives and other advantages of the present invention will be realized and attained by means of the elements and combinations particularly pointed out in the description and appended claims.

[Solution to Problem]
[0024]
To achieve these and other advantages, and in accordance with the purposes of the present invention, as embodied and broadly described herein, the present invention relates to a
1. An anti dengue virus (DENV) monoclonal antibody or an antigen-binding fragment thereof, the monoclonal antibody or the antigen-binding fragment thereof comprising a neutralization activity against serotypes of DENV-1, DENV-2, DENV-3 and DENV-4, wherein the monoclonal antibody comprises a human monoclonal antibody or a humanized monoclonal antibody.
2. An antiDENV human monoclonal antibody according to item 1, wherein the human monoclonal antibody is produced by a hybridoma made by fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of DENV infection with a fusion partner cell capable of efficient cell fusion.
3. The antiDENV human monoclonal antibody according to item 2, wherein the fusion partner cell is a SPYMEG cell.
4. The anti-DENV monoclonal antibody or antigen-binding fragment thereof according to item 1 comprising an IgG, a Fab, a Fab', a F(ab')2, a scFv, or a dsFv.
5. An antidengue virus(DENV) monoclonal antibody or antigen-binding fragment thereof, comprising a heavy chain variable region and a light chain variable region any one of (a) to (gg):

(a)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:1 of CDR1, SEQ ID NO:2 of CDR2, and SEQ ID NO:3 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:100 of CDR1, SEQ ID NO:101 of CDR2, and SEQ ID NO:102 of CDR3;

(b)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:4 of CDR1, SEQ ID NO:5 of CDR2, and SEQ ID NO:6 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:103 of CDR1, SEQ ID NO:104 of CDR2, and SEQ ID NO:105 of CDR3;

(c)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:7 of CDR1, SEQ ID NO:8 of CDR2, and SEQ ID NO:9 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:106 of CDR1, SEQ ID NO:107 of CDR2, and SEQ ID NO:108 of CDR3;

(d)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:10 of CDR1, SEQ ID NO:11 of CDR2, and SEQ ID NO:12 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:109 of CDR1, SEQ ID NO:110 of CDR2, and SEQ ID NO:111 of CDR3;

(e)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:13 of CDR1, SEQ ID NO:14 of CDR2, and SEQ ID NO:15 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:112 of CDR1, SEQ ID NO:113 of CDR2, and SEQ ID NO:114 of CDR3;

(f)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:16 of CDR1, SEQ ID NO:17 of CDR2, and SEQ ID NO:18 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:115 of CDR1, SEQ ID NO:116 of CDR2, and SEQ ID NO:117 of CDR3;

(g)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:19 of CDR1, SEQ ID NO:20 of CDR2, and SEQ ID NO:21 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:118 of CDR1, SEQ ID NO:119 of CDR2, and SEQ ID NO:120 of CDR3;

(h)a first amino acid sequence of a complementarity-determining region (CDR) of the

heavy chain variable region comprising SEQ ID NO:22 of CDR1, SEQ ID NO:23 of CDR2, and SEQ ID NO:24 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:121 of CDR1, SEQ ID NO:122 of CDR2, and SEQ ID NO:123 of CDR3;

(i)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:25 of CDR1, SEQ ID NO:26 of CDR2, and SEQ ID NO:27 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:124 of CDR1, SEQ ID NO:125 of CDR2, and SEQ ID NO:126 of CDR3;

(j)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:28 of CDR1, SEQ ID NO:29 of CDR2, and SEQ ID NO:30 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:127 of CDR1, SEQ ID NO:128 of CDR2, and SEQ ID NO:129 of CDR3;

(k)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:31 of CDR1, SEQ ID NO:32 of CDR2, and SEQ ID NO:33 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:130 of CDR1, SEQ ID NO:131 of CDR2, and SEQ ID NO:132 of CDR3;

(l)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:34 of CDR1, SEQ ID NO:35 of CDR2, and SEQ ID NO:36 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:133 of CDR1, SEQ ID NO:134 of CDR2, and SEQ ID NO:135 of CDR3;

(m)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:37 of CDR1, SEQ ID NO:38 of CDR2, and SEQ ID NO:39 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:136 of CDR1, SEQ ID NO:137 of CDR2, and SEQ ID NO:138 of CDR3;

(n)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:40 of CDR1, SEQ ID NO:41 of CDR2, and SEQ ID NO:42 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:139 of CDR1, SEQ ID NO:140 of CDR2, and SEQ ID NO:141 of CDR3;

(o)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:43 of CDR1, SEQ ID NO:44 of

CDR2, and SEQ ID NO:45 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:142 of CDR1, SEQ ID NO:143 of CDR2, and SEQ ID NO:144 of CDR3;
(p)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:46 of CDR1, SEQ ID NO:47 of CDR2, and SEQ ID NO:48 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:145 of CDR1, SEQ ID NO:146 of CDR2, and SEQ ID NO:147 of CDR3;
(q)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:49 of CDR1, SEQ ID NO:50 of CDR2, and SEQ ID NO:51 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:148 of CDR1, SEQ ID NO:149 of CDR2, and SEQ ID NO:150 of CDR3;
(r)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:52 of CDR1, SEQ ID NO:53 of CDR2, and SEQ ID NO:54 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:151 of CDR1, SEQ ID NO:152 of CDR2, and SEQ ID NO:153 of CDR3;
(s)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:55 of CDR1, SEQ ID NO:56 of CDR2, and SEQ ID NO:57 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:154 of CDR1, SEQ ID NO:155 of CDR2, and SEQ ID NO:156 of CDR3;
(t)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:58 of CDR1, SEQ ID NO:59 of CDR2, and SEQ ID NO:60 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:157 of CDR1, SEQ ID NO:158 of CDR2, and SEQ ID NO:159 of CDR3;
(u)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:61 of CDR1, SEQ ID NO:62 of CDR2, and SEQ ID NO:63 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:160 of CDR1, SEQ ID NO:161 of CDR2, and SEQ ID NO:162 of CDR3;
(v)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:64 of CDR1, SEQ ID NO:65 of CDR2, and SEQ ID NO:66 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:163 of CDR1, SEQ ID NO:164 of CDR2, and SEQ ID NO:165 of CDR3;

(x)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:70 of CDR1, SEQ ID NO:71 of CDR2, and SEQ ID NO:72 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:169 of CDR1, SEQ ID NO:170 of CDR2, and SEQ ID NO:171 of CDR3;

(y)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:73 of CDR1, SEQ ID NO:74 of CDR2, and SEQ ID NO:75 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:172 of CDR1, SEQ ID NO:173 of CDR2, and SEQ ID NO:174 of CDR3;

(z)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:76 of CDR1, SEQ ID NO:77 of CDR2, and SEQ ID NO:78 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:175 of CDR1, SEQ ID NO:176 of CDR2, and SEQ ID NO:177 of CDR3;

(aa)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:79 of CDR1, SEQ ID NO:80 of CDR2, and SEQ ID NO:81 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:178 of CDR1, SEQ ID NO:179 of CDR2, and SEQ ID NO:180 of CDR3;

(bb)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:82 of CDR1, SEQ ID NO:83 of CDR2, and SEQ ID NO:84 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:181 of CDR1, SEQ ID NO:182 of CDR2, and SEQ ID NO:183 of CDR3;

(cc)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:85 of CDR1, SEQ ID NO:86 of CDR2, and SEQ ID NO:87 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:184 of CDR1, SEQ ID NO:185 of CDR2, and SEQ ID NO:186 of CDR3;

(dd)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:88 of CDR1, SEQ ID NO:89 of CDR2, and SEQ ID NO:90 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising

SEQ ID NO:187 of CDR1, SEQ ID NO:188 of CDR2, and SEQ ID NO:189 of CDR3;
(ee)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:91 of CDR1, SEQ ID NO:92 of CDR2, and SEQ ID NO:93 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:190 of CDR1, SEQ ID NO:191 of CDR2, and SEQ ID NO:192 of CDR3;
(ff)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:94 of CDR1, SEQ ID NO:95 of CDR2, and SEQ ID NO:96 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:193 of CDR1, SEQ ID NO:194 of CDR2, and SEQ ID NO:195 of CDR3;
(gg)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:97 of CDR1, SEQ ID NO:98 of CDR2, and SEQ ID NO:99 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:196 of CDR1, SEQ ID NO:197 of CDR2, and SEQ ID NO:198 of CDR3;
, wherein the monoclonal antibody comprises a human monoclonal antibody or a humanized monoclonal antibody.

6. The antiDENV human monoclonal antibody according to item 5 comprising an IgG, a Fab, a Fab', a F(ab')2, a scFv, or a dsFv.

7. A method for producing an antidengue virus (DENV) human monoclonal antibody comprising:

1)producing a hybridoma by fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of DENV infection with a fusion partner cell capable of efficient cell fusion;

2)obtaining an anti-DENV human monoclonal antibody from the hybridoma.

8. A method for producing an antiDENV human monoclonal antibody according to item 7, wherein the fusion partner cell is a SPYMEG cell.

9. A method for producing a hybridoma comprising fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of dengue virus (DENV) infection with a fusion partner cell capable of efficient cell fusion.

10. The method for producing hybridoma according to item 9, wherein the fusion partner cell is a SPYMEG cell.

11. The method for producing a hybridoma according to item 9, wherein an antiDENV human monoclonal antibody obtained from the hybridoma comprises a neutralization activity against serotypes of DENV-1, DENV-2, DENV-3 and DENV-4.

[0025]
It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide a further explanation of the present invention, as claimed.

[Advantageous Effects of Invention]
[0026]
We observed the perfect effect of HuMAb using D23-1G7C2 as a representative on the lethality by DENV injection using suckling mice. Further studies are needed to characterize their positive and negative effects in other in vivo evaluation systems, such as macaque monkeys, marmosets, and mice (Bernardo et al., Clin Vaccine Immunol 16:1829-1831 (2009); Zompi et al., Viruses 4:62-82 (2012); Sukupolvi-Petty et al., J Virol 84: 9227-9239 (2010); Omatsu et al., J Gen Virol 92: 2272-2280 (2011); Omatsu et al., J Med Primatol, in press (2012)) in order to apply them to the possible development of therapeutic antibody.

[Brief Description of Drawings]
[0027]
[Fig. 1]
FIG. 1 illustrates summary of HuMAbs and their cross-reactivity to DENV-1 to -4 in IF and VN assays. A total of 121 acute-phase HuMAbs and 15 convalescent-phase HuMAbs were examined for cross-reactivity and cross-neutralization against DENV serotypes in IF and VN assays, respectively. Culture fluids of HuMAb-producing hybridoma clones were used. The HuMAbs were classified as groups 1 to 10 and groups A to X according to their cross-reactivity against the four serotypes of DENV in IF assay and VN assay, respectively. Individual groups are shown by different colors. Vero cells individually infected with DENV-1 to -4 were used as target cells in these assays. The data from VN assay are shown by the degree of neutralization ("-", <50%; "+", 50% to <90%; and "++", 90% neutralization).

[Fig. 2]
FIG. 2 illustrates representation of the percentages of HuMAbs obtained from individual patients for their cross-reactivity with DENV-1 to -4 by IF and VN assays. A total of 121 acute-phase HuMAbs (75 from D23, 25 from D30, five from D32, and 16 from D33) and 15 convalescent-phase HuMAbs (four from D22, five from D25, two from

D26, two from D27, and two from D28) were examined for their cross-reactivity and cross-neutralization against four serotypes of DENV. Culture fluids from the HuMAb-producing individual hybridoma clones were used for these assays. The HuMAbs were classified into groups 1 to 10 and groups A to X according to their cross-reactivity with the four serotypes of DENV as assessed by IF assay and VN assay, respectively. Individual groups are shown by different colors. Vero cells individually infected with DENV-1 to -4 were used as target cells in these assays. The data from VN assays are shown by the degree of neutralization ("+", 50% to <90%; and "++", 90% neutralization).

[Fig. 3]

FIG. 3 shows staining profiles of HuMAbs by IF assay. The HuMAbs in the culture fluids of hybridoma clones producing DENV serotype-specific (D28-2B11D10 in group 1 and D23-4A7D6 in group 2), cross-reactive with two serotypes (D28-2B11F9 in group 3 and D23-1B11A5 in group 4), and cross-reactive with three serotypes (D25-4D3D2 in group 7 and D23-3E6D7 in group 8), and cross-reactive with all four serotypes (D22-1B7G2 in group 10) antibodies were used for IF. Vero cells mock-infected with PBS or individually infected with DENV-1 to -4 were used as target cells. As a positive control, 4G2 anti-flavivirus E mouse MAb was used.

[Fig. 4]

FIG. 4 shows a correlation between IF and VN results. A total of 121 acute-phase HuMAbs and 15 convalescent-phase HuMAbs are shown separately to highlight the correlation between IF and VN ("-", <50%; "+", 50% to <90%; and "++", 90% neutralization) according to their cross-reactivity with different DENV serotypes (groups 1 to 10 according to the IF assay and groups A to X according to the VN assay). Culture fluids of HuMAb-producing hybridoma clones were used. Individual groups are shown by different colors, as in Figure 1. Vero cells individually infected with DENV-1 to -4 were used as target cells in these assays.

[Fig. 5]

FIG. 5 shows binding limits of 18 HuMAbs to four serotypes of DENV by indirect IF assay. Vero cells in 96-well microplate were infected with DENV. After incubation for 2 days, the cells were fixed with formaldehyde, then reacted with the serial 10-fold dilutions of the purified individual HuMAbs (10.0 micrograms/ml). As the titer for the reactivity to individual serotypes of DENV by indirect IF assay, the final antibody

concentration showing positive reaction is shown in figure.

[Fig. 6]
FIG. 6 shows VN activity of 18 HuMAbs to four serotypes of DENV. Vero cells in 96-well microplate were infected with individual serotypes of DENV which had been incubated with serial 2-fold dilutions of the purified HuMAbs (25.0 micrograms/ml) at 37centigrade for 30 min, and incubated at 37 centigrade for overnight. Finally, the cells were fixed and infected cells were detected by IF assay with 4G2.

[Fig. 7-1]
FIGs. 7 show VN and ADE activities of 18 HuMAbs to DENV-2. For VN assay, Vero cells were infected with DENV2 (16681 strain) which had been incubated with serial 2-fold dilutions of the purified HuMAbs (25.0 micrograms/ml) at 37 centigrade for 30 min, and incubated at 37 centigrade for overnight. Finally, the cells were fixed and infected cells were detected by IF assay with 4G2. For ADE assay, THP-1 cells were infected with DENV-2 (16681 strain) which had been incubated with serial 10-fold dilutions of the purified HuMAbs (10.0 micrograms/ml) at 37 centigrade for 30 min, then incubated for 3 days. Total RNA was extracted from the infected cells and applied to one-step real-time PCR for estimating viral genome copies. Vertical green line indicates 1 microgram/ml of HuMAb in both figures for VN and ADE results. Horizontal red line in figures for ADE results indicates the basal level (x1) in the control (PBS).
FIG. 7-1 shows 4G2, 5E4, D23-1A10H7 and D23-1B3B9.

[Fig. 7-2]
FIG. 7-2 shows D23-1C2D2, D23-1G7C2, D23-1H5A11 and D23-3A10G2.

[Fig. 7-3]
FIG. 7-3 shows D23-4A6F9, D23-4F5E1, D23-4H12C8 and D23-5E6B1.

[Fig. 7-4]
FIG. 7-4 shows D23-5G2D2, D23-5G8E3, D23-1C1G4 and D30-1E7B8.

[Fig. 7-5]
FIG. 7-5 shows D30-3A1E2, D30-33B6C7, D32-2D1G5 and D32-2H8G1.

[Fig. 8]

FIG.8 shows ADE activities of representative two HuMAbs to four serotypes of DENV. For ADE assays, four serotypes of DENV (Mochizuki strain of DENV-1; 16681 strain of DENV-2; H87 strain of DENV-3; and H241 strain of DENV-4) were used as in Fig. 7. The infection was performed at an MOI of 0.05.

[Fig. 9]
FIG.9 shows IF binding activity of representative HuMAbs to clinical isolates of DENV. Representative HuMAbs, D23-1A10H7, D23-1B3B9, and D23-1G7C2 were examined for the detection limit by IF assay to clinical isolates DV1-1 to DV1-5, DV2-1 to DV2-5, DV3-1 to DV3-5, and DV4-1 to DV4-5.

[Fig. 10]
FIG.10 shows VN activity of representative HuMAbs to clinical isolates of DENV. Representative HuMAbs, D23-1A10H7, D23-1B3B9, and D23-1G7C2 were examined for VN50 assay to clinical isolates DV1-1 to DV1-5, DV2-1 to DV2-5, DV3-1 to DV3-5, and DV4-1 to DV4-5.

[Fig. 11]
FIG.11 shows the results of in vivo therapeutic efficiency of HuMAbs in suckling mice.

[Fig. 12]
FIG.12 shows examples of the pictures of Immunofluorescent assay of positive control, negative control, Hybridoma clones DMSc-17, DMSc-14 and DMSc-36.

[Fig. 13]
FIG.13 shows illustration of 22 clones showing VN reduction to DENV1, DENV2, DENV3, DENV4 higher than 80%. All clones showed VN to DENV1 and DENV2 higher than 90%. The 10 clones showed VN to DENV3 and 16 clones showed VN to DENV4 higher than 90%.

[Description of Embodiments]
[0028]
The accompanying drawings, which are incorporated into and constitute a part of the specification, illustrate specific embodiments of the present teachings, and taken in conjunction with the detailed description of the specific embodiments, serve to explain the principles of the present teachings.

[0029]
The present invention provides antibodies against dengue and methods of using the same to treat a dengue infection.

[0030]
1. An anti dengue virus (DENV) monoclonal antibody or an antigen-binding fragment thereof, the monoclonal antibody or the antigen-binding fragment thereof comprising a neutralization activity against serotypes of DENV-1, DENV-2, DENV-3 and DENV-4, wherein the monoclonal antibody comprises a human monoclonal antibody or a humanized monoclonal antibody.
2. An antiDENV human monoclonal antibody according to item 1, wherein the human monoclonal antibody is produced by a hybridoma made by fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of DENV infection with a fusion partner cell capable of efficient cell fusion.
3. The antiDENV human monoclonal antibody according to item 2, wherein the fusion partner cell is a SPYMEG cell.
4. The anti-DENV monoclonal antibody or antigen-binding fragment thereof according to item 1 comprising an IgG, a Fab, a Fab', a F(ab')2, a scFv, or a dsFv.
5. An antidengue virus(DENV) monoclonal antibody or antigen-binding fragment thereof, comprising a heavy chain variable region and a light chain variable region any one of (a) to (gg):
(a)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:1 of CDR1, SEQ ID NO:2 of CDR2, and SEQ ID NO:3 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:100 of CDR1, SEQ ID NO:101 of CDR2, and SEQ ID NO:102 of CDR3;
(b)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:4 of CDR1, SEQ ID NO:5 of CDR2, and SEQ ID NO:6 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:103 of CDR1, SEQ ID NO:104 of CDR2, and SEQ ID NO:105 of CDR3;
(c)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:7 of CDR1, SEQ ID NO:8 of CDR2, and SEQ ID NO:9 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising

SEQ ID NO:106 of CDR1, SEQ ID NO:107 of CDR2, and SEQ ID NO:108 of CDR3;
(d)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:10 of CDR1, SEQ ID NO:11 of CDR2, and SEQ ID NO:12 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:109 of CDR1, SEQ ID NO:110 of CDR2, and SEQ ID NO:111 of CDR3;
(e)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:13 of CDR1, SEQ ID NO:14 of CDR2, and SEQ ID NO:15 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:112 of CDR1, SEQ ID NO:113 of CDR2, and SEQ ID NO:114 of CDR3;
(f)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:16 of CDR1, SEQ ID NO:17 of CDR2, and SEQ ID NO:18 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:115 of CDR1, SEQ ID NO:116 of CDR2, and SEQ ID NO:117 of CDR3;
(g)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:19 of CDR1, SEQ ID NO:20 of CDR2, and SEQ ID NO:21 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:118 of CDR1, SEQ ID NO:119 of CDR2, and SEQ ID NO:120 of CDR3;
(h)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:22 of CDR1, SEQ ID NO:23 of CDR2, and SEQ ID NO:24 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:121 of CDR1, SEQ ID NO:122 of CDR2, and SEQ ID NO:123 of CDR3;
(i)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:25 of CDR1, SEQ ID NO:26 of CDR2, and SEQ ID NO:27 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:124 of CDR1, SEQ ID NO:125 of CDR2, and SEQ ID NO:126 of CDR3;
(j)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:28 of CDR1, SEQ ID NO:29 of CDR2, and SEQ ID NO:30 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:127 of CDR1, SEQ ID NO:128 of CDR2, and SEQ ID NO:129 of CDR3;

(k)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:31 of CDR1, SEQ ID NO:32 of CDR2, and SEQ ID NO:33 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:130 of CDR1, SEQ ID NO:131 of CDR2, and SEQ ID NO:132 of CDR3;
(l)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:34 of CDR1, SEQ ID NO:35 of CDR2, and SEQ ID NO:36 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:133 of CDR1, SEQ ID NO:134 of CDR2, and SEQ ID NO:135 of CDR3;
(m)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:37 of CDR1, SEQ ID NO:38 of CDR2, and SEQ ID NO:39 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:136 of CDR1, SEQ ID NO:137 of CDR2, and SEQ ID NO:138 of CDR3;
(n)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:40 of CDR1, SEQ ID NO:41 of CDR2, and SEQ ID NO:42 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:139 of CDR1, SEQ ID NO:140 of CDR2, and SEQ ID NO:141 of CDR3;
(o)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:43 of CDR1, SEQ ID NO:44 of CDR2, and SEQ ID NO:45 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:142 of CDR1, SEQ ID NO:143 of CDR2, and SEQ ID NO:144 of CDR3;
(p)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:46 of CDR1, SEQ ID NO:47 of CDR2, and SEQ ID NO:48 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:145 of CDR1, SEQ ID NO:146 of CDR2, and SEQ ID NO:147 of CDR3;
(q)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:49 of CDR1, SEQ ID NO:50 of CDR2, and SEQ ID NO:51 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:148 of CDR1, SEQ ID NO:149 of CDR2, and SEQ ID NO:150 of CDR3;
(r)a first amino acid sequence of a complementarity-determining region (CDR) of the

heavy chain variable region comprising SEQ ID NO:52 of CDR1, SEQ ID NO:53 of CDR2, and SEQ ID NO:54 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:151 of CDR1, SEQ ID NO:152 of CDR2, and SEQ ID NO:153 of CDR3;

(s)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:55 of CDR1, SEQ ID NO:56 of CDR2, and SEQ ID NO:57 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:154 of CDR1, SEQ ID NO:155 of CDR2, and SEQ ID NO:156 of CDR3;

(t)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:58 of CDR1, SEQ ID NO:59 of CDR2, and SEQ ID NO:60 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:157 of CDR1, SEQ ID NO:158 of CDR2, and SEQ ID NO:159 of CDR3;

(u)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:61 of CDR1, SEQ ID NO:62 of CDR2, and SEQ ID NO:63 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:160 of CDR1, SEQ ID NO:161 of CDR2, and SEQ ID NO:162 of CDR3;

(v)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:64 of CDR1, SEQ ID NO:65 of CDR2, and SEQ ID NO:66 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:163 of CDR1, SEQ ID NO:164 of CDR2, and SEQ ID NO:165 of CDR3;

(x)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:70 of CDR1, SEQ ID NO:71 of CDR2, and SEQ ID NO:72 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:169 of CDR1, SEQ ID NO:170 of CDR2, and SEQ ID NO:171 of CDR3;

(y)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:73 of CDR1, SEQ ID NO:74 of CDR2, and SEQ ID NO:75 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:172 of CDR1, SEQ ID NO:173 of CDR2, and SEQ ID NO:174 of CDR3;

(z)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:76 of CDR1, SEQ ID NO:77 of

CDR2, and SEQ ID NO:78 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:175 of CDR1, SEQ ID NO:176 of CDR2, and SEQ ID NO:177 of CDR3;
(aa)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:79 of CDR1, SEQ ID NO:80 of CDR2, and SEQ ID NO:81 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:178 of CDR1, SEQ ID NO:179 of CDR2, and SEQ ID NO:180 of CDR3;
(bb)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:82 of CDR1, SEQ ID NO:83 of CDR2, and SEQ ID NO:84 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:181 of CDR1, SEQ ID NO:182 of CDR2, and SEQ ID NO:183 of CDR3;
(cc)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:85 of CDR1, SEQ ID NO:86 of CDR2, and SEQ ID NO:87 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:184 of CDR1, SEQ ID NO:185 of CDR2, and SEQ ID NO:186 of CDR3;
(dd)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:88 of CDR1, SEQ ID NO:89 of CDR2, and SEQ ID NO:90 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:187 of CDR1, SEQ ID NO:188 of CDR2, and SEQ ID NO:189 of CDR3;
(ee)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:91 of CDR1, SEQ ID NO:92 of CDR2, and SEQ ID NO:93 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:190 of CDR1, SEQ ID NO:191 of CDR2, and SEQ ID NO:192 of CDR3;
(ff)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:94 of CDR1, SEQ ID NO:95 of CDR2, and SEQ ID NO:96 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:193 of CDR1, SEQ ID NO:194 of CDR2, and SEQ ID NO:195 of CDR3;
(gg)a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:97 of CDR1, SEQ ID NO:98 of CDR2, and SEQ ID NO:99 of CDR3; and

a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:196 of CDR1, SEQ ID NO:197 of CDR2, and SEQ ID NO:198 of CDR3;
, wherein the monoclonal antibody comprises a human monoclonal antibody or a humanized monoclonal antibody.
6. The antiDENV human monoclonal antibody according to item 5 comprising an IgG, a Fab, a Fab', a F(ab')2, a scFv, or a dsFv.
7. A method for producing an antidengue virus (DENV) human monoclonal antibody comprising:
1)producing a hybridoma by fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of DENV infection with a fusion partner cell capable of efficient cell fusion;
2)obtaining an anti-DENV human monoclonal antibody from the hybridoma.
8. A method for producing an antiDENV human monoclonal antibody according to item 7, wherein the fusion partner cell is a SPYMEG cell.
9. A method for producing a hybridoma comprising fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of dengue virus (DENV) infection with a fusion partner cell capable of efficient cell fusion.
10. The method for producing hybridoma according to item 9, wherein the fusion partner cell is a SPYMEG cell.
11. The method for producing a hybridoma according to item 9, wherein an antiDENV human monoclonal antibody obtained from the hybridoma comprises a neutralization activity against serotypes of DENV-1, DENV-2, DENV-3 and DENV-4.

[0031]
Anti-dengue antibodies and polypeptides containing antigen binding fragments thereof are provided as well as methods, uses, compositions, and kits employing the same. A method of forming an antibody specific to a dengue or a polypeptide or a fragment thereof is provided. Such a method can contain providing a nucleic acid encoding a dengue antigen polypeptide or a polypeptide containing an immunologically specific epitope thereof; expressing the polypeptide containing the antigen amino acid sequence or a polypeptide containing an immunologically specific epitope thereof from the isolated nucleic acid; and generating an antibody specific to the polypeptide obtained or a polypeptide containing an antigen binding fragment thereof. An antibody or polypeptide containing an antigen binding fragment thereof produced by the aforementioned method is provided. An isolated antibody or isolated polypeptide containing an antigen binding fragment thereof that specifically binds a dengue antigen

is provided. Such an antibody can be generated using any acceptable method(s) known in the art. The antibodies as well as kits, methods, and/or other aspects of the present invention employing antibodies can include one or more of the following: a polyclonal antibody, a monoclonal antibody, a chimeric antibody, a single-chain antibody, a monovalent antibody, a diabody, and/or a humanized antibody.

[0032]
Naturally occurring antibody structural units typically contain a tetramer. Each such tetramer can be composed of two identical pairs of polypeptide chains, each pair having one full-length light" (for example, about 25 kDa) and one full- length "heavy" chain (for example, about 50-70 kDa). The amino-terminal portion of each chain typically includes a variable region of about 100 to 110 or more amino acids that typically is responsible for antigen recognition. The carboxy-terminal portion of each chain typically defines a constant region that may be responsible for effector function. Human light chains are typically classified as kappa and lambda light chains. Heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. IgG has several subclasses, including, but not limited to, IgGl, IgG2, IgG3, and IgG4. IgM has subclasses including, but not limited to, IgMl and IgM2. IgA is similarly subdivided into subclasses including, but not limited to, IgAl and IgA2. In light and heavy chains, the variable and constant regions can be joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 or more amino acids. See, e.g., Fundamental Immunology Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N. Y. (1989)) (incorporated by reference in its entirety for all purposes). The variable regions of each light/heavy chain pair typically form the antigen binding site.

[0033]
The variable regions typically exhibit the same general structure of relatively conserved framework regions (FR) joined by three hyper variable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair typically are aligned by the framework regions, which can enable binding to a specific epitope. From N-terminal to C-terminal, both light and heavy chain variable regions typically contain the domains FRl, CDRl, FR2, CDR2, FR3, CDR3 and FR4. The assignment of amino acids to each domain is typically in accordance with the definitions of Kabat Sequences of Proteins of Immunological Interest (National Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk J. MoI. Biol.

196:901-917 (1987); Chothia et al., Nature 342:878-883 (1989).

[0034]
"Antibody fragments" include a portion of an intact antibody, such as the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab1, F(ab')2, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng. 8(10): 1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen. "Fv" is an antibody fragment which contains a complete antigen-recognition and -binding site. This region includes a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. A single variable domain (or half of an Fv containing only three CDRs specific for an antigen) can recognize and bind an antigen. "Single-chain Fv" or "sFv" antibody fragments include the VH and VL domains of the antibody, wherein these domains are present in a single polypeptide chain. The Fv polypeptide can further contain a polypeptide linker between the VH and VL domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer- Verlag, New York, pp. 269-315 (1994).

[0035]
Antibodies can be used as probes, therapeutic treatments and other uses. Antibodies can be made by injecting mice, rabbits, goats, or other animals with the translated product or synthetic peptide fragments thereof. These antibodies are useful in diagnostic assays or as an active ingredient in a pharmaceutical composition.

[0036]
The antibody or polypeptide administered can be conjugated to a functional agent to form an immunoconjugate. The functional agent can be a cytotoxic agent such as a chemotherapeutic agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate), an antibiotic, a nucleolytic enzyme, or any combination thereof. Chemotherapeutic agents can be used in the generation of immunoconjugates, e.g.,

methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes, and/or fragments thereof, such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Enzymatically active toxins and fragments thereof that can be used include, for example, diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricotheeenes. Any appropriate radionucleotide or radioactive agent known in the art or are otherwise available can be used to produce radioconjugated antibodies.

[0037]

Conjugates of the antibody and cytotoxic agent can be made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol)propionate (SPDP); iminothiolane (IT); bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL); active esters (such as disuccinimidyl suberate); aldehydes (such as glutareldehyde); bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine); bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine); diisocyanates (such as tolyene 2,6-diisocyanate); bis-active fluorine compounds (such as l,5-difluoro-2,4-dinitrobenzene); maleimidocaproyl (MC); valine-citrulline, dipeptide site in protease cleavable linker (VC); 2-amino-5-ureido pentanoic acid PAB=p-aminobenzylcarbamoyl ("self immolative" portion of linker) (Citrulene); N-methyl-valine citrulline where the linker peptide bond has been modified to prevent its cleavage by cathepsin B (Me); maleimidocaproyl-polyethylene glycol, attached to antibody cysteines; N-Succinimidyl 4-(2-pyridylthio)pentanoate (SPP); and N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-l carboxylate (SMCC). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science, 238: 1098 (1987). Carbon-14-labeled l-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacctic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody, see WO 94/11026. The antibody can be conjugated to a "receptor" (such as streptavidin) for utilization in tumor pre-targeting wherein the antibody- receptor

conjugate is administered to the subject, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) that is conjugated to a cytotoxic agent (e.g., a radionucleotide).

[0038]
The antibodies of the present invention can be coupled directly or indirectly to a detectable marker by techniques well known in the art. A detectable marker is an agent detectable, for example, by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Useful detectable markers include, but are not limited to, fluorescent dyes, chemiluminescent compounds, radioisotopes, electron-dense reagents, enzymes, colored particles, biotin, or dioxigenin. A detectable marker often generates a measurable signal, such as radioactivity, fluorescent light, color, or enzyme activity. Antibodies conjugated to detectable agents may be used for diagnostic or therapeutic purposes. Examples of detectable agents include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance can be coupled or conjugated either directly to the antibody or indirectly, through an intermediate such as, for example, a linker known in the art, using techniques known in the art. See, e.g., U.S. Patent No. 4,741,900, describing the conjugation of metal ions to antibodies for diagnostic use. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, and acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferin, and aequorin.

[0039]
Antibodies useful in practicing the present invention can be prepared in laboratory animals or by recombinant DNA techniques using the following methods. Polyclonal antibodies can be raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the gene product molecule or fragment thereof in combination with an adjuvant such as Freund's adjuvant (complete or incomplete). To enhance immunogenicity, it can be useful to first conjugate the gene product molecule or a

fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl, etc. Alternatively, immunogenic conjugates can be produced recombinantly as fusion proteins.

[0040]
Animals can be immunized against the immunogenic conjugates or derivatives (such as a fragment containing the target amino acid sequence) by combining about 1 mg or about 1 microgram of conjugate (for rabbits or mice, respectively) with about 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. Approximately 7 to 14 days later, animals are bled and the serum is assayed for antibody titer. Animals are boosted with antigen repeatedly until the titer plateaus. The animal can be boosted with the same molecule or fragment thereof as was used for the initial immunization, but conjugated to a different protein and/or through a different cross-linking agent. In addition, aggregating agents such as alum can be used in the injections to enhance the immune response.

[0041]
The antibody administered can include a chimeric antibody. The antibody administered can include a humanized antibody. The antibody administered can include a completely humanized antibody. The antibodies can be humanized or partially humanized. Non-human antibodies can be humanized using any applicable method known in the art. A humanized antibody can be produced using a transgenic animal whose immune system has been partly or fully humanized. Any antibody or fragment thereof of the present invention can be partially or fully humanized. Chimeric antibodies can be produced using any known technique in the art. See, e.g., U.S. Patent Nos. 5,169,939; 5,750,078; 6,020,153; 6,420,113; 6,423,511; 6,632,927; and 6,800,738.

[0042]
The antibody administered can include a monoclonal antibody, that is, the anti-dengue antibodies of the present invention that can be monoclonal antibodies. Monoclonal antibodies can be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975). In a hybridoma method, a mouse,

hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes can be immunized in vitro. Monoclonal antibodies can be screened as are described, for example, in Harlow & Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Press, New York (1988); Goding, Monoclonal Antibodies, Principles and Practice (2d ed.) Academic Press, New York (1986). Monoclonal antibodies can be tested for specific immunoreactivity with a translated product and lack of immunoreactivity to the corresponding prototypical gene product.

[0043]
Monoclonal antibodies can be prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g., by fusion with myeloma cells. The clones are then screened for those expressing the desired antibody. The monoclonal antibody preferably does not cross-react with other gene products. After the desired hybridoma cells are identified, the clones can be subcloned by limiting dilution procedures and grown by standard methods. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells can be grown in vivo as ascites in a mammal. The monoclonal antibodies secreted by the subclones can be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0044]
The monoclonal antibodies can also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the present invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the present invention can serve as a preferred source of such DNA. Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also can be modified, for example, by substituting the coding sequence for human heavy and light

chain constant domains in place of the homologous murine sequences or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the present invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody. Preparation of antibodies using recombinant DNA methods such as the phagemid display method, can be accomplished using commercially available kits, as for example, the Recombinant Phagemid Antibody System available from Pharmacia (Uppsala, Sweden), or the SurfZAP™ phage display system (Stratagene Inc., La Jolla, Califorinia).

[0045]
Also included in the present invention are hybridoma cell lines, transformed B cell lines, and host cells that produce the monoclonal antibodies of the present invention; the progeny or derivatives of these hybridomas, transformed B cell lines, and host cells; and equivalent or similar hybridomas, transformed B cell lines, and host cells.

[0046]
The antibodies can be diabodies. The term "diabodies' refers to small antibody fragments with two antigen-binding sites, which fragments include a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (Vn-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains can be forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

[0047]
The antibody administered can include a single-chain antibody. The antibodies can be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain can be truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking. In vitro methods are also suitable for preparing monovalent antibodies. Digestion of

antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

[0048]
The antibodies can be bispecific. Bispecific antibodies that specifically bind to one protein and that specifically bind to other antigens relevant to pathology and/or treatment are produced, isolated, and tested using standard procedures that have been described in the literature. [See, e.g., Pluckthun & Pack, Immunotechnology, 3:83-105 (1997); Carter, et al., J. Hematotherapy, 4:463-470 (1995); Renner & Pfreundschuh, Immunological Reviews, 1995, No. 145, pp. 179-209; Pfreundschuh U.S. Patent No. 5,643,759; Segal, et al., J. Hematotherapy, 4:377-382 (1995); Segal, et al., Immunobiology, 185:390-402 (1992); and Bolhuis, et al., Cancer Immunol. Immunother., 34:1-8 (1991)].

[0049]
The antibodies disclosed herein can be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art. such as described in Epstein et al., Proc. Natl. Acad. Sci. USA, 82: 3688 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA 77: 4030 (1980); and U.S. Patent Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556. Particularly useful liposomes can be generated by the reverse-phase evaporation method with a lipid composition containing phosphatidylcholine, cholesterol, and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes can be extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., J. Biol. Chem., 257:286-288 (1982) via a disulfide-interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon et al, J. National Cancer Inst., 81(19): 1484 (1989).

[0050]
Two or more dengue antagonists can act synergistically to treat or reduce a dengue infection or a symptom of the same, for example, fever. A dengue antagonist can be one or more anti-dengue antibody alone or in combination with one or more other dengue antagonist, for example, a small drug pharmaceutical, or other anti-dengue therapy. Two or more anti-dengue antibodies, or at least one anti-dengue antibody and one or more additional therapies can act synergistically to treat or reduce the

susceptibility to the at least one inflammatory condition. Two or more therapies, including one or more anti-dengue antibody, can be administered in synergistic amounts. Accordingly, the administration of two or more therapies can have a synergistic effect on the decrease in one or more symptoms of a dengue infection, whether administered simultaneously, sequentially, or in any combination. A first therapy can increase the efficacy of a second therapy greater than if second therapy was employed alone, or a second therapy increases the efficacy of a first therapy, or both. The effect of administering two or more therapies can be such that the effect on decreasing one or more symptoms of a dengue infection is greater than the additive effect of each being administered alone. When given in synergistic amounts, one therapy can enhance the efficacy of one or more other therapy on the decrease in one or more symptoms of a dengue infection, even if the amount of one or more therapy alone would have no substantial effect on one or more symptom of a dengue infection. Measurements and calculations of synergism can be performed as described in Teicher, "Assays for In Vitro and In Vivo Synergy," in Methods in Molecular Medicine, vol. 85: Novel Anticancer Drug Protocols, pp. 297-321 (2003) and/or by calculating the combination index (CI) using CalcuSyn software.

[0051]
Exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. [See, e.g., Fingl et. al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. I.] The attending physician can determine when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician can also adjust treatment to higher levels if the clinical response were not adequate, precluding toxicity. The magnitude of an administrated dose in the management of disorder of interest will vary with the severity of the disorder to be treated and the route of administration. The severity of the disorder can, for example, be evaluated, in part, by standard prognostic evaluation methods. The dose and dose frequency, can vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above can be used in veterinary medicine.

[0052]
Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the present invention. With proper choice of

carrier and suitable manufacturing practice, the compositions relevant to the present invention, in particular, those formulated as solutions, can be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds relevant to the present invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, tablets, dragees, solutions, suspensions and the like, for oral ingestion by a patient to be treated.

[0053]
The therapeutic agent can be prepared in a depot form to allow for release into the body to which it is administered is controlled with respect to time and location within the body (see, for example, U.S. Patent No. 4,450,150). Depot forms of therapeutic agents can be, for example, an implantable composition containing the therapeutic agent and a porous or non-porous material, such as a polymer, wherein the therapeutic agent is encapsulated by or diffused throughout the material and/or degradation of the non-porous material. The depot is then implanted into the desired location within the body and the therapeutic agent is released from the implant at a predetermined rate.

[0054]
The therapeutic agent that is used in the present invention can be formed as a composition, such as a pharmaceutical composition containing a carrier and a therapeutic compound. Pharmaceutical compositions containing the therapeutic agent can include more than one therapeutic agent. The pharmaceutical composition can alternatively contain a therapeutic agent in combination with other pharmaceutically active agents or drugs.

[0055]
The carrier can be any suitable carrier. For example, the carrier can be a pharmaceutically acceptable carrier. With respect to pharmaceutical compositions, the carrier can be any of those conventionally used with consideration of chemico-physical considerations, such as solubility and lack of reactivity with the active compound(s), and by the route of administration. In addition to, or in the alternative to, the following described pharmaceutical compositions, the therapeutic compounds of the present inventive methods can be formulated as inclusion complexes, such as cyclodextrin inclusion complexes, or liposomes.

[0056]

The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, and diluents; are well-known to those skilled in the art and are readily available to the public. The pharmaceutically acceptable carrier can be chemically inert to the active agent(s) and one which has no detrimental side effects or toxicity under the conditions of use. The choice of carrier can be determined in part by the particular therapeutic agent, as well as by the particular method used to administer the therapeutic compound. There are a variety of suitable formulations of the pharmaceutical composition of the present invention. The following formulations for oral, aerosol, parenteral, subcutaneous, transdermal, transmucosal, intestinal, intramedullary injections, direct intraventricular, intravenous, intranasal, intraocular, intramuscular, intraarterial, intrathecal, intraperitoneal, rectal, and vaginal administration are exemplary and are in no way limiting. More than one route can be used to administer the therapeutic agent, and in some instances, a particular route can provide a more immediate and more effective response than another route. Depending on the specific disorder being treated, such agents can be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa. (1990).

[0057]

Formulations suitable for oral administration can include (a) liquid solutions, such as an effective amount of the inhibitor dissolved in diluents, such as water, saline, or orange juice; (b) capsules, sachets, tablets, lozenges, and troches, each containing a predetermined amount of the active ingredient, as solids or granules; (c) powders; (d) suspensions in an appropriate liquid; and (e) suitable emulsions. Liquid formulations can include diluents, such as water and alcohols, for example, ethanol, benzyl alcohol, and the polyethylene alcohols, either with or without the addition of a pharmaceutically acceptable surfactant. Capsule forms can be of the ordinary hard or soft shelled gelatin type containing, for example, surfactants, lubricants, and inert fillers, such as lactose, sucrose, calcium phosphate, and corn starch. Tablet forms can include one or more of lactose, sucrose, mannitol, corn starch, potato starch, alginic acid, microcrystalline cellulose, acacia, gelatin, guar gum, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, calcium stearate, zinc stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, disintegrating

agents, moistening agents, preservatives, flavoring agents, and other pharmacologically compatible excipients. Lozenge forms can contain the inhibitor in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles containing the inhibitor in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, gels, and the like containing, in addition to, such excipients as are known in the art.

[0058]
Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers can be added.

[0059]
The therapeutic agent, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They also can be formulated as pharmaceuticals for non-pressurized preparations, such as in a nebulizer or an atomizer. Such spray formulations also may be used to spray mucosa. Topical formulations are well known to those of skill in the art. Such formulations are particularly suitable in the context of the invention for application to the skin.

[0060]
Injectable formulations are in accordance with the present invention. The parameters for effective pharmaceutical carriers for injectable compositions are well-known to those of ordinary skill in the art [see, e.g., Pharmaceutics and Pharmacy Practice, J. B. Lippincott Company, Philadelphia, PA, Banker and Chalmers, eds., pages 238250 (1982), and ASHP Handbook on Injectable Drugs, Toissel, 4th ed., pages 622 630 (1986)]. For injection, the agents of the present invention can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[0061]
Formulations suitable for parenteral administration can include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The therapeutic agent can be administered in a physiologically acceptable diluent in a pharmaceutical carrier, such as a sterile liquid or mixture of liquids, including water, saline, aqueous dextrose and related sugar solutions, an alcohol, such as ethanol or hexadecyl alcohol, a glycol, such as propylene glycol or polyethylene glycol, poly(ethyleneglycol) 400, glycerol, dimethylsulfoxide, ketals such as 2,2-dimethyl-l,3- dioxolane-4-methanol, ethers, oils, fatty acids, fatty acid esters or glycerides, or acetylated fatty acid glycerides with or without the addition of a pharmaceutically acceptable surfactant, such as a soap or a detergent, suspending agent, such as pectin, carbomers, methylcellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agents and other pharmaceutical adjuvants.

[0062]
Oils, which can be used in parenteral formulations, include petroleum, animal, vegetable, or synthetic oils. Specific examples of oils include peanut, soybean, sesame, cottonseed, corn, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters.

[0063]
Suitable soaps for use in parenteral formulations include fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents include (a) cationic detergents such as, for example, dimethyl dialkyl ammonium halides, and alkyl pyridinium halides, (b) anionic detergents such as, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates, (c) nonionic detergents such as, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylenepolypropylene copolymers, (d) amphoteric detergents such as, for example, alkyl-beta-aminopropionates, and 2-alkyl-imidazoline quaternary ammonium salts, and (e) mixtures thereof.

[0064]
The parenteral formulations can contain from about 0.5% to about 25% by weight of the drug in solution. Preservatives and buffers can be used. In order to minimize or eliminate irritation at the site of injection, such compositions may contain one or more nonionic surfactants having a hydrophilic-lipophilic balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulations will typically range from about 5% to about 15% by weight. Suitable surfactants include polyethylene glycol sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol. The parenteral formulations can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid excipient, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

[0065]
The therapeutic agent can be made into suppositories by mixing with a variety of bases, such as emulsifying bases or water-soluble bases. Formulations suitable for vaginal administration can be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

[0066]
Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents can be encapsulated into liposomes. Liposomes are spherical lipid bilayers with aqueous interiors. Molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intra-cellularly. Materials and methods described for one aspect of the present invention can also be employed in other aspects of the present invention. For example, a material such a nucleic acid or antibody described for use in screening assays can also be employed as therapeutic agents and vice versa.

[0067]
Anti-dengue antibodies of the present invention can be administered to a subject before, during, and/or after diagnosing the patient as having a dengue infection. Dengue infection is caused by any one of four distinct but closely related dengue virus (DENV) serotypes (called DENV-1, -2, -3, and -4). These dengue viruses are single-stranded RNA viruses that belong to the family Flaviviridae and the genus Flavivirus-a family which includes other medically significant vector-borne viruses (for example, West Nile virus, Yellow Fever virus, Japanese Encephalitis virus, St. Louis Encephalitis virus, and the like). Dengue viruses are arboviruses (arthropod-borne virus) that are transmitted primarily to humans through the bite of an infected Aedes species mosquito. Transmission may also occur through transfusion of infected blood or transplantation of infected organs or tissues. Human transmission of dengue is also known to occur after occupational exposure in healthcare settings (for example, needle stick injuries) and cases of vertical transmission have been described in the literature (that is, transmission from a dengue infected pregnant mother to her fetus in utero or to her infant during labor and delivery).

[0068]
Infection with any of the four dengue serotypes can produce the full spectrum of illness and severity. The spectrum of illness can range from a mild, non-specific febrile syndrome to classic dengue fever (DF), to the severe forms of the disease, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Severe forms typically manifest after a two to seven day febrile phase and are often heralded by clinical and laboratory warning signs. Management of dengue can include timely and judicious use of supportive care, including administration of isotonic intravenous fluids or colloids, and close monitoring of vital signs and hemodynamic status, fluid balance, and hematologic parameters.

[0069]
DHF can usually be distinguished from DF as it progresses through its three predictable pathophysiological phases. A febrile phase can include viremia-driven high fevers. A critical/plasma leak phase can include sudden onset of varying degrees of plasma leak into the pleural and abdominal cavities. A convalescence or reabsorption phase can include a sudden arrest of plasma leak with concomitant reabsorption of extravasated plasma and fluids. The anti-dengue antibodies of the present invention

can be administered during any phase or combination of phases.

[0070]
Dengue infected patients are either asymptomatic or they can have one of three clinical presentations: undifferentiated fever, dengue fever with or without hemorrhage, or dengue hemorrhagic fever or dengue shock syndrome. As many as one half of all dengue infected individuals are asymptomatic, that is, they have no clinical signs or symptoms of disease.

[0071]
With undifferentiated fever, the first clinical course is a relatively benign scenario where the patient experiences fever with mild non-specific symptoms that can mimic any number of other acute febrile illnesses. They may not meet case definition criteria for DF. Dengue fever with or without hemorrhage patients are typically older children or adults and they can present within two to seven days of high fever (occasionally biphasic) and have two or more of the following symptoms: severe headache, retro-orbital eye pain, myalgias, arthralgias, a diffuse erythematous maculo-papular rash, and mild hemorrhagic manifestation. Subtle, minor epithelial hemorrhage, in the form of petechiae, are often found on the lower extremities (but may occur on buccal mucosa, hard and soft palates and or subconjunctivae as well), easy bruising on the skin, or the patient may have a positive tourniquet test. Other forms of hemorrhage such as epistaxis, gingival bleeding, gastrointestinal bleeding, or urogenital bleeding can also occur, but are rare. Leukopenia is frequently found and may be accompanied by varying degrees of thrombocytopenia. Children may also have nausea and vomiting. Patients with DF do not generally develop substantial plasma leak (as in DHF and DSS) or extensive clinical hemorrhage. Serological testing for anti-dengue IgM antibodies or molecular testing for dengue viral RNA or viral isolation can confirm the diagnosis. Clinical presentation of DF and the early phase of DHF are similar. With close monitoring of key indicators, the development of DHF can be detected at the time of defervescence so that early and appropriate therapy can be initiated.

[0072]
Dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS) constitutes another clinical presentation. There can be three phases of DHF: the febrile phase, the critical (plasma leak) phase, and the convalescent (reabsorption) phase. Early in the course of illness, patients with DHF can present much like DF, but they may also have

hepatomegaly without jaundice (later in the febrile phase). The hemorrhagic manifestations that occur in the early course of DHF most frequently consist of mild hemorrhagic manifestations as in DF. Less commonly, epistaxis, bleeding of the gums, or frank gastrointestinal bleeding occur while the patient is still febrile (gastrointestinal bleeding may commence at this point, but commonly does not become apparent until a melenic stool is passed much later in the course). Dengue viremia is typically highest in the first three to four days after onset of fever but then falls quickly to undetectable levels over the next few days. The level of viremia and fever usually follow each other closely, and anti-dengue IgM anti-bodies increase as fever abates.

[0073]
About the time when the fever abates, the patient enters a period of highest risk for developing the severe manifestations of plasma leak and hemorrhage. At this time, evidence of hemorrhage and plasma leak into the pleural and abdominal cavities can be monitored and appropriate therapies can be implemented replacing intravascular losses and stabilizing effective volume. If left untreated, this can lead to intravascular volume depletion and cardiovascular compromise. Evidence of plasma leak includes sudden increase in hematocrit (20% or more increase from baseline), presence of ascites, a new pleural effusion on lateral decubitus chest x-ray, or low serum albumin or protein for age and sex. Patients with plasma leak can be monitored for early changes in hemodynamic parameters consistent with compensated shock such as increased heart rate (tachycardia) for age especially in the absence of fever, weak and thready pulse, cool extremities, narrowing pulse pressure (systolic blood pressure minus diastolic blood pressure <20 mmHg), delayed capillary refill (>2 seconds), and decrease in urination (i.e., oliguria). Patients exhibiting signs of increasing intravascular depletion, impending or frank shock, or severe hemorrhage can be admitted to an appropriate level intensive care unit for monitoring and intravascular volume replacement. Once a patient experiences frank shock he or she can be categorized as having DSS. Prolonged shock is the main factor associated with complications that can lead to death including massive gastrointestinal hemorrhage. Interestingly, many patients with DHF/DSS remain alert and lucid throughout the course of the illness, even at the tipping point of profound shock.

[0074]
Anticipatory management and monitoring indicators can be used in effectively administering therapies as the patient enters the critical phase. New-onset leucopenia

(WBC <5,000 cells/mm3) with a lymphocytosis and an increase in atypical lymphocytes indicate that the fever will likely dissipate within the next 24 hours and that the patient is entering into the critical phase. Indicators that suggest the patient has already entered the critical phase include sudden change from high (>38.0 centigrade) to normal or subnormal temperatures, thrombocytopenia (100,000 or less cells/mm^3) with a rising or elevated hematocrit (20% or more increase from baseline), new hypoalbuminemia or hypocholesterolemia, new pleural effusion or ascites, and signs and symptoms of impending or frank shock.

[0075]
The critical period can last less than 24 to 48 hours. Most of the complications that arise during this period-such as hemorrhage and metabolic abnormalities (for example, hypocalcemia, hypoglycemia, hyperglycemia, lactic acidosis, and hyponatremia) are frequently related to prolonged shock. The principal objective during this period can be to prevent prolonged shock and support vital systems until plasma leak subsides. Careful attention can be paid to the type of intravenous fluid (or blood product if transfusion is needed) administered, the rate, and the volume received over time. Frequent monitoring of intravascular volume, vital organ function, and the patient's response can be performed. Monitoring for overt and occult hemorrhage can be performed. Transfusion of volume-replacing blood products can be implemented if substantial hemorrhage is suspected during this phase.

[0076]
The convalescent (reabsorption) phase can begin when the critical phase ends and is characterized when plasma leak stops and reabsorption begins. During this phase, fluids that leaked from the intravascular space (i.e., plasma and administered intravenous fluids) during the critical phase are reabsorbed. Indicators suggesting that the patient is entering the convalescent phase include sense of improved well-being reported by the patient, return of appetite, stabilizing vital signs (widen pulse pressure, strong palpable pulse), bradycardia, hematocrit levels returning to normal, increased urine output, and appearance of the characteristic convalescence rash of dengue (i.e., a confluent sometimes pruritic, petechial rash with multiple small round islands of unaffected skin). At this point, care can be taken to recognize signs indicating that the intravascular volume has stabilized (i.e., that plasma leak has halted) and that reabsorption has begun. Modifying the rate and volume of intravenous fluids (and often times discontinuing intravenous fluids altogether) to avoid fluid overload as the

extravasated fluids return to the intravascular compartment can be undertaken. Complications that arise during convalescent (reabsorption) phase are frequently related to the intravenous fluid management. Fluid overload may result from use of hypotonic intravenous fluids or over use or continued use of isotonic intravenous fluids during the convalescence phase.

[0077]
For the routine diagnosis of suspected dengue patients, an acute-phase serum specimen can be collected for serology at least 7 days before onset of fever and paired with convalescent serum drawn at least 7 days after the acute phase, optimally 14-21 days after onset of fever (Halstead, Annu. Rev. Entomol., 53:273-291 (2008); Kurosu et al., Biochem. Biophys. Res. Commun. 394:398-404 (2010)).

[Examples]
[0078]
The present invention will be further clarified by the following examples, which are intended to be exemplary of, but not limiting, the present invention.
[Example 1]
[0079]
This study is the first to report the significance of using of PBMCs from acute-phase patients for preparing complex-type neutralizing HuMAbs. In this study, blood specimens (about 10 ml in each) for cell fusion were collected from nine Thai dengue patients at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University (Tables 1). The participants were selected based on clinical diagnosis and the results of a rapid test with immunochromatography (SD BIOLINE Dengue Duo kit, SD, Kyonggi-do, Korea). Blood samples were also obtained from four acute-phase patients (around 1 week after onset of fever) and five convalescent-phase patients (around two weeks after onset of fever). The research protocols for human samples were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University.

[0080]
SPYMEG (Kubota-Koketsu et al., Biochem. Biophys. Res. Commun., 387:180-185 (2009)) was used as fusion partner cells, to develop hybridomas producing specific HuMAbs. SPYMEG cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37

centigrade. Vero cells were maintained in minimum essential medium (MEM) with 10% FBS in a 5% CO_2 incubator at 37 centigrade. Mosquito-derived cell line C6/36 was maintained in Leibovitz's L-15 medium with 10% FBS and 0.3% tryptose phosphate broth in an incubator at 28 centigrade.

[0081]

The DENVs used in this study were Mochizuki strain DENV-1, 16681 and New Guinea C (NGC) strains DENV-2, H87 strain DENV-3, and H241 strain DENV-4. The culture supernatants from C6/36 cells infected with individual strains were used as viral stocks. Virus titer was estimated as focus-forming unit (FFU), as described previously (Kurosu et al., Biochem. Biophys. Res. Commun. 394:398-404 (2010)).

[0082]

Reverse transcriptase (RT)-polymerase chain reaction (PCR) for DENV serotyping was performed as follows. Total RNA was extracted from patient plasma using a QIAamp Viral RNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. This RNA was used as the template for reverse transcription by SuperScript III cDNA synthesis kit (Invitrogen, Carlsbad, California). The oligonucleotide primer pairs previously reported for serotyping (Yenchitsomanus et al., Southeast Asian J. Trop. Med. Public Health, 27: 228-236 (1996)) were used for amplification of the DENV E gene, including most parts of domain III: the 1st PCR primers DEUL (5'-TGGCTGGTGCACAGACAATGGTT-3') SEQ ID NO: 199/ DEUR (5'-GCTGTGTCACCCAGAATGGCCAT-3') SEQ. ID NO. 200, that are common to all DENV serotypes and the 2nd PCR primers D1L (5'-GGGGCTTCAACATCCCAAGAG-3') SEQ ID NO: 201/ D1R (5'-GCTTAGTTTCAAAGCTTTTTCAC-3') SEQ. ID NO. 202, D2L (5'-ATCCAGATGTCATCAGGAAAC-3') SEQ ID NO: 203/ D2R (5'-CCGGCTCTACTCCTATGATG-3') SEQ. ID NO. 204, D3L (5'-CAATGTGCTTGAATACCTTTGT-3') SEQ ID NO: 205/ D3R (5'-GGACAGGCTCCTCCTTCTTG-3') SEQ. ID NO. 206, and D4L (5'-GGACAACAGTGGTGAAAGTCA-3') SEQ ID NO: 207/ D4R (5'-GGTTACACTGTTGGTATTCTCA-3') SEQ. ID NO. 208, which are specific to individual serotypes of DENV, DENV-1, -2, -3, and -4, respectively.

[0083]

Hybridomas were prepared as follows. About 10 milliliters of blood were obtained from individual patients and the PBMCs were prepared by centrifugation through

Ficoll-PaqueTM PLUS (GE Healthcare, Uppsala, Sweden) for 40 min at 1,700 rpm (520 g). The PBMCs were fused with SPYMEG cells at a ratio of 10:1 with polyethylene glycol 1500 (Roche Diagnostics Japan, Tokyo, Japan). Fused cells were cultured in DMEM supplemented with 15% FBS and 3% BM-condimed (Roche Diagnostics Japan, Tokyo, Japan) in a 96-well microplate for 10-14 days in the presence of hypoxanthine-aminopterin-thymidine (HAT). The first screening of the culture media for antibody specificity against DENV was performed by indirect immunofluorescence (IF) assay. Wells producing specific antibody were next subjected to cell cloning by limiting dilution. After 10-14 days, the second screening was also performed by IF assay.

[0084]

The purification of HuMAbs by protein G affinity column chromatography was performed as follows. HuMAbs were purified from hybridoma in a large-scaled culture of 1 liter using serum-free medium (Hybridoma SFM, Life Technologies). Antibody IgG in the culture fluids was purified by column chromatography of protein G (HiTrap Protein G HP Columns, GE health care), according to the manual recommended by the company. After purification, the purified HuMAbs was dialyzed by Slide-A-Lyzer Dialysis Cassettes, 10K MWCO, 0.5 - 3 ml Capacity (Thermo scientific) and filtrated by syringe filter (0.2 m pore size). The concentration of IgG was measured by BCA method using Pierce(R) BCA™ Protein Assay Kit (Thermo scientific).

[0085]

The IF assay was prepared as follows. Vero cells, at 2.5 x 10^4 per well in a 96-well microplate, were mock-infected or infected with DENV. After incubation for 16-24 hr, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 1% Triton X-100 in PBS. Undiluted hybridoma culture fluids were used for the HuMAbs. Also, the purified HuMAb of 10.0 micrograms/ml were serially two-fold diluted and these dilutions were used. Vero cells in 96-well microplate were incubated with the hybridoma culture fluids or the serial dilutions. As a positive control, cells were incubated with 4G2, anti-flavivirus E mouse MAb (Falconar et al., Arch Virol 144: 2313-2330 (1999)). The bound antibody was visualized by reaction with an AlexaFluor 488-conjugated anti-human and anti-mouse secondary antibody (1:1,000; Invitrogen). The reciprocal of the final dilution showing positive reaction by indirect IF was used as binding titer of individual HuMAbs.

[0086]

The VN assay was conducted on culture media of individual hybridoma clones, as described previously (Okuno et al., Biken J 21: 137-147 (1978)). The 25 microlitters of undiluted hybridoma culture supernatant, or DMEM supplemented with 15% FBS (as a negative control) was mixed with 100 FFU of individual DENV serotypes (25 microlitters). After incubation for 15 min, the mixture was used to infect Vero cells in a 96-well microplate. After inoculation at 37 centigrade for 2 hr, 100 microlitters of MEM with 3% FBS was added. After incubation at 37 centigrade overnight, the cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. The plate was stained with 4G2 at 4 centigrade overnight, as for the IF assay. The bound antibody was visualized by further reaction with an AlexaFluor 488-conjugated anti-mouse antibody (1:1,000; Invitrogen). The assays were performed in duplicate and the results expressed as averages. VN activity of HuMAbs in the culture medium from hybridoma clones was expressed as "-" (<50%), "+" (50% to <90%), or "++" (90% reduction in FFU), compared with the negative control. In addition, 25 microlitters of purified HuMAb with variable concentrations of IgG (2-fold serial dilutions of 25.0 micrograms/ml) or PBS (as a negative control) was mixed with 100 FFU of individual DENV serotypes (25 microlitters). After incubation at 37 centigrade for 15 min, the mixture was used to infect Vero cells in a 96-well microplate. After inoculation at 37 centigrade for 2 hr, supernatant was removed. And 100 microlitters of MEM with 1% carboxymethylcellulose, 2% FBS was added. After incubation at 37 centigrade overnight, the cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. The plate was stained with 4G2 at 4 centigrade overnight, as for the IF assay. The bound antibody was visualized by further reaction with an AlexaFluor 488-conjugated anti-mouse antibody (1:1,000; Invitrogen). The assays were performed in triplicate and the results expressed as averages and standard deviation. The neutralization activity was expressed as the concentration showing a 50% reduction in FFU compared with the negative control calculated by Behrens-Karber method (Klassen CD (1991) Principles of Toxicology. In Pharmacological Basis of Therapeutics, pp. 49-61. Edited by Gilman AG, Tall TW, Nies AS, Taylor P. 8th edition. McGraw-Hill) (referred to as the VN50).

[0087]

The antibody-dependent enhancement (ADE) assay was performed as follows. The purified HuMAbs (serial 10-fold dilutions of 10.0 micrograms/ml) were incubated with DV1 to DV4 at 37 centigrade for 30 min. Then, THP-1 cells without FBS condition

were inoculated with the HuMAb-DENV mixed solution and incubated at 37 centigrade for further 1hr. After FBS addition at a final concentration of 2%, the THP-1 solution with HuMAb and DENV were cultured at 37 centigrade for 3 days. Total RNA extracted from the collected infected cells using TRIzol(R) reagent (life technologies) was applied to one-step real-time PCR. One-step real-time PCR was performed as described previously (Shu et al., J Clin Microbiol. 41:2408-2416 (2003)). Briefly, the oligonucleotide primer pairs were used for One-step real-time PCR: DV-F (5'-CAATATGCTGAAACGCGAGAGAAA-3') (SEQ. ID NO. 209),/DV-N (5'-CCCCATCTATTCAGAATCCCTGCT-3') (SEQ. ID NO. 210),were designed to give amplicons from all serotyped of DENV and GAPDH-F (5'-ACCACAGTCCATGCCATCAC-3') (SEQ. ID NO. 211),/GAPDH-R (5'-TCCACCACCCTGTTGCTGTA-3') (SEQ. ID NO. 212),were designed to give amplicons from GAPDH. PrimeScript(R) One Step RT-PCR Kit Ver. 2 (Takara) was used for this assay according to the manual recommended by the company. Real-time PCR was performed using CFX96 Real-Time PCR Detection System (BIORAD). The data derived from Real-time PCR was analyzed bydelta-delta Ct analysis method (Schmittgen et al., Nat Protoc 3:1101-1108 (2008)) that used Ct value of GAPDH as internal control.

[0088]

The following expression vectors were used for DENV proteins. The CMV4-HA vector was used for the molecular cloning of a fusion form of the E and prM (prM-E) and E protein of DENV genes. A double-stranded oligo DNA derived from annealing FW-CMV3-HA-stop-linker (GGCCGCAGGCGATATCTACCCCTACGACGTGCCCGACTACGCCTGAG) SEQ. ID NO. 213)and RV-CMV3-HA-stop-linker (GATCCTCAGGCGTAGTCGGGCACGTCGTAGGGGTAGATATCGCCTGC) SEQ. ID NO. 214) was inserted between the NotI and BamH1 sites on the pFLAG-CMV™-4 Expression Vector (Sigma). The individual coding regions for the E derived from DENV-2 NGC strain and for the prM-E derived from the DENV-2 16681 and DENV-2 NGC strains were amplified with PrimeSTAR GXL DNA polymerase (Takara) with gene-specific primers (NGC env Fw NotI, GGCGCGGCCGCCATGCGTTGCATAGGAATATCAAATA (SEQ. ID NO. 215); NGC env Rv EcoRV, GCGGATATCTCAGGCCTGCACCATAACTCCCAAAT (SEQ. ID NO. 216); prM-NotI Fw, GGAGCGGCCGCGTTCCATTTAACCACACGTAACGG (SEQ. ID NO. 217); and Env-EcoRV Rv, GGCGATATCGGCCTGCACCATGACTCCCAAATAC (SEQ. ID

NO. 218)) according to the manufacturer's instructions. The amplified DNA fragments were digested with NotI and EcoRV and ligated with the NotI- and EcoRV-digested CMV4-HA Expression Vector. The pcDNA3-C-Flag vector was used for the molecular cloning of the prM and C DENV genes. The mammalian expression plasmid pcDNA3 (Invitrogen) was digested with restriction enzymes (EcoRV and XhoI), and double-stranded oligoDNA (5'- ATCGACTACAAAGACGATGACGACAAGCT(SEQ. ID NO. 219) and 5'- TCGATCAAAGCTTGTCGTCATCGTCTTTGTAGTCGATATC-3'(SEQ. ID NO. 220)) was inserted between the EcoRV and XhoI sites. The coding regions for the prM protein from the 16681 and NGC strains of DENV-2 were amplified with PrimeSTAR GXL DNA polymerase (Takara) with gene-specific primers (DV2 prM F BglII, CGAGATCTGCCACCATGTTCCATTTAACCACACGTAAC(SEQ. ID NO. 221); and DV2 prM R SmaI, GCCCCGGGGATATCTGTCATTGAAGGAGTGACAGC(SEQ. ID NO. 222)) following the manufacturer's instructions. The amplified DNA fragments were digested with BglII and SmaI and ligated with the BamHI- and EcoRV-digested pcDNA3-C-Flag vector. The DENV-2 C gene was amplified with PrimeSTAR Max DNA polymerase (Takara) with gene-specific primers (D2 C F, GGATCTCCAGCCATGAATGACCAACGGAAAAAGGCG(SEQ. ID NO. 223); and D2 C R, GTCGATGGGGATATCTCTGCGTCTCCTATTCAAGATG(SEQ. ID NO. 224)) following the manufacturer's instructions. The amplified DNA fragments were cloned into plasmids with the In-Fusion Advantage PCR cloning kit (Takara). The NS1 gene was cloned as reported previously (Kurosu et al., Biochem Biophys Res Commun 362: 1051-1056 (2007). Briefly, specific primer sets (GGCGGATCCGCCATGGCCGATAGTGGTTGCGTTGTGAGC (SEQ. ID NO. 225)and GCGCTCGAGTCAGGCTGTGACCAAGGAGTTGACC(SEQ. ID NO. 226)) were used to amplify the NGC NS1-specific gene. The amplified sequence was inserted into the BamHI/XhoI sites of pGEX-6P-1 (Invitrogen). 293T cells transfected with individual plasmids were used as viral antigens for the identification of viral proteins recognized by HuMAbs by IF.

[0089]

HuMAbs were isotyped as follows. The HuMAbs obtained were isotyped by Human IgG ELISA Quantitation set, Human IgM ELISA Quantitation set, and Human IgA ELISA Quantitation set (Bethyl Laboratories, Inc., Montgomery, Texas). The fluids of individual hybridoma clone cultures were used for this isotyping. Further, for subclassing of the HuMAbs, ELISA microplates Maxsorp (Nunc, Copenhagen, Denmark) were coated overnight at 4 centigrade with 50 microlitters of goat

anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.05 M Sodium Bicarbonate buffer, pH 8.6 (1 microgram/ml). After washing 5 times with 300 microlitters of PBS-0.1% Tween-20, the wells were incubated with 300 microlitters of 0.5 % BSA in PBS blocking buffer for 1 hr. at 37 centigrade. After washing again, the wells were incubated with 50 microlitters of hybridoma supernatant or control serum for 2 hrs. at 37 centigrade. After washing again, the wells were incubated with 50 microlitters anti-IgG1 HRP (SouthernBiotech, Birmingham, Alabama) (1:2000), anti-IgG2 HRP (SouthernBiotech) (1:2000), anti-IgG3 HRP (SouthernBiotech) (1:2000), and anti-IgG4 HRP (SouthernBiotech) (1:2000) for 1 hr at 37 centigrade. The wells were washed 5 times, followed by incubation with 50 microlitters TMB peroxidase substrate (KPL, Gaithersburg, MD) at room temperature in the dark. After 20 minutes, the reaction was stopped with 2 N H2SO4 solution. Color was read at 450 nm in an ELISA microplate reader (Corona Electric, Tokyo, Japan). All samples were run in triplicate.

[0090]

In vivo evaluation of HuMAbs in suckling mouse was performed as follows. BALB/c suckling mice were purchased from Japan SLC Inc., Shizuoka, Japan. Two-days-old suckling mice were intracerebrally inoculated with 20 microlitters containing 20,000 FFU of DENV-2 (16681 strain) per head which had been incubated with the purified 1 microgram and 0.2 microgram of HuMAb per head, or PBS as a negative control, for 30 minutes at the ice-cold condition.

[0091]

Sequencing of HuMAb variable regions were performed as follows. Total RNA was extracted from the hybridoma cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). It was subjected to RT-PCR using a PrimeScript RT reagent Kit (Takara) with an oligo(dT) primer. The coding region of the HuMAb H- and L-chains was amplified by PCR, with the following primers: 5'-ATGAAACACCTGTGGTTCTTCCTCCT-3' (SEQ. ID NO. 227) H-chain-forward and 5'-CCTTGGTGTTGCTGGGCTTGTGAT-3' (SEQ. ID NO. 228) H-chain-reverse; 5'-ATGGCCTGGWYYCCTCTCYTYCTS-3' (SEQ. ID NO. 229) L-chain-forward and 5'-TGGCAGCTGTAGCTTCTGTGGGACT-3' (SEQ. ID NO. 230) L-chain-reverse. PCR products were ligated into pGEM T-Easy vector (Promega, Madison, Wisconsin), and their sequences were analyzed using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City,

California).

[0092]
In this study, the preparation of hybridomas producing HuMAbs against DENV using specimens from Thai patients was examined. The PBMC samples were obtained from patients in the acute (around one week after onset of fever) and convalescent phases (around two weeks after onset of fever). In the first trial, a total of nine samples from Thai patients were used: three patients (D30, D32, and D33) in the acute phase, ranging between 6-8 days after onset of fever; four patients (D22, D25, D27, and D28) in the convalescent phase, 12-15 days after onset of fever; and one patient in both acute (D23; 5 days after onset of fever) and convalescent phases (D26; 19 days after onset of fever) (Tables 1). D22, D27, and D30, were clinically diagnosed as DHF infections and the others were all DF. D22, D27, D28, D30. There were no significant differences in gender (P=0.798), age (P=0.856), or disease severity (P=0.685) between the patients in the acute and convalescent phases. Data obtained from the rapid test kits showed that all acute-phase patients were positive for both anti-dengue IgG and IgM, indicating that these patients had secondary DENV infections.

[0093]
[Table 1]

Table 1. Summary of patients' background and HuMAbs obtained in this study.

Patient	Gender	Age	Diagnosis	Blood collection[2]		Rapid test[3]		PCR	Hybridoma clone	Isotyping of HuMAb			
				Days	Phase	IgG	IgM	serotyping	obtained	IgG	IgA	IgM	None[4]
D23[1]	Female	33	DF	5	Acute	+	+	DENV-2	75	70	3	0	2
D30	Female	23	DHF grade 1	8	Acute	+	+	DENV-2	25	22	3	0	0
D32	Male	19	DF	6	Acute	+	+	DENV-2	5	5	0	0	0
D33	Male	31	DF	8	Acute	+	+	DENV-2	16	14	2	0	0
D22	Female	25	DHF grade 3	12	Convalescent	+	+	NT[5]	4	3	0	0	1
D25	Male	27	DF	14	Convalescent	+	+	NT	5	5	0	0	0
D26[1]	Female	33	DF	19	Convalescent	+	+	NT	2	2	0	0	0
D27	Male	21	DHF grade 2	13	Convalescent	+	+	NT	2	2	0	0	0
D28	Female	23	DF	15	Convalescent	+	+	NT	2	1	0	0	1

[1]D23 and D26 were derived from the same patient at acute and convalescent phases, respectively.

[2]Blood were collected at days after the onset of fever: 5-8days for acute and 12-19 days for convalescent phase.

[3]Rapid test for D22, D25-D28 was performed with the plasma from these patients at their acute phase.

[4]HuMAbs not reacted with any of IgG, IgA, nor IgM.

[5]Not tested, because enough amounts of the plasma from the patients at acute phase for RT-PCR were not available.

[0094]

The acute-phase plasma samples were also subjected to RT-PCR, for DENV serotyping. The first PCR was performed with primers DEUL and DEUR, which are common to all DENV serotypes. The resulting products were then subjected to a second PCR with primers specific to individual serotypes of DENV: D1L and D1R for DENV-1; D2L and D2R for DENV-2; D3L and D3R for DENV-3; and D4L and D4R for DENV-4. The resulting products were detected only by D2L and D2R, but not by other primer sets, in all samples from the 4 acute-phase patients (data not shown), indicating that all 4 patients were cases of secondary infection with DENV-2. About patients, the blood samples from whom were available for hybridoma preparation at convalescent phase, the plasma samples from their acute phase were used only for rapid test that showed all positive for both of anti-dengue IgG and IgM, indicating that these patients were also secondarily infected cases.

[0095]

Hybridomas were prepared as follows. The PBMC fractions prepared from four acute-phase patients and five convalescent-phase patients were used to prepare hybridoma by fusion with SPYMEG cells. Fused cells were seeded and cultured for 10-14 days in HAT selection medium in 96-well microplates. The first screening of individual well culture fluids revealed unexpectedly high efficiencies. With the addition of DENV-infected cells, wells with acute-phase PBMCs were more frequently IF-positive (734 wells) than convalescent-phase PBMCs (57 wells). Of these wells, 272 IF-positive acute-phase PBMCs wells and 29 convalescent-phase ones were selected for cell cloning as the wells exhibited stronger reactions and contained clear discrete colony formations. As summarized in Tables 1 (Tables 2 for the data of individual HuMAbs), after secondary screening by IF after incubation for 2 weeks, 121 acute-phase and 15 convalescent-phase hybridomas showing stable proliferation and production of anti-DENV MAbs were obtained. Isotyping showed IgG-type in 91.7% (111/121) of HuMAbs from acute-phase cells and 86.6% (13/15) of HuMAbs from convalescent-phase cells. IgA-type was detected only in 6.6% (8/121) of HuMAbs from acute-phase cells. There were no positive cases for IgM-type. There were no positive reactions in four clones for any of IgG, IgA, and IgM under the conditions examined using the culture fluids of hybridoma clones.

[0096]

Cross-reactivity of HuMAbs was determined as follows. The HuMAbs obtained as

described above were characterized for their serological reactivity to all four DENV serotypes by IF and VN assays. HuMAbs in the fluids of individual hybridoma cell cultures were used for these assays. As shown in Figure 1, the HuMAbs were classified into groups 1 to 10 and groups A to X based on their cross-reactivity with the four serotypes of DENV in IF and VN assays, respectively: group A showed no VN activity to any of four serotypes; groups 1-2 and groups B-E showed specific reactions with a single serotype; groups 3-6 and groups F-H showed cross-reactions with two serotypes; groups 7-9 and groups I-O showed cross-reactions with three serotypes; and group 10 and groups P-X showed cross-reactions with all four serotypes. The IF assay revealed that 109 of 121 clones (90.1%) derived from acute-phase patients were cross-reactive with all four serotypes (Figure 1): 65 of 75 clones (86.7%) from D23, 23 of 25 clones (92.0%) from D30, five of five clones (100%) from D32, and 16 of 16 clones (100%) from D33 (Figure 2 and Table 2). By contrast, only seven of 15 clones (46.7%) derived from convalescent-phase patients were shown to be cross-reactive with all four serotypes (Figure 1): three of four clones (75.0%) from D22, two of five clones (40.0%) from D25, one of two clones (50.0%) from D26, one of two clones (50.0%) from D27, and neither of the two clones from D28 (0%) (Figure 2 and Table 2). Thus, obtaining HuMAbs cross-reactive with all four serotypes was significantly more efficient using PBMCs from acute-phase patients, as compared to convalescent-phase patients (P=0.008). The IF profiles of several representative HuMAbs by IF are shown in Figure 3: D28-2B11D10 and D23-4A7D6 were serotype-specific, D28-2B11F9 and D23-1B11A5 were cross-reactive with two serotypes, D25-4D3D2 and D23-3E6D7 were cross-reactive with three serotypes, and D22-1B7G2 were cross-reactive with all four serotypes. Mouse MAb 4G2 was used as a positive control for antibodies reactive with all four serotypes (corresponding to group 10). Next, we examined the VN activity of HuMAbs. The culture fluids from individual hybridoma clones were reacted with DENV-1 to -4. Under these conditions, the control 4G2 showed a 90% reduction in FFU compared with the negative control (DMEM with 15% FBS) in all four serotypes of DENV and, therefore, this MAb was classified into group X.

[0097]

[Table 2-1]

Table 2. Summarized results of the cross-reactivity of the individual HuMAbs.

Patienst	Hybridoma	IF reactivity					VN activity[1]					Isotype	Target protein
		DENV-1	DENV-2	DENV-3	DENV-4	JEV	DENV-1	DENV-2	DENV-3	DENV-4	JEV		
Acute D23	D23-1A2B6	+	+	+	+	+	+	++	+	++	-	IgG	E
	D23-1A5D7	+	+	+	+	+	-	+	-	-	-	IgG	E
	D23-1A8H5	+	+	-	+	+	-	+	-	-	+	IgG	E
	D23-1A10H7	+	+	+	+	+	++	++	++	++	-	IgG	E
	D23-1B1E5	+	+	+	+	+	-	++	+	+	+	IgG	E
	D23-1B3B9	+	+	+	+	+	+	++	++	++	+	IgG	E
	D23-1B8F7	+	+	+	+	+	-	+	-	+	-	IgG	E
	D23-1B10C7	+	+	+	+	+	-	++	+	+	+	IgG	E
	D23-1B11A5	+	-	-	+	-	-	+	-	-	+	ND[2]	Other[3]
	D23-1C1G4	+	+	+	+	+	++	++	++	++	-	IgG	E
	D23-1C2D2	+	+	+	+	+	++	++	++	++	+	IgG	E
	D23-1C3B11	+	+	+	+	-	-	-	+	-	-	IgG	Other[3]
	D23-1C6F6	+	+	+	+	+	+	++	+	++	+	IgG	E
	D23-1D4E2	+	+	+	+	+	-	++	+	+	-	IgG	E
	D23-1D5G10	+	+	+	+	-	-	-	-	-	-	IgG	prM
	D23-1D8B3	+	+	+	+	+	-	-	-	-	-	IgG	Other[3]
	D23-1E3B1	+	+	+	+	+	+	++	+	+	-	IgG	E
	D23-1E12E8	+	+	+	+	-	-	++	+	+	-	IgG	E
	D23-1G1H8	+	+	+	+	+	+	++	+	++	-	IgG	E
	D23-1G5C8	+	+	+	+	+	+	++	+	+	+	IgG	E
	D23-1G7C2	+	+	+	+	+	++	++	+	++	+	IgG	E
	D23-1G10A3	+	+	+	+	+	+	++	+	++	-	IgG	E
	D23-1H5A11	+	+	+	+	+	++	++	++	++	+	IgG	E
	D23-1H11C2	+	+	+	+	-	-	-	-	-	-	IgG	prM
	D23-2A5B5	+	+	+	+	-	-	-	-	+	-	IgG	prM
	D23-2A8D2	+	+	+	+	-	-	-	-	-	+	IgG	NS1
	D23-2A8G5	+	+	+	-	-	-	-	-	-	-	IgG	NS1
	D23-2A11B7	+	+	+	+	+	+	++	+	+	-	IgG	E
	D23-2A12B9	-	+	-	+	-	-	-	-	-	-	IgA	Other[3]
	D23-2C3D8	+	+	+	+	-	+	-	-	-	-	IgG	prM
	D23-2C11G6	+	+	+	+	+	+	++	+	+	-	IgG	E
	D23-2D12C6	+	+	+	+	+	+	++	+	+	+	IgG	E
	D23-2E1D7	+	+	+	+	+	-	++	-	+	-	IgG	E
	D23-2E10D7	+	+	+	+	+	+	++	+	+	-	IgG	E
	D23-2F2B1	+	+	+	+	+	+	++	-	+	-	IgG	E
	D23-2G4E7	+	+	+	+	+	-	++	+	+	-	IgG	E
	D23-3A2F9	+	+	+	+	-	-	-	-	-	-	IgG	prM
	D23-3A10G12	+	+	+	+	+	++	++	++	++	+	IgG	E
	D23-3B2G9	+	+	+	+	+	+	++	+	-	+	IgG	E
	D23-3C5A9	+	+	+	+	-	-	-	-	-	-	IgG	prM
	D23-3E6D7	+	+	-	+	+	+	++	+	++	+	IgG	E
	D23-4A6F9	+	+	+	+	+	+	++	++	++	+	IgG	E
	D23-4A11A2	+	+	+	+	+	+	++	+	++	+	IgG	E
	D23-4A7D6	-	-	-	+	-	-	-	-	-	-	ND[2]	Other[3]
	D23-4B9H3	+	+	+	+	+	+	++	+	++	-	IgG	E
	D23-4B12C5	+	+	+	+	+	++	++	+	+	-	IgG	E
	D23-4C5D7	+	+	+	+	+	+	++	+	++	-	IgG	E

[0098]

[Table 2-2]

Table 2 (Continued-1). Summarized results of the cross-reactivity of the individual HuMAbs.

Patient		Hybridoma	IF reactivity					VN activity[1]					Isotype	Target protein
			DENV-1	DENV-2	DENV-3	DENV-4	JEV	DENV-1	DENV-2	DENV-3	DENV-4	JEV		
Acute (Continued)	D23	D23-4D6F9	+	+	+	+	+	+	++	+	+	-	IgG	E
		D23-4D9G5	+	+	+	+	-	-	-	-	-	-	IgG	prM
		D23-4D10E9	+	+	+	+	+	++	++	+	++	+	IgG	E
		D23-4E9A10	+	+	+	+	+	+	++	+	+	+	IgG	E
		D23-4F5E1	+	+	+	+	+	++	++	+	++	+	IgG	E
		D23-4F10H7	+	+	+	+	-	-	+	+	-	-	IgG	E
		D23-4H12C8	+	+	+	+	+	+	++	++	+	-	IgG	E
		D23-5A2C10	+	+	+	+	+	+	++	+	+	+	IgG	E
		D23-5A4E8	+	+	+	+	+	+	++	+	+	-	IgG	E
		D23-5A5A6	+	+	+	+	-	+	+	-	+	-	IgG	Other[3]
		D23-5A7F6	+	+	+	+	+	-	++	+	+	-	IgG	E
		D23-5A8B1	+	+	+	+	-	-	-	-	+	-	IgG	prM
		D23-5B5E1	+	+	+	+	+	+	++	+	+	-	IgG	E
		D23-5B9C9	+	+	+	+	+	+	++	+	+	-	IgG	E
		D23-5C1G7	+	+	+	+	+	+	++	+	+	+	IgG	E
		D23-5C3B2	+	+	+	+	-	+	+	+	-	-	IgG	E
		D23-5C7G1	+	+	+	-	-	-	-	-	-	-	IgG	NS1
		D23-5D6D1	+	+	+	+	+	+	++	+	+	-	IgG	E
		D23-5E3G3	-	+	+	-	-	+	++	++	-	-	IgG	E
		D23-5E6B1	+	+	+	+	+	++	++	++	++	-	IgG	E
		D23-5E8B8	+	+	+	+	+	++	++	+	++	-	IgG	E
		D23-5F3B8	+	+	+	+	+	+	++	+	+	+	IgG	E
		D23-5F4B3	+	+	+	+	+	+	++	+	+	+	IgG	E
		D23-5G2D2	+	+	+	+	+	++	++	+	++	+	IgG	E
		D23-5G4B6	-	+	-	-	-	-	-	-	-	-	IgA	Other[3]
		D23-5G7F10	-	+	-	+	-	-	-	-	-	-	IgA	Other[3]
		D23-5G8E3	+	+	+	+	+	++	++	++	++	+	IgG	E
		D23-5G10G8	+	+	+	+	-	-	++	+	+	-	IgG	E
	D30	D30-1A4F4	+	+	+	+	+	+	+	+	+	+	IgG	E
		D30-1B5D8	+	+	+	+	+	+	+	+	+	+	IgG	E
		D30-1D10C7	+	+	+	+	+	+	+	+	+	-	IgG	E
		D30-1E7B8	+	+	+	+	+	++	++	++	++	+	IgG	E
		D30-1E12C3	+	+	+	+	+	+	+	+	++	-	IgG	E
		D30-1G1E6	+	+	+	+	+	+	++	+	++	+	IgG	E
		D30-1G7D6	+	+	+	+	+	+	++	+	++	+	IgG	E
		D30-1G11D7	+	+	+	+	+	+	+	+	+	-	IgG	E
		D30-2B1G5	+	+	+	-	+	-	+	+	+	+	IgG	NS1[4]
		D30-2B2G7	+	+	+	+	+	+	+	+	+	-	IgG	E
		D30-2C1B8	+	+	+	+	+	+	+	+	+	+	IgG	E
		D30-2C12B4	+	+	+	+	+	+	+	+	+	-	IgG	E
		D30-2D4D10	+	+	+	+	+	-	-	-	-	+	IgG	E
		D30-2G8B10	+	+	+	+	+	+	+	+	-	-	IgG	E
		D30-2G8H12	-	+	+	+	-	-	-	-	+	+	IgG	Other[3]

[0099]

[Table 2-3]

Table 2 (Continued-2). Summarized results of the cross-reactivity of the individual HuMAbs.

Patient		Hybridoma	IF reactivity					VN activity[1]					Isotype	Target protein
			DENV-1	DENV-2	DENV-3	DENV-4	JEV	DENV-1	DENV-2	DENV-3	DENV-4	JEV		
Acute (Continued)	D30	D30-2G12F10	+	+	+	+	+	+	+	+	++	+	IgG	E
		D30-3A1E2	+	+	+	+	+	++	++	++	++	+	IgG	E
		D30-3B1H9	+	+	+	+	+	+	+	+	+	+	IgG	E
		D30-3B5B3	+	+	+	+	+	+	+	+	+	-	IgG	E
		D30-3B6C7	+	+	+	+	+	+	++	+	++	-	IgG	E
		D30-3B10E7	+	+	+	+	+	++	++	++	++	+	IgA	E
		D30-3G8F5	+	+	+	+	+	+	+	+	+	-	IgG	E
		D30-3H3G2	+	+	+	+	+	+	+	+	+	+	IgA	E
		D30-3H5C9	+	+	+	+	+	+	++	+	+	-	IgG	E
		D30-3H9F8	+	+	+	+	+	+	+	+	+	+	IgA	E
	D32	D32-1B5G5	+	+	+	+	-	-	+	+	+	-	IgG	E
		D32-2D1G5	+	+	+	+	+	++	++	++	++	+	IgG	E
		D32-2D9C2	+	+	+	+	+	-	-	-	-	-	IgG	E
		D32-2H8G1	+	+	+	+	+	+	++	++	++	+	IgG	E
		D32-5B12F7	+	+	+	+	+	+	+	+	+	+	IgG	E
	D33	D33-1A10D8	+	+	+	+	+	-	-	+	-	-	IgG	Other[3]
		D33-1B6H10	+	+	+	+	+	-	+	-	-	-	IgG	E
		D33-1C8D2	+	+	+	+	+	+	+	+	+	+	IgG	E
		D33-1D3D10	+	+	+	+	+	+	+	+	+	-	IgG	E
		D33-1F9B11	+	+	+	+	+	+	+	+	-	-	IgG	E
		D33-2H1G4	+	+	+	+	+	-	-	-	-	+	IgG	E
		D33-2H9D10	+	+	+	+	+	+	+	+	+	+	IgA	E
		D33-3A4A6	+	+	+	+	+	-	-	-	-	-	IgG	E
		D33-3A5C7	+	+	+	+	+	-	+	+	-	-	IgG	E
		D33-3A7B4	+	+	+	+	+	+	+	+	+	-	IgG	E
		D33-3D8A9	+	+	+	+	+	-	+	+	+	+	IgG	E
		D33-3D12D4	+	+	+	+	+	+	+	+	-	+	IgA	E
		D33-3E4H10	+	+	+	+	+	+	+	+	+	+	IgG	E
		D33-3G12C3	+	+	+	+	+	-	-	+	-	-	IgG	E
		D33-4C10C10	+	+	+	+	+	+	+	+	+	-	IgG	E
		D33-5D12B11	+	+	+	+	+	-	-	-	-	-	IgG	E
Convalescent	D22	D22-1B7G2	+	+	+	+	-	-	-	-	-	-	IgG	prM
		D22-2G4E3	+	+	+	+	+	-	-	-	-	-	IgG	E
		D22-3H2G5	-	+	-	-	-	-	-	-	-	-	ND[2]	Other[3]
		D22-3H10F10	+	+	+	+	+	-	-	-	-	-	IgG	Other[3]
	D25	D25-2B11C3	+	+	+	-	-	-	-	-	-	-	IgG	NS1
		D25-2B11G11	+	+	+	+	+	-	+	+	+	+	IgG	NS1[5]
		D25-4D3D2	+	+	+	-	-	-	-	-	-	+	IgG	NS1
		D25-4D4C3	+	+	+	-	-	-	-	-	-	+	IgG	NS1
		D25-4D4F10	+	+	+	+	-	-	+	+	+	-	IgG	NS1[6]
	D26	D26-5A2G2	+	+	+	+	+	+	++	+	++	-	IgG	E
		D26-5A2B12	+	+	+	-	-	-	-	-	-	+	IgG	NS1
	D27	D27-1E8A3	+	+	+	+	-	-	+	-	+	-	IgG	prM
		D27-1E8A4	+	+	+	-	-	-	-	-	-	+	IgG	NS1
	D28	D28-2B11D10	-	+	-	-	-	-	-	-	-	-	ND[2]	Other[3]
		D28-2B11F9	+	+	-	-	-	-	-	-	-	-	IgG	NS1

[1] "-" for <50%, "+" for <=50% and <90%, and "++" for <=90% FFU reduction compared with the negative control.

[2] No reaction with any of IgG, IgA nor IgM.

[3] No reaction at least with prM-E, E, prM, and NS1.

[4] In addition to NS1, E protein was also weakly reactive with this HuMAb.

[5] In addition to NS1, E and prM proteins were also weakly reactive with this HuMAb.

[6] In addition to NS1, prM protein was also weakly reactive with this HuMAb.

[0100]

On the other hand, 103 of 121 acute-phase clones (85.1%) and four of 15 convalescent-phase clones (26.7%) showed a 50% reduction in viral replication (Figure 1). A 90% reduction in viral replication was detected in 62 of 121 acute-phase clones (51.2%) and one of 15 convalescent-phase clones (6.7%) (Figure 1). A total of 70 acute-phase clones (57.9%) and one convalescent-phase clone (6.7%) showed neutralization activity (a 50% reduction in viral replication) against all four serotypes, while only 11 acute-phase (9.1%) and no convalescent-phase clones (0%) showed neutralization activity (a 90% reduction in viral replication) against all four serotypes (Figure 1 and Table 2).

[0101]

The correlations between IF and VN results (50% and 90% reduction) for individual HuMAbs are shown in Figure 4. The obtained HuMAbs were highly heterogeneous. HuMAbs with neutralization activities to all four serotypes of DENV were more efficiently obtained using acute-phase PBMCs. There were inconsistencies between the IF and VN data regarding the four HuMAbs: one from patient D23 belonging to group 4-C (in the IF and VN assays, respectively), one from patient D23 belonging to group 5-K, one from patient D23 belonging to group 8-U, and one from patient D30 belonging to group 7-N.

[0102]

Tables 2-4 shows summarized results of viral protein recognized by HuMAbs. 293T cells transfected with expression vectors for the DENV-2 prM, E, NS1, and C proteins, or for the prM-E fusion protein, were used as targets for the identification of viral proteins recognized by individual HuMAbs by IF. Summarized data on viral proteins recognized by individual HuMAbs classified in groups 1 to 10 by IF assay and in groups A to X by VN assay are shown in Tables 3 and 4, respectively (the results from individual HuMAbs are shown in Table 2). Of the acute-phase HuMAbs, 99 were reactive with E, eight with prM, four with NS1, and none with C. Culture fluid from the remaining 10 hybridoma clones was not reactive with the E, prM, NS1, or C proteins ("Other"). Of the convalescent-phase HuMAbs, two were reactive with E, two with prM, eight with NS1, and none with C, and the remaining three were not reactive with any of the proteins assayed ("Other"). Interestingly, five HuMAbs obtained from D25 were all reactive against NS1 (Table 2). Tables 3 and 4 summarize the viral proteins recognized by HuMAbs broken down according to reactivity group (groups 1 to 10 by IF assay and groups A to X by VN assay). The 98 HuMAbs recognizing E (96 of 99 HuMAbs from the acute-phase and two of two HuMAbs from the convalescent-phase) were all in group 10 (cross-reactive with all four serotypes) according to the IF assay (Table 3). Of these, 70 acute-phase and one convalescent-phase HuMAbs showed 50% VN activity against all four DENV serotypes (groups P to X). Of the 70 acute-phase HuMAbs, 11 also showed 90% VN activity against all four DENV serotypes (group X).

[0103]

[Table 3]

Table 3. Target viral proteins of HuMAbs categorized by IF results.

	DENV serotype				IF							
					Acute				Convalescent			
	1	2	3	4	E	prM	NS1	Other[1]	E	prM	NS1	Other[1]
1	-	+	-	-				1 (0)[5]				2 (0)
2	-	-	-	+				1 (0)				
3	+	+	-	-							1 (0)	
4	+	-	-	+				1 (0)				
5	-	+	+	-	1 (0)							
6	-	+	-	+				2 (0)				
7	+	+	+	-			3[2] (1[2])				5 (0)	
8	+	+	-	+	2 (2)							
9	-	+	+	+				1 (0)				
10	+	+	+	+	96 (91)	8 (0)	1 (0)	4 (2)	2 (2)	2 (0)	2[3,4] (1[3])	1 (1)

[1] No reaction at least with prM-E, E, prM, and NS1.

[2] The HuMAb (D30-2B1G5) is also reactive with E weakly.

[3] The HuMAb (D25-2B11G11) is also reactive with E and prM weakly.

[4] The HuMAb (D25-4D4F10) is also reactive with prM weakly.

[5] Number of HuMAb clones showing positive reaction with JEV are shown in parenthesis.

[0104]

[Table 4]

Table 4. Target viral proteins categorized by Nt results

	DENV serotype				VN							
					Acute				Convalescent			
	1	2	3	4	E	prM	NS1	Other[1]	E	prM	NS1	Other[1]
A[5]	-	-	-	-	5 (2)[6]	5 (0)	3 (1)	5 (0)	1 (0)	1 (0)	6 (4)	3 (0)
B	+	-	-	-		1 (0)						
C	-	+	-	-	3 (1)			1 (1)				
D	-	-	+	-	1 (0)			2 (0)				
E	-	-	-	+		2 (0)		1 (1)				
F	-	+	+	-	2 (0)							
G	-	+	-	+	1 (0)					1 (0)		
H	-	++	-	+	1 (0)							
I	+	+	+	-	4 (1)							
J	+	++	+	-	1 (1)							
K	+	++	++	-	1 (0)							
L	+	+	-	+				1 (0)				
M	+	++	-	+	1 (0)							
N	-	+	+	+	2 (1)		1^2 (1^2)				$2^{3,4}$ (1^3)	
O	-	++	+	+	7 (2)							
P	+	+	+	+	19 (10)							
Q	+	++	+	+	17 (7)							
R	+	+	+	++	2 (1)							
S	++	++	+	+	1 (0)							
T	+	++	++	+	1 (0)							
U	+	++	+	++	11 (5)				1 (0)			
V	++	++	+	++	5 (4)							
W	+	++	++	++	3 (3)							
X	++	++	++	++	11 (8)							

[1] No reaction at least with prM-E, E, prM, and NS1.

[2] The HuMAb (D30-2B1G5) is also reactive with E weakly.

[3] The HuMAb (D25-2B11G11) is also reactive with E and prM weakly.

[4] The HuMAb (D25-4D4F10) is also reactive with prM weakly.

[5] HuMAbs showing positive reactions with DENV by IF, but no VN activity to any serotypes of DENV.

[6] Number of HuMAb clones showing positive reaction with JEV are shown in parenthesis.

[0105]

Tables 2-4 show a summary of the cross-reactivity of HuMAbs with Japanese Encephalitis Virus (JEV). We next examined the possible cross-reactivity of these

HuMAbs with JEV-infected cells by IF. Interestingly, many HuMAb clones showed positive reactions with JEV: 96 of 121 (79.3%) HuMAbs from the acute phase and four of 15 (26.7%) HuMAbs from the convalescent phase (Table 2 shows data for the individual HuMAbs). Most HuMAbs were classified into group 10: 91 recognizing DENV E and two recognizing "Other" from the acute phase; and two recognizing DENV E, one recognizing NS1, and one recognizing "Other" from convalescent phase. Three HuMAbs from acute phase were reactive with JEV and belonged to groups other than group 10: two in group 8 recognized DENV E and one in group 7 recognized NS1. Next, the HuMAbs were examined for VN activity against JEV. A total of 55 HuMAb clones showed 50% VN activity against JEV, although none showed 90% VN activity. As summarized in Table 4, 50 (46 recognizing E, two recognizing NS1, and two recognizing "Other") of the above 55 HuMAbs were from acute phase and remained five HuMAbs recognizing NS1 were from convalescent phase (Table 2 shows the data for individual HuMAbs). Interestingly, VN activity against JEV was observed not only for HuMAbs cross-reactive with all four DENV serotypes (38 in groups P to X from the acute phase), but also for HuMAbs cross-reactive with two or three DENV serotypes (six from the acute phase and one from the convalescent phase in groups F to O), for DENV serotype-specific HuMAbs (three from the acute phase in groups B to E), and for HuMAbs with no VN activity to any of the DENV serotypes (three from the acute phase and four from the convalescent phase in group A). Selection of 18 hybridoma clones producing stronger neutralizing antibodies to all four serotypes of DENV. A total of 18 hybridoma clones producing specific HuMAbs against DENV were selected as producers of antibody with higher neutralizing titers from a total of 136 hybridoma clones, 121 obtained from 4 Thai patients in the acute phase and 15 from 5 Thai patients in the convalescent phase, according to the results by viral neutralizing activity assay using the culture fluids of individual hybridomas against the laboratory strains of DENV-1 to DENV-4 serotypes (Setthapramote et al., Biochem Biophy Res Commun 423: 867-872 (2012)). All these 18 clones selected as the producers of HuMAbs showing >85% inhibition of viral replication in all four serotypes were derived from three patients in the acute phase (Table 5). All the three patients were adults: 13 HuMAbs prepared with the PBMCs at day 5 after the onset of dengue fever in the female patient D23 of 33 years-old; 3 HuMAbs prepared with the PBMCs at day 8 after the onset of dengue hemorrhagic fever in the female patient D30 of 23 years-old; and 2 HuMAbs prepared with the PBMCs at day 6 after the onset of dengue fever in the male patient D32 of 19 years-old. All these HuMAbs are IgG1, except for D30-1E7B8 of IgG4, and recognize DENV E protein. Two other HuMAbs showing similar strong neutralizing activity

against all 4 serotypes were removed from the selected HuMAbs, because D30-3B10E7 is IgA, which is difficult to purify, and a hybridoma clone producing D23-4D10E8 produced reduced amount of antibody when we recovered the hybridoma clone from freezed condition.

[0106]

[Table 5]

Table 5. Background of HuMAbs obtained by fusion with patient's PBMCs and their activities against DENV-1 to DENV-4.

HuMAb clone	Patient	Disease Days after fever onset	DENV by RT-PCR	IF assay				VN assay (%)				IgG Isotype
				DENV1	DENV2	DENV3	DENV4	DENV1	DENV2	DENV3	DENV4	
D23-1A10H7	D23 Female 33 years-old	DF Day 5	2nd with DENV2	+	+	+	+	94.9	100	92.0	93.4	IgG_1
D23-1B3B9				+	+	+	+	89.7	100	90.5	94.3	IgG_1
D23-1C1G4				+	+	+	+	97.4	100	91.2	94.3	IgG_1
D23-1C2D2				+	+	+	+	97.4	100	94.9	95.3	IgG_1
D23-1G7C2				+	+	+	+	97.4	100	89.1	93.4	IgG_1
D23-1H5A11				+	+	+	+	100	100	93.4	94.3	IgG_1
D23-3A10G12				+	+	+	+	97.4	100	92.7	97.2	IgG_1
D23-4A6F9				+	+	+	+	87.2	100	93.4	90.5	IgG_1
D23-4F5E1				+	+	+	+	92.3	100	89.8	91.5	IgG_1
D23-4H12C8				+	+	+	+	87.2	100	92.0	87.7	IgG_1
D23-5E6B1				+	+	+	+	92.3	100	92.0	91.5	IgG_1
D23-5G2D2				+	+	+	+	92.3	100	85.4	92.4	IgG_1
D23-5G8E3				+	+	+	+	94.9	100	92.7	95.3	IgG_1
D30-1E7B8	D30. Female 23 years-old	DHF (grade 1) Day 8	2nd with DENV2	+	+	+	+	92.4	93.9	91.0	92.0	IgG_4
D30-3A1E2				+	+	+	+	97.0	97.2	90.5	94.9	IgG_1
D30-3B6C7				+	+	+	+	87.1	93.3	85.2	94.9	IgG_1
D32-2D1G5	D32 Male 19 years-old	DF Day 6	2nd with DENV2	+	+	+	+	90.1	93.3	91.5	90.6	IgG_1
D32-2H8G1				+	+	+	+	86.3	93.3	93.6	94.2	IgG_1

[0107]

Table 6s show a summary of sequences of the HuMAbs. The variable regions of IgG in individual above 18 HuMAbs, CDR1, CDR2, and CDR3 were cloned and sequenced.

[0108]

[Table 6-1]

Table 6. 1 (A) The deduced amino acid sequences of CDRs in the V_H and V_L of 18 HuMAbs.

HuMAb clone	CDR1[a]	CDR2[a]	CDR3[a]
D23-1A 10H7	NYAIS (SEQ ID:No. 1) RASQGNSNAVA (SEQ ID:No. 100)	GVIPTLHTTNYVERFQG (SEQ ID:No. 2) GASRLER (SEQ ID:No. 101)	SRYYYDSDASNYGMDV (SEQ ID:No. 3) QQYSSIPYT (SEQ ID:No. 102)
D23-1 B3B9	RYIMN (SEQ ID:No. 4) ASSTGAVTSGHYAN (SEQ ID:No. 103)	WIDTKTGNPTYAQAFT (SEQ ID:No. 5) RTDKKHS (SEQ ID:No. 104)	TLSGYSADWPEDY (SEQ ID:No. 6) LLYRDGGVV (SEQ ID:No. 105)
D23-1 C1G4	SYGVS (SEQ ID:No. 7) SGSSSNIGNNYVY (SEQ ID:No. 106)	WISAYNGDTNFAQKFQG (SEQ ID:No. 8) SNNQRPS (SEQ ID:No. 107)	GPDYESSDSPWFDY (SEQ ID:No. 9) ATWDDSLSRPV (SEQ ID:No. 108)
D23-1 C2D2	SYYWS (SEQ ID:No. 10) QASQDIKNHLN (SEQ ID:No. 109)	NFHYSGSTNDNPSLKS (SEQ ID:No. 11) DASNLGT (SEQ ID:No. 110)	VAKLFGSATYGMDV (SEQ ID:No. 12) HQYENLPIT (SEQ ID:No. 111)
D23-1 G7C2	TYAIS (SEQ ID:No. 13) TGTSGDVGGDSLVS (SEQ ID:No. 112)	GIIPLYKQSTYAQKFRG (SEQ ID:No. 14) EDHKRPS (SEQ ID:No. 113)	LIAVAGSEGAGSFDI (SEQ ID:No. 15) CSYAISSVRVL (SEQ ID:No. 114)

[a] The upper and lower sequences indicate the CDRs of heavy chain (V_H) and light (V_L) chains.

[0109]

[Table 6-2]

Table 6. 2 (A) The deduced amino acid sequences of CDRs in the V_H and V_L of 18 HuMAbs.

D23-1 H5A11	TYAMS (SEQ ID:No. 16) SGSSSNIGSNFVY (SEQ ID:No. 115)	GIGDSGHSIYYADSVKG (SEQ ID:No. 17) SDNQRPS (SEQ ID:No. 116)	GSQWPGDY (SEQ ID:No. 18) ATWDDSLSGPV (SEQ ID:No. 117)
D23-3 A10G12	NYAMT (SEQ ID:No. 19) SGSSSNIANNYVY (SEQ ID:No. 118)	AIGGSGHSIYYGDSVKG (SEQ ID:No. 20) STNQRPS (SEQ ID:No. 119)	GSQWPGDY (SEQ ID:No. 21) AAWDDSLSAPL (SEQ ID:No. 120)
D23-4 A6F9	SNAMS (SEQ ID:No. 22) RASQGISSWLA (SEQ ID:No. 121)	GVSNSGGDTYYADSVKGR (SEQ ID:No. 23) ATSSLQS (SEQ ID:No. 122)	TLWTVGSKGGFDY (SEQ ID:No. 24) QQANSFPWT (SEQ ID:No. 123)
D23-4 F5E1	NSAMS (SEQ ID:No. 25) RSSQTILYSSNNNNYLA (SEQ ID:No. 124)	AFSGSGGSTYYADSVKG (SEQ ID:No. 26) WASTRES (SEQ ID:No. 125)	VTGGWSDY (SEQ ID:No. 27) QQYYSSPPT (SEQ ID:No. 126)
D23-4 H12C8	TYPMN (SEQ ID:No. 28) ASSTGAVTSGYYPN (SEQ ID:No. 127)	WIDTNTGNPTYAQAFTG (SEQ ID:No. 29) STSSKHS (SEQ ID:No. 128)	LSGYSADWPEDY (SEQ ID:No. 30) LLYYRGGVV (SEQ ID:No. 129)
D23-5 E6B1	RYIMN (SEQ ID:No. 31) ASSTGAVTSGNYAN (SEQ ID:No. 130)	WIDTKTGNPTYAQAFTG (SEQ ID:No. 32) RTDKKHS (SEQ ID:No. 131)	LSGYSADWPEDY (SEQ ID:No. 33) LLYRDGGVV (SEQ ID:No. 132)
D23-5 G2D2	KYAIN (SEQ ID:No. 34) RASQGNSNSLA (SEQ ID:No. 133)	GIIPILTITTYAQRFQG (SEQ ID:No. 35) AASRLHS (SEQ ID:No. 134)	STYYYDGSDLTYGMDV (SEQ ID:No. 36) QQYYSIPYT (SEQ ID:No. 135)

[a] The upper and lower sequences indicate the CDRs of heavy chain (V_H) and light (V_L) chains.

[0110]

[Table 6-3]

Table 6. 3 (A) The deduced amino acid sequences of CDRs in the V_H and V_L of 18 HuMAbs.

D23-5 G8E3	DYAMH (SEQ ID:No. 37) TGNSSNIGAGYDVH (SEQ ID:No. 136)	VISHVGTSTYQAESYYA (SEQ ID:No. 38) SNTNRPS (SEQ ID:No. 137)	YYCARRGDYSSSAENFQH (SEQ ID:No. 39) QSYDSSLFACV (SEQ ID:No. 138)
D30-1 E7B8	NYAIS (SEQ ID:No. 40) RASQSVSSYLG (SEQ ID:No. 139)	AIIPMSRTTDYAQKFRG (SEQ ID:No. 41) DASNRAT (SEQ ID:No. 140)	HRAVAGGDSDHDENNWFGP (SEQ ID:No. 42) QQRDNWPYT (SEQ ID:No. 141)
D30-3 A1E2	DYYMI (SEQ ID:No. 43) TGRSSNIGAGHDVH (SEQ ID:No. 142)	YISNSGDMTYHADSVKG (SEQ ID:No. 44) GNNIRPS (SEQ ID:No. 143)	GMTGFTTSNTESFDL (SEQ ID:No. 45) QSYDGSLSGLV (SEQ ID:No. 144)
D30-3 B6C7	GSSFY (SEQ ID:No. 46) ASSTGAVTSDYFPN (SEQ ID:No. 145)	IGSIDYRGNTYYNPSLKS (SEQ ID:No. 47) HTGKKHS (SEQ ID:No. 146)	PGGLISDEAMAGYFDY (SEQ ID:No. 48) LLYHVDAHLV (SEQ ID:No. 147)
D32-2 D1G5	SYSIS (SEQ ID:No. 49) TGSSRDFGDYDHVS (SEQ ID:No. 148)	GTIPFFGRASYAQKFQG (SEQ ID:No. 50) DVTKRPS (SEQ ID:No. 149)	AGPIAATGVQYEMDV (SEQ ID:No. 51) CSYAGSYIWV (SEQ ID:No. 150)
D32-2 H8G1	SSGMH (SEQ ID:No. 52) RASQNIAKYLN (SEQ ID:No. 151)	VVSFDGSKTYYADSAKG (SEQ ID:No. 53) AASSLQS (SEQ ID:No. 152)	GGGRFSGSGNYYYYGMDV (SEQ ID:No. 54) LQTYTAPLT (SEQ ID:No. 153)

[a] The upper and lower sequences indicate the CDRs of heavy chain (V_H) and light (V_L) chains.

[0111]

FIG.7 show a summary of VN and ADE activities of 18 HuMAbs to four serotypes of DENV. We examined the titers of above-selected 18 HuMAbs by indirect IF assay, VN assay, and ADE assay. Enough amounts of HuMAbs were purified by protein G column chromatography from the culture fluids from individual hybridoma clones that were adapted to serum-free medium and cultured in a large scale. Serial dilutions of the purified HuMAbs from individual hybridoma clones were reacted with DENV-1 to DENV-4. The serial 2-fold dilutions of 25.0 micrograms/ml were used for reaction with viral proteins in infected Vero cells by indirect IF assay. The lowest dilutions of the HuMAbs to individual DENV serotypes were used for the binding activities to viral protein. We used 4G2 murine anti-DENV E MAb as a positive control and PBS and 5E4 HuMAb of IgG1 that was prepared with the PBMCs from an influenza-vaccinated donor and shows strong neutralization against influenza A virus H1N1 2009 [Yasugi et al., unpublished] as negative controls. As shown in Figure 5, stronger binding activities to DENV were observed in all the 18 HuMAbs than 4G2, especially to DENV-1 and DENV-2. D23-5G8E3 showed slightly lower activities against DENV-3 and DENV-4, and D23-4H12C8 showed slightly lower activity against DENV-4, compared with those with 4G2. Next, the serial 2-fold dilutions of HuMAbs were used for IF binding titer and VN50 titer (Figure 7) to individual DENV-1 to DENV-4 in Vero cells. In addition, ADE assay for the HuMAbs with THP-1 cells to individual serotypes of DENV were performed. Figure 7 shows the activities of individual HuMAbs in the concentrations ranging from 25.0 to 0.20 micrograms/ml by serial 2-fold dilution of 25.0 micrograms/ml for VN assay to DENV-2 in Vero cells and in the concentrations ranging from 10.0 to 0.0001 micrograms/ml by serial 10-fold dilution of 10.0 micrograms/ml for ADE assay to DENV-2 in THP-1 cells. As controls, 4G2 murine MAb as a positive control and 5E4 human anti-influenza MAb as a negative control were used. The results for VN and ADE were shown in individual HuMAbs. Although the HuMAb concentrations showing the highest ADE activity were different with each other, all the HuMAbs showed ADE activities. In Figure 8, profiles of ADE activities for the representative HuMAbs D23-1B3B9 and D23-1G7C2 are shown. As a control antibody, we used 4G2 murine MAb. Interestingly, ADE activities for these HuMAbs against DENV-1 were lower than that of 4G2 and those against DENV-2 and -4 were almost similar as that of 4G2. The ADE activities of the HuMAbs againstDENV-1 were almost none, similarly as 4G2. Above data were obtained using representative laboratory strains belonging to individual four serotypes of DENV. Next, we examined the reactivities of 18 HuMAbs to a total of 20 clinical isolates, i.e., five in each serotype of DENV, which were prepared

from the blood samples from patients at 2007 to 2010 (Table 7). The reactivities of HuMAbs in the culture fluids of individual hybridomas to these 20 clinical isolates were examined by indirect IF assay (Table 7). All HuMAbs showed reactivities to all the clinical isolates examined, although D23-1C1G4 to DV1-1 to -5 and D23-1C1G4 and D23-1H5A11 to DV4-3 showed weak reactivities. When we comparatively examined the detection limits by IF assay (Figure 9) and VN50 (Figure 10) of the three representative HuMAbs, D23-1A10H7, D23-1B3B9, and D23-1G7C2 to these clinical isolates. The results showed essentially stronger activities in both activities by IF assay (Figure 9) and VN assay (Figure 10) compared with the controlled DENV-2, 16681 (Figures 5 and 6).

[0112]

[Table 7]

Table 7. Summarized results of HuMAbs on their reactivity with DENV clinical isolates by indirect IF assay[a].

DV serotype	Clinical isolate	Isolated year	D23													D30			D32	
			1A 10H7	1B 3B9	1C 1G4	1C 2D2	1G 7C2	1H 5A11	3A 10G12	4A 6F9	4F 5E1	4H 12C8	5E 6B1	5G 2D2	5G 8E3	1E 7B8	3A 1E2	3B 6C7	2D 1G5	2H 8G1
DV1	DV1-1	2007	+	+	Weak	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV1-2	2008	+	+	Weak	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV1-3	2008	+	+	Weak	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV1-4	2008	+	+	Weak	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV1-5	2008	+	+	Weak	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DV2	DV2-1	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV2-2	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV2-3	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV2-4	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV2-5	2009	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DV3	DV3-1	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV3-2	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV3-3	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV3-4	2009	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV3-5	2010	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DV4	DV4-1	2007	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DV4-2	2007	+	+	Weak	+	+	Weak	+	+	+	+	+	+	+	+	+	+	+	+
	DV4-3	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D4-4	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D4-5	2008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

a The culture fluid of hybridoma producing HuMAbs was used for the reactivities with Vero cells infected with each clinical isolates and fixed with glutaraldehyde by indirect IF assay.

[0113]

FIG. 1 illustrates summary of HuMAbs and their cross-reactivity to DENV-1 to -4 in IF and VN assays. A total of 121 acute-phase HuMAbs and 15 convalescent-phase HuMAbs were examined for cross-reactivity and cross-neutralization against DENV serotypes in IF and VN assays, respectively. Culture fluids of HuMAb-producing hybridoma clones were used. The HuMAbs were classified as groups 1 to 10 and groups A to X according to their cross-reactivity against the four serotypes of DENV in IF assay and VN assay, respectively. Individual groups are shown by different colors. Vero cells individually infected with DENV-1 to -4 were used as target cells in these assays. The data from VN assay are shown by the degree of neutralization ("-", <50%; "+", 50% to <90%; and "++", 90% neutralization).

[0114]

FIG. 2 illustrates representation of the percentages of HuMAbs obtained from individual patients for their cross-reactivity with DENV-1 to -4 by IF and VN assays. A total of 121 acute-phase HuMAbs (75 from D23, 25 from D30, five from D32, and 16 from D33) and 15 convalescent-phase HuMAbs (four from D22, five from D25, two from D26, two from D27, and two from D28) were examined for their cross-reactivity and cross-neutralization against four serotypes of DENV. Culture fluids from the HuMAb-producing individual hybridoma clones were used for these assays. The HuMAbs were classified into groups 1 to 10 and groups A to X according to their cross-reactivity with the four serotypes of DENV as assessed by IF assay and VN assay, respectively. Individual groups are shown by different colors. Vero cells individually infected with DENV-1 to -4 were used as target cells in these assays. The data from VN assays are shown by the degree of neutralization ("+", 50% to <90%; and "++", 90% neutralization).

[0115]

FIG. 3 shows staining profiles of HuMAbs by IF assay. The HuMAbs in the culture fluids of hybridoma clones producing DENV serotype-specific (D28-2B11D10 in group 1 and D23-4A7D6 in group 2), cross-reactive with two serotypes (D28-2B11F9 in group 3 and D23-1B11A5 in group 4), and cross-reactive with three serotypes (D25-4D3D2 in group 7 and D23-3E6D7 in group 8), and cross-reactive with all four serotypes (D22-1B7G2 in group 10) antibodies were used for IF. Vero cells mock-infected with PBS or individually infected with DENV-1 to -4 were used as target cells. As a positive

control, 4G2 anti-flavivirus E mouse MAb was used.

[0116]
FIG. 4 shows a correlation between IF and VN results. A total of 121 acute-phase HuMAbs and 15 convalescent-phase HuMAbs are shown separately to highlight the correlation between IF and VN ("-", <50%; "+", 50% to <90%; and "++", 90% neutralization) according to their cross-reactivity with different DENV serotypes (groups 1 to 10 according to the IF assay and groups A to X according to the VN assay). Culture fluids of HuMAb-producing hybridoma clones were used. Individual groups are shown by different colors, as in Figure 1. Vero cells individually infected with DENV-1 to -4 were used as target cells in these assays.

[0117]
FIG. 5 shows binding limits of 18 HuMAbs to four serotypes of DENV by indirect IF assay. Vero cells in 96-well microplate were infected with DENV. After incubation for 2 days, the cells were fixed with formaldehyde, then reacted with the serial 10-fold dilutions of the purified individual HuMAbs (10.0 micrograms/ml). As the titer for the reactivity to individual serotypes of DENV by indirect IF assay, the final antibody concentration showing positive reaction is shown in figure.

[0118]
FIG. 6 shows VN activity of 18 HuMAbs to four serotypes of DENV. Vero cells in 96-well microplate were infected with individual serotypes of DENV which had been incubated with serial 2-fold dilutions of the purified HuMAbs (25.0 micrograms/ml) at 37 centigrade for 30 min, and incubated at 37 centigrade for overnight. Finally, the cells were fixed and infected cells were detected by IF assay with 4G2.

[0119]
FIGs. 7 show VN and ADE activities of 18 HuMAbs to DENV-2. For VN assay, Vero cells were infected with DENV2 (16681 strain) which had been incubated with serial 2-fold dilutions of the purified HuMAbs (25.0 micrograms/ml) at 37 centigrade for 30 min, and incubated at 37 centigrade for overnight. Finally, the cells were fixed and infected cells were detected by IF assay with 4G2. For ADE assay, THP-1 cells were infected with DENV-2 (16681 strain) which had been incubated with serial 10-fold dilutions of the purified HuMAbs (10.0 micrograms/ml) at 37 centigrade for 30 min, then incubated for 3 days. Total RNA was extracted from the infected cells and applied to one-step

real-time PCR for estimating viral genome copies. Vertical green line indicates 1 microgram/ml of HuMAb in both figures for VN and ADE results. Horizontal red line in figures for ADE results indicates the basal level (x1) in the control (PBS).FIG. 7-1 shows 4G2, 5E4, D23-1A10H7 and D23-1B3B9.FIG. 7-2 shows D23-1C2D2, D23-1G7C2, D23-1H5A11 and D23-3A10G2.FIG. 7-3 shows D23-4A6F9, D23-4F5E1, D23-4H12C8 and D23-5E6B1.FIG. 7-4 shows D23-5G2D2, D23-5G8E3, D23-1C1G4 and D30-1E7B8.FIG. 7-5 shows D30-3A1E2, D30-33B6C7, D32-2D1G5 and D32-2H8G1.

[0120]
FIG.8 shows ADE activities of representative two HuMAbs to four serotypes of DENV. For ADE assays, four serotypes of DENV (Mochizuki strain of DENV-1; 16681 strain of DENV-2; H87 strain of DENV-3; and H241 strain of DENV-4) were used as in Fig. 7. The infection was performed at an MOI of 0.05.

[0121]
FIG.9 shows IF binding activity of representative HuMAbs to clinical isolates of DENV. Representative HuMAbs, D23-1A10H7, D23-1B3B9, and D23-1G7C2 were examined for the detection limit by IF assay to clinical isolates DV1-1 to DV1-5, DV2-1 to DV2-5, DV3-1 to DV3-5, and DV4-1 to DV4-5.

[0122]
FIG.10 shows VN activity of representative HuMAbs to clinical isolates of DENV. Representative HuMAbs, D23-1A10H7, D23-1B3B9, and D23-1G7C2 were examined for VN50 assay to clinical isolates DV1-1 to DV1-5, DV2-1 to DV2-5, DV3-1 to DV3-5, and DV4-1 to DV4-5.

[0123]
FIG.11 shows the results of in vivo therapeutic efficiency of HuMAbs in suckling mice.

[0124]
FIG.12 shows examples of the pictures of Immunofluorescent assay of positive control, negative control, Hybridoma clones DMSc-17, DMSc-14 and DMSc-36.

[0125]
FIG.13 shows illustration of 22 clones showing VN reduction to DENV1, DENV2, DENV3, DENV4 higher than 80%. All clones showed VN to DENV1 and DENV2

higher than 90%. The 10 clones showed VN to DENV3 and 16 clones showed VN to DENV4 higher than 90%.

[0126]
The HuMAbs to neutralize DENV-1 to DENV-4 by in vitro assay as above were next examined for their in vivo therapeutic efficacy by animal model using suckling mice. The mice (n=10) were intracerebrally inoculated with the representative HuMAbs, D23-1B3B9 and D23-1G7C2 (1 microgram or 0.2 micrograms per head) that had been incubated with 20,000 FFU of DENV-2 16681 per head for 30 min in ice-cold condition. PBS in place of the solution containing HuMAb was used as a negative control. During our observation for 25 days, one in 10 mouse treated with HuMAbs were died, although all mice treated with PBS were died around 13 to 15 days after virus inoculation (Figure 11).

[0127]
A total of 136 hybridoma clones producing specific HuMAbs against DENV were obtained using PBMCs from nine blood samples from eight patients. The four acute-phase patients were all secondarily infected with DENV-2. These samples efficiently generated hybridomas producing specific and robust HuMAbs [121 clones: 99 recognizing E, eight recognizing prM, four recognizing NS1, none recognizing C, and 10 recognizing other unknown viral protein(s)], compared with those from the five convalescent-phase patients (15 clones: two recognizing E, two recognizing prM, eight recognizing NS1, none recognizing C, and three recognizing other unknown proteins). In addition, 90.1% (109/121) of the acute-phase HuMAb clones were cross-reactive with all four serotypes of DENV by IF. Most of these (88.1%; 96/109) recognized the viral E protein, while only 7.3% (8/109) recognized prM, 0.9% (1/109) recognized NS1, and 3.7% (4/109) recognized other viral proteins which were not identified in this study. By contrast, 46.7% (7/15) of the convalescent-phase HuMAbs were cross-reactive with all four serotypes of DENV by IF: 28.6% (2/7) recognized E, 28.6% (2/7) recognized prM, 28.6% (2/7) recognized NS1, and 14.3% (1/7) recognized other unknown proteins. VN assays also revealed that a greater proportion of HuMAbs prepared from acute-phase PBMCs were able to neutralize all four serotypes of DENV [57.9% (70/121) acute-phase clones, versus 6.7% (1/15) convalescent-phase clones]. Antibodies at the acute phase showed complex cross-reactivity with all four DENV serotypes, with much stronger VN activity not only against DENV-2, which was replicating in the patient, but also against the other serotypes of DENV and against JEV. Of note, 18 hybridoma clones that were

selected as producers of HuMAbs to neutralize all four serotypes of DENV at higher rates had similar characterization: stronger binding activity to DENV antigen and stronger VN activity to all four serotypes of DENV than control 4G2 murine neutralizing MAb; and lower ADE activities to DENV-1 to DENV-4 than 4G2.

[0128]
There are several options for methods to prepare HuMAbs: humanization of murine MAbs, the creation of chimeras of human and murine MAbs, HuMAb preparation by phage display, immortalization of antibody-producing cells by EBV, and cell-to-cell fusion of human antibody-producing cells with myeloma cells (Marasco et al., Nat Biotech 25: 1421-1434 (2007)). In this study, a cell-to-cell fusion procedure was used with newly-developed fusion partner cells, called SPYMEG cells, with which we have also prepared several HuMAbs against a seasonal influenza virus from healthy volunteers inoculated with influenza vaccine (Kubota-Koketsu et al., Biochem Biophys Res Commun 387: 180-185 (2009)). Thus, this procedure may allow for the generation HuMAbs at a high rate.

[0129]
PBMC samples in this study were collected from patients at the acute phase (5-8 days after the onset of fever) or at the convalescent phase (12-19 days for convalescent phase) of secondary infection. This study enabled us to compare the efficiency of obtaining HuMAbs at each stage. From the acute-phase PBMCs, 81.8% anti-E, 6.6% anti-prM, and 3.3% anti-NS1 HuMAbs were obtained, while 13.3% anti-E, 13.3% anti-prM, and 53.3% anti-NS1 HuMAbs were obtained from convalescent-phase PBMCs. Several groups have used PBMCs from convalescent-phase, but not acute-phase, patients to prepare HuMAbs by immortalizing patient-derived B cells with EBV. Dejnirattisai et al. (Dejnirattisai et al., Science 328: 745-748 (2010)) prepared anti-E and anti-prM HuMAbs using B cells from seven DENV-infected patients 15-24 days after defervescence. They observed that 89% of anti-E HuMAbs were cross-reactive with all four serotypes. Surprisingly, their studies resulted in the preparation of more anti-prM than anti-E HuMAbs. The anti-prM HuMAbs were also highly cross-reactive with all four serotypes of DENV (94%) and potently promoted ADE. In contrast to anti-E HuMAbs (which showed 64% cross-reactivity), only 3% of anti-prM HuMAbs cross-reacted with JEV. Also using the immortalization method, Bettramello et al. (Beltramello et al., Cell Host Microbe 8: 271-283 (2010)) used B cells from three primarily-infected patients at 200 days to 8 years after infection and from two

secondarily-infected patients at 212-510 days after infection. Since domain III of the E protein is the main target of anti-flavivirus neutralizing antibodies in mice (Oliphant et al., J Virol 81: 11828-11839 (2007)), they also performed a large screen to gain insights into the domain specificity and cross-reactivity of E domain III-specific antibodies isolated from two patients, one with a primary infection (donor 13) and the other with a secondary infection (donor 12). From donor 13, 18 of 152 HuMAbs were reactive with domain III (13 type-specific, one cross-reactive with three serotypes, and four cross-reactive with all four serotypes). From donor 12, 138 of 313 HuMAbs were reactive with domain III (44 type-specific, 31 cross-reactive with two or three serotypes, and 63 cross-reactive with all four serotypes). A study by de Alwis et al. (de Alwis et al., PLoS Negl Trop Dis 5: e1188 (2011)) showed the preparation of anti-DENV HuMAbs using memory B cells from two patients who had been infected with DENV 1-8 years previously. Most of the HuMAbs they obtained were weakly neutralizing and did not recognize the E protein. Thus, the efficiency of preparation of DENV complex cross-reactive neutralizing HuMAbs was significantly higher in secondary infection cases. Indeed, Beltramello et al. differentiated their HuMAbs into two categories: those that recognized the DENV E domain III and showed complex cross-reactive neutralization activity, and those that recognized domain I/domain II and were more broadly cross-reactive but showed lower neutralization activity. Furthermore, our data in this study is the first to report the efficacy of using PBMCs from acute-phase patients for preparing HuMAbs with strong VN activity against all four DENV serotypes by assay using Vero cells.

[0130]

It was an unexpected finding that acute-phase PBMCs were more efficient in the production of DENV-specific HuMAbs than convalescent-phase PBMCs, as neutralizing antibody titers tended to be slightly higher in convalescent-phase patients. This finding is similar to the findings of Wrammert et al. (Wrammert et al., Nature 453: 667-671 (2008)), who demonstrated a similar phenomenon for HuMAbs against the influenza virus in vaccinated donors. That study found a rapid and robust induction of influenza-specific IgG+ antibody-secreting plasma cells, which accounted for up to 6% of the peripheral blood B cells at the peak of the response, approximately 7 days after vaccination. However, the influenza-specific IgG+ memory B cells fell to an average of 1% of all B cells by 14-21 days after vaccination. Generally, reports show a difference in the B cell phenotype between acute- and convalescent-phase patients with infectious diseases (Leyendeckers et al., Eur J Immunol 29: 1406-1417 (1999)).

[0131]
Consequently, many HuMAbs showing neutralizing activity could be obtained in the acute phase. In addition, VN assay of the HuMAbs obtained in this study classified them into heterogeneous groups: serotype-specific HuMAbs and cross-reactive HuMAbs with two, three, and all four serotypes of DENV. These HuMAbs will also be highly useful as probes to understand the complex mechanisms through which the same antibodies mediate neutralization and ADE of heterologous DENV serotypes. Further epitope mapping studies of these HuMAbs would help shed light on this important issue.

[0132]
All of the 18 HuMAbs showed strong neutralization, much higher against all four serotypes of DENV than 4G2 that we used as a positive control of murine anti-DENV E protein. In contrast, these HuMAbs showed ADE activities to DENV-1 to DENV-4 that were much lower than that of 4G2. Essentially, the VN activities of these HuMAbs were similar even when we examined against clinical isolates of DENV-1 to DENV-4 in Thailand. Several antibodies showed stronger VN activities than those to the laboratory strains. This seems to be derived from the origins for the HuMAbs, the PBMCs from patients who admitted to the Tropical Medicine hospital at Mahidol University from July to August 2010 (Puiprom et al., Biochem Biophys Res Commun 413: 136-142 (2011)). The clinical isolates were prepared from patients' blood samples who admitted to the same hospital from 2007 to 2010. Therefore, HuMAbs recognized DENVs that seem to be highly close to the clinical isolates we used in this study.

[0133]
Previous reports showed that antibodies from primary infections were more type-specific, while those from secondary infections were more heterogeneous and wide-ranging in their ability to cross-react with various serotypes (Beltramello et al., Cell Host Microbe 8: 271-283 (2010); Wahala et al., Virology 392: 103-113 (2009). The 18 HuMAbs in this study were all derived from the antibody-producing lymphocytes in 3 patients at acute phase of secondarily infected with DENV-2. Since such HuMAbs with stronger VN activity were not obtained from 5 patients in convalescent phase, such antibodies could be quickly generated only at acute phase after DENV-2 secondary infection, which could be a significant role as a boost stimulation of immune lymphocytes. Therefore, all the three patients could be primarily infected with DENV-2 serotype other than DENV-2 and therefore the HuMAbs used in this study

produced by priming the serotypes other than DENV-2 that seemed to be boost-stimulated by DENV-2 secondary infection. Interestingly, D23-1G7C2 we used for ADE assay to all four serotypes as a representative showed much higher ADE activity against DENV-2 and more against DENV-4 than those against DENV-1 and DENV-3. These profiles of ADE activities in this HuMAb were clearly different from those in control Murine MAb, 4G2. Another interesting hypothesis is that the epitope region recognized by most of HuMAbs in this study could be the central domain for transient production of common neutralizing antibodies during the period of their battle with DENV agent, because these HuMAbs could not be obtained by fusion of the PBMCs from any of 5 patients at convalescent phase (around 2 weeks after the onset of disease).

[0134]

Finally, we observed the perfect effect of HuMAb using D23-1G7C2 and D23-1B3B9 as representatives on the lethality by DENV injection using suckling mice. Further studies are needed to characterize their positive and negative effects in other in vivo evaluation systems, such as macaque monkeys, marmosets, and mice (Bernardo et al., Clin Vaccine Immunol 16:1829-1831 (2009); Zompi et al., Viruses 4:62-82 (2012); Sukupolvi-Petty et al., J Virol 84: 9227-9239 (2010); Omatsu et al., J Gen Virol 92: 2272-2280 (2011); Omatsu et al., J Med Primatol, in press (2012)) in order to apply them to the possible development of therapeutic antibody.

[0135]

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[Example 2]

[0136]

In the second trial, the blood specimens (1-8 ml in each) used for cell fusion were collected from 8 infant Thai patients with dengue illness at Pranangklao Hospital (Table 8). The participants were selected based on the clinically diagnosis and results of IgG and IgM levels. The research protocols for human samples were approved by the Ethics Committee of the Department of Medical Sciences, Ministry of Public Health, Thailand.

[0137]

In this study, SPYMEG (Kubota-Koketsu et al., Biochem Biophys Res Commun 387: 180-185 (2009)) was used as fusion partner cells to develop hybridomas producing specific HuMAbs. SPYMEG cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% of fetal bovine serum (FBS) in a 5% CO_2 incubator at 37 centigrade. Vero cells were maintained in Vero cells were maintained in Advanced Dulbecco's modified Eagle medium (Advanced DMEM) with 10% FBS in a 5% CO_2 incubator at 37 centigrade. Mosquito-derived cell line C6/36 was maintained in Leibovitz's L-15 medium with 10% FBS and 0.3% tryptose phosphate broth in an incubator at 28 centigrade.

[0138]

DENVs used in this study were the 16007 strain of DENV-1, the New Guinea C (NGC) strain of DENV-2, the H87 strain of DENV-3, and the H241 strain of DENV-4. The culture supernatants from C6/36 cells infected with individual strains were used as

viral stocks. Virus titer was estimated as focus-forming unit (FFU), as described previously (Kurosu et al., Biochem Biophys Res Commun 394: 398-404 (2010)).

[0139]

Serotypes of Dengue infection were detected by RT-PCR from plasma on the first day visit of the patients at the hospital. Total RNA was extracted from the plasma of patients using a QIAamp Viral RNA kit (QIAGEN, Hilden, Germany) or NucloSpin RNA kit (MN, Germany) by following the manufacturer's protocol. The RNA was used as the template for reverse transcription by using a one-step RT-PCR kit (QIAGEN, Hilden, Germany). The oligonucleotide primer pairs previously reported for serotyping (Yenchitsomanus et al., Southeast Asian J Trop Med Public Health 27: 228-236 (1996)) were used for amplification of DENV E gene including most part of domain III: the 1st PCR primers DUL (5'-TGGCTGGTGCACAGACAATGGTT-3' (SEQ. ID NO. 231)) /DUR (5'- GCTGTGTCACCCAGAATGGCCAT-3' (SEQ. ID NO. 232)) that are common to all the serotypes of DENV and the 2nd PCR primers D1L (5'-GGGGCTTCAACATCCCAAGAG-3' (SEQ. ID NO. 233)) / D1R (5'-GCTTAGTTTCAAAGCTTTTTCAC-3' (SEQ. ID NO. 234)), D2L (5'-ATCCAGATGTCATCAGGAAAC-3' (SEQ. ID NO. 235))/ D2R (5'-CCGGCTCTACTCCTATGATG-3' (SEQ. ID NO. 236)), D3L (5'-CAATGTGCTTGAATACCTTTGT-3' (SEQ. ID NO. 237)) / D3R (5'-GGACAGGCTCCTCCTTCTTG-3' (SEQ. ID NO. 238)) and D4L (5'-GGACAACAGTGGTGAAAGTCA-3' (SEQ. ID NO. 239)) / D4R (5'-GGTTACACTGTTGGTATTCTCA-3' (SEQ. ID NO. 240)), that are specific to individual serotypes of DENV, DENV-1, -2, -3, and -4, respectively.

[0140]

Hybridomas were prepared as follows. About 1-8 milliliters of blood were obtained from individual patients and the PBMCs were prepared by centrifugation through Ficoll-PaqueTM PLUS (GE Healthcare, Uppsala, Sweden) for 40 min at 1,200x g. The PBMCs were fused with SPYMEG cells at a ratio of 10:1 with polyethylene glycol 1500 (Roche Diagnostics Japan, Tokyo, Japan). Fused cells were cultured in DMEM supplemented with 15% FBS and 3% BM-condimed (Roche Diagnostics Japan, Tokyo, Japan) in 96-well tissue culture plate for 10 to 14 days in the presence of hypoxanthine-aminopterin-thymidine (HAT; Invitrogen, USA or Sigma Aldrich, USA). The 1st screening of the culture media for antibody specificity against infected serotype of DENV was performed by indirect immunofluorescent (IF) assay. Wells producing

specific antibody were next subjected to cell cloning by limiting dilution. After 10 to 14 days, the 2nd screening was also performed by IF assay.

[0141]

The IF assay was performed as follows. Vero cells at 2.0 x 10^4 per well in a 96-well microplate were infected with DENV at MOI 0.5 and the plate were incubated at 37oC, 5% CO_2. The incubation period for DENV-1 and DENV-2 is 72 hr, and for DENV-3, DENV-4 is 48 hr. After incubation the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) at RT for 40-60 min and then perrmeabilized with 1% Triton X-100 in PBS at RT for 10 min. Microplates were washed with PBS 3 times and 50 microlitters of PBS were added to protect cell dehydration. To perform the assay, 50 microlitters of supernatant of hybridoma culture, positive and negative control diluted plasma were added to each well and incubated at 37oC for 1 h. Wells were then washed with PBS twice and 50 microlitters of conjugate FITC- anti-human IgG (dilution 1:500 in PBS) were placed into the wells and then incubated at 37oC for 45 min. Unbound antibody were washed out the same as described above and 50 microlitters of PBS were laid on the surface of the wells. The bound antibody was visualized under a fluorescent microscope.

[0142]

VN assay was carried out as follows. The 50 microlitters of undiluted supernatant of hybridoma culture or 2- fold serially diluted purified antibodies from 25 micrograms/ml to 0.20 micrograms/ml, and also advanced DMEM as a negative control, were mixed with 50-100 FFU of individual serotypes of DENV (50 microlitters) and incubated at 37 centigrade, 5% CO_2 for 15 min. Vero cells in a 96-well microplate were then neutralized with the mixture. After inoculation at 37 centigrade for 1 hr 30 min, 100 microlitters of overlay medium (4% CMC mixed with 2x MEM pH 7.0 and 2%FBS, 1% of 200 mMol L-glutamine) was added. After incubation at 37 centigrade, 5% CO_2, (3 days for DENV-1, DENV-2 and 2 days for DENV-3, DENV-4), the overlay medium was discarded and the plates were washed with PBS, and then the cells were fixed with 3.7% formaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS. The infected cells were reacted with 4G2 that is anti-flavivirus mouse MAb at 37 centigrade, 1 hr. The bound antibody was reacted with 1:500 of diluted conjugate HRP-rabbit anti-mouse in diluents buffer (PBS, 1%FBS, 0.05% Tween-20), and incubated at 37 centigrade, 1 hr. The plates were empty and washed with PBS and then 50 microlitters of substrate solution [0.1% of 3, 3' DAB (Sigma, USA), 0.4% of 3%H_2O_2) was added and

incubated in a dark box at RT. The color reaction was observed under a phase-contrast microscope. The assays were performed in triplicate and the results are expressed as their average. The neutralization activity from purified antibodies is expressed as the highest dilution showing more than 50% FFU reduction, compared with the negative control, named VN50. On the other hand, the neutralization activity of the culture medium from hybridoma clone is expressed as the percentage of FFU reduction, compared with the negative control.

[0143]

Immunoglobulin isotypes/subtypes of antibodies secreted from hybridoma clones were determined by using Bio-Plex ProTM Assays Immunoglobulin Isotyping kit (Bio-Rad, USA). The assay is able to determine IgG1, IgG2, IgG3, IgG4, IgA, IgE and IgM at the same time. The protocol was followed the manufacturer's instruction. Fifty l of culture supernatant from each hybridoma clone, known concentration of standard, and control were added into the well containing magnetic polystyrene color-coded bead labeled with different antibody specifically directed against all immunoglobulin isotypes/subtypes. After 30 min for reaction, the plate was washed to remove unbound proteins and a biotinylated detection antibody specific for a different epitope on the immunoglobulin class/subclass was added to the beads. This led to sandwich formation of antibodies around specific immunoglobulin isotypes/subtypes. The reaction was detected by adding streptavidin-phycoerythrin which bound to biotylated detection antibodies. The reaction mixture of each well were identified and quantitated based on bead color and fluorescence. The results were read and calculated by Bio-Plex Manager TH software using standard curve derived from each isotype standard.

[0144]

Antibody from each hybridoma clone was determined their specific target against 4 viral antigens, envelop (E), prM (premembrane), NS1, and C (capsid) protein by IF assay. Vectors for expression of the DENV-2 prM, E, NS1, C proteins and prM-E fusion were kindly provided from Prof. Kazuyoshi Ikuta Laboratory Osaka University, Japan. All expressed proteins were FLAG fusion proteins. The 293T cells were transfected with individual plasmids by using LipofectamineTM reagent (Invitrogen, USA). The transfected cells were used as viral antigens for determination of antibody specificity. To do so, smear of air-dried cells were made on multispots microscope slides and fixed with acetone containing 40% methanol. The slides can be stored at -80 centigrade until use. Ten microliters of culture supernatant of HuMAbs were placed onto the

slides and incubated for 60 min at RT. Slides were washed 3 times with PBS and then stained with FITC- conjugated rabbit anti-human IgG (Dako, Denmark) at dilution of 1:400 for 45 min at RT. After washing to remove unbound protein, the slides were mounted in PBS containing 10% glycerol and specific binding targets were examined under a fluorescent microscope.

[0145]

CDR regions of heavy chains and light chains from 8 hybridoma clones showing strong neutralization to all Dengue serotypes were sequenced. To do so, RNA was extracted from the hybridoma cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and subjected to RT-PCR using a SuperScript III First-Strand Synthesis Kit (Invitrogen) with an oligo(dT) primer. The coding region of H-chain and L-chain of HuMAb were PCR amplified separately using the following primer: 5'-ATG GAC TGG ACC TGG AGG ATC CTC-3' (SEQ. ID NO. 241), 5'-ATG GAC ATA CTT TGT TCC ACG CTC CT-3' (SEQ. ID NO. 242), 5'-ATG AAA CAC CTG TGG TTC TTC CTC CT-3' (SEQ. ID NO. 243), 5'-ATG TCT GTC TCC TTC CTC ATC TTC CT-3' (SEQ. ID NO. 244), 5'-ATG GAG TTT GGG CTG AGC TGG GTT-3' (SEQ. ID NO. 245), 5'-ATG GGG TCA ACC GCC ATC CTC GC-3' (SEQ. ID NO. 246) for 6 family H-chain forward and 5'-CCT TGG TGT TGC TGG GCT TGT GAT-3' (SEQ. ID NO. 247) for H-chain reverse; 5'-ATG GAC ATG AGG GTC CCC GCT CAG-3' (SEQ. ID NO. 248), 5'-ATG AGG CTC CCT GCT CAG CTC CTG-3' (SEQ. ID NO. 249), 5'-ATG GAA RCC CCA GCG CAG CTT CTC-3'(SEQ. ID NO. 250), 5'-ATG GTG TTG CAG ACC CAG GTC TTC AT-3' (SEQ. ID NO. 251), 5'-ATG GGG TCC CAG GTT CAC CTC CTC-3' (SEQ. ID NO. 252) for 5 family kappa L-chain forward and 5'-GAG TTA CCC GAT TGG AGG GCG TTA T-3'(SEQ. ID NO. 253) for kappa L-chain reverse; 5'-ATG GCC TGG WYY CCT CTC YTY CTS-3'(SEQ. ID NO. 254), 5'-ATG GCC TGG ATG ATG CTT CTC CTC G-3'(SEQ. ID NO. 255), 5'-ATG SCC TGG GCT CYK CTS CTC CTS-3'(SEQ. ID NO. 256), 5'-ATG GCC TGG RYC YCM YTC YWC CTM-3'(SEQ. ID NO. 257) for 4 family lambda L-chain forward and 5'-TGG CAG CTG TAG CTT CTG TGG GAC T-3'(SEQ. ID NO. 258) for lambda L-chain reverse. PCR products were ligated into pGEM T-Easy vector (Promega, Madison, WI) and their sequences were analyzed using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

[0146]

In this study, we prepared hybridomas producing HuMAbs against DENV by the use of the blood specimens from 8 infant Thai patients (Table 8) aged between 6 to 14 years old.

Among them 3 patients were clinically diagnosed as Dengue haemorrhagic fever (DHF) grade I and 5 patients were diagnosed as Dengue fever (DF). The patients were made an appointment for PBMCs donors 1-3 days after the first visit. The PBMC samples were collected at 4 to 12 days after onset of fever, 7 samples were obtained from patients at acute phase, and 1 from patient at convalescent phase (12 days after onset of fever). The data of IgM and IgG levels, determined from blood samples collected on the day of hybridoma preparation, showed that all patients were infected secondarily with DENV. Further, plasma samples from the first visit were also subjected to RT-PCR for serotyping of DENV. The results showed that 3 volunteers were infected with DENV-1, 1 with DENV-2, 1 with DENV-3 and the other 3 samples provided negative results to all serotypes.

[0147]
[Table 8]

Table 8 Summary of patients' background and HuMAbs obtained in this study

Patient	Gender	Diagnosis	Diagnosis	Blood collection[3]		ELISA[7]		PCR[8] serotype	Hybridoma obtained
				Day	Phase	IgG (unit)	IgM (unit)		
A	Male	12	DHF[1] grade I	12	Conv[4]	10	69	DENV-3	1
B	Male	11	DF	5	Acute[5]	35	62	Negative	2
C	Female	13	DF	4	Acute	90	6	Negative	1
D	Female	6	DHF grade I	8	Acute	138	100	DENV-1	14
E	Male	9	DHF grade I	6	Acute	29	63	DENV-2	15
F	Male	14	DF[2]	4	Acute	90	27	DENV-1	3
G	Female	11	DF	6	Acute	109	41	DENV-1	4
H	Female	6	DF	7	Acute	61	132	Negative	3

[1]Dengue Hemorrhagic fever
[2]Dengue Fever
[3] Blood collection on the day of hybridoma preparation
[4] Convalescent blood sample collected after 10 days of onset
[5] Acute blood sample collected between 5 to 9 days after onset
[7] Results from samples collected at the hybridoma preparation day
[8] Resultsfrom samples collected at the first visit day to hospital

[0148]
The PBMC fractions prepared from patients' blood were applied for the preparation of hybridoma by fusion with SPYMEG cells. The culture fluids of individual wells in 96-well microplates in which fused cells were seeded and cultured for 10-14 days in HAT selection medium were subjected to the 1st screening by IF. The cells in the wells showing positive signal by IF were next subjected to cell cloning by limiting dilution. After 2 weeks, the culture fluids of the wells containing a single colony were against subjected to the 2nd screening by IF. Consequently, eight fusion experiments with the PBMCs from 8 Thai infant patients secondarily infected with either DENVs produced a total of 43 hybridoma clones producing specific HuMAbs to DENV by IF as shown in Table 8. Of all hybridma clones, patient A, patient B, patient C, patient D, patient E, patient F, patient G and patient H provided 1, 2, 1, 14, 15, 3, 4, 3 clones, respectively.

[0149]
The 43 HuMAbs obtained as mentioned above were characterized their serological reactivity to all 4 DENV serotypes by IF and VN assays. We used HuMAbs in the fluids of individual hybridoma cell cultures for these assays. The summary of 43 clones with their serological activities is shown in Table 9. The IF score and VN activity were varied up to each clone, IF score ranged from negative to 3+ and VN were from 0 to 100%. Examples of IF assay are given in Figure 12. By IF assay, most clones showed cross reactive to all DENV serotypes and IF results could be differentiated into 8 patterns, (I, II, III, IV, V, VI, VII and VIII) as shown in Table 10. It was found that 29 clones (67.4%) belong to Pattern I that cross reacted to all DENV serotypes. Clones characteristics as Patterns II, III, IV, V, VI, VII and VIII are 2, 2, 2, 1, 3, 2, 2, respectively. As summarized in Table 11 and Figure 13, a total 22 hybridoma clones produced HuMAbs with strong neutralization (>80% reduction of DENV replication) against all DENV serotypes. Especially, 10 HuMAbs (DMSc- 4, 5, 14, 24, 28, 31, 34, 36, 38, 41) showed VN reduction activity higher than 90% to all serotypes and 12 HuMAbs (DMSc-1, 7, 8. 13, 16, 17, 21, 30, 33, 37. 39. 40) showed VN reduction rate higher than 80 % to all serotypes. Interestingly, all clones showed strong VN to DENV1 and DENV2 higher than 90%, 10 clones and 16 clones showed VN reduction DENV-3 and DENV4 higher than 90%, respectively. In this study, as a whole, HuMAbs showed the lowest neutralization to DENV-3.

[0150]

[Table 9-1]

Table 9-1 Summarized results of 43 HuMAbs, cross reactivities and viral neutralization reduction to DENV-1 to DENV-4

Patient	No.	Hybridoma clone	Code	IFA				VN (% reduction)			
				DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4
A (M, 12 yr)	1	15H4	DMSC-1	+	++	+	+	100	100	82.3	98.7
B (M, 11 yr)	2	24C2	DMSC-2	+	+++	+	++	69.6	96.0	79.5	83.3
	3	24C3	DMSC-3	-	+	+	-	0	15.1	37.0	25.0
C (F, 13 yr)	4	73E3	DMSC-4	++	++	++	++	98.2	100	90.8	92.7
D (F, 6 yr)	5	74B11	DMSC-5	+	+++	++	+++	100	100	93.9	91.0
	6	74H1	DMSC-6	-	+	-	-	23.3	5.6	18.7	0
	7	74H2	DMSC-7	++	+++	+	++	93.3	99.6	79.7	91.8
	8	74H10	DMSC-8	++	++	++	++	96.4	100	88.4	91.8
	9	75D11	DMSC-9	+++	+++	++	+++	92.8	99.0	73.6	84.8
	10	75E4	DMSC-10	+	+	+	-	100	100	16.7	38.7
	11	76B3	DMSC-11	+	+	++	-	18.7	82.7	60.1	57.4
	12	76C1	DMSC-12	-	-	+	-	9.5	27.9	40.2	42.7
	13	76E3	DMSC-13	++	++	+++	++	97.7	99.6	86.5	94.8
	14	76F3	DMSC-14 (NIH 11)	++	+++	+	++	99.5	99.6	90.2	93.0
	15	76F6	DMSC-15	++	+++	+	++	93.6	100	58.9	90.2
	16	76H6	DMSC-16	++	+++	++	++	96.6	99.6	79.0	93.4
	17	77G2	DMSC-17 (NIH 9)	+++	+++	++	+++	98.7	100	88.4	95.3
	18	77E10	DMSC-18	±	+	+	±	80.7	100	71.6	89.5
E (M, 9 yr)	19	78A6	DMSC-19	-	+	+++	-	10.2	31.6	9.1	0.8
	20	78C4	DMSC-20	-	+	-	-	20.0	2.4	42.7	37.8
	21	78C7	DMSC-21	++	+++	++	++	99.5	100	83.9	90.8
	22	78D5	DMSC-22	+++	+++	++++	++	99.2	100	69.6	94.3
	23	78E2	DMSC-23	-	-	+++	-	49.5	4.8	0	4.8
	24	78E3	DMSC-24	±	++	+	++	99.5	100	93.9	98.8
	25	78E5	DMSC-25	++	++	-	-	89.2	95.6	0	43.4
	26	78E6	DMSC-26	+++	++	+++	+++	82.5	100	79.7	83.8

[0151]

[Table 9-2]

Table 9-2 Summarized results of 43 HuMAbs, cross reactivities and viral neutralization reduction to DENV-1 to DENV-4

Patient	No.	Hybridoma clone	Code	IFA				VN (% reduction)			
				DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4
E (M, 9 yr)	27	78F2	DMSC-27	++	+++	-	++	100	99.4	70	97.4
	28	78F3	DMSC-28 (NIH 11)	++	++	+	++	99.6	100	96.3	96.0
	29	78F4	DMSC-29	+	++	-	-	91.7	100	27.5	72.7
	30	78F5	DMSC-30	++	+++	+	+++	99.4	100	88.3	95.4
	31	78G2	DMSC-31	++	+++	++	+++	99.5	100	90.2	95.3
	32	78G4	DMSC-32	+	+	-	+	94.3	100	37.7	87.7
	33	78H6	DMSC-33	++	++	++	++	91.9	97.4	85.2	97.0
F (M, 14 yr)	34	80C2	DMSC-34	++	++	++	++	100	100	90.8	96.5
	35	80D5	DMSC-35	+	++	+	+	67.0	94.3	66.7	85.5
	36	80E5	DMSC-36 (NIH 15)	++	++	++	++	99.5	99.6	92.6	96.0
G (F, 11 yr)	37	84A12	DMSC-37	++	++	++	++	91.1	100	87.3	94.9
	38	84C10	DMSC-38	++	+++	++	+++	100	100	91.3	93.6
	39	85C9	DMSC-39	+++	+++	++	++	100	98.9	80.2	93.0
	40	85H7	DMSC-40	+++	+++	+	++	96.9	100	85.8	91.3
H (F, 6 yr)	41	87G5	DMSC-41	±	++	+	+	100	100	95.7	100
	42	88F2	DMSC-42	-	+	±	±	10.5	2.7	36.6	22.8
	43	88B9	DMSC-43	-	+	+	-	42.2	37.9	73.6	28.0
	22	78D5	DMSC-22	+++	+++	++++	++	99.2	100	69.6	94.3
	23	78E2	DMSC-23	-	-	+++	-	49.5	4.8	0	4.8
	24	78E3	DMSC-24	±	++	+	++	99.5	100	93.9	98.8
	25	78E5	DMSC-25	++	++	-	-	89.2	95.6	0	43.4
	26	78E6	DMSC-26	+++	++	+++	+++	82.5	100	79.7	83.8

[0152]

[Table 10]

Patterns of IFA of 43 clones obtained in this study

Pattern	IF				No. of clone	Clone name
	DENV-1	DENV-2	DENV-3	DENV-4		
I	+	+	+	+	29	DMSc-1, 2, 4, 5, 7, 8, 9,13, 14, 15, 16, 17, 18, 21, 22, 24, 26,28, 30, 31, 33, 34, 35, 36, 37, 38, 39, 40, 41
II	+	+	+	-	2	DMSc-10, 11
III	+	+	-	-	2	DMSc25, 29
IV	+	+	-	+	2	DMSc-27, 32
V	-	+	+	+	1	DMSc-42
VI	-	+	+	-	3	DMSc-3, 19, 43
VII	-	+	-	-	2	DMSc-6, 20
VIII	-	-	+	-	2	DMSc-12, 23

Pattern I = ++++, Pattern II= +++-, Pattern III=++--, Pattern IV = ++-+,

[0153]

Results of imunoglobulin isotypes and specific-binding targets of 22 clones are given in Table 11. It was found that all antibodies secreted from all clones belong to IgG1 and specific to envelope protein.

[0154] [Table 11]

Table 11. Summarized results of 22 HuMAbs, showed cross reactivity and viral neutralization reduction to DENV-1 to DENV-4, Ig isotypes, and their specific target protein

Patient	No.	Hybridoma clone	Code	IFA				NT (% reduction)				Ig isotype	Target protein
				DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4		
A	1	15H4	DMSC-1	+	++	+	+	100	100	82.3	98.7	IgG1	E
C	2	73E3	DMSC-4	++	++	++	++	98.2	100	90.8	92.7	IgG1	E
D	3	74B11	DMSC-5	+	+++	++	+++	100	100	93.9	91.0	IgG1	nd
	4	74H2	DMSC-7	++	+++	+	++	93.3	99.6	79.7	91.8	IgG1	E
	5	74H10	DMSC-8	++	++	++	++	96.4	100	88.4	91.8	IgG1	E
	6	76E3	DMSC-13	++	++	+++	++	97.7	99.6	86.5	94.8	IgG1	E
	7	76F3	DMSC-14 (NIH 11)	++	+++	+	++	99.5	99.6	90.2	93.0	IgG1	E
	8	76H6	DMSC-16	++	+++	++	++	96.6	99.6	79.0	93.4	IgG1	nd
	9	77G2	DMSC-17 (NIH 9)	+++	+++	++	+++	98.7	100	88.4	95.3	IgG1	E
E	10	78C7	DMSC-21	++	+++	++	++	99.5	100	83.9	90.8	IgG1	nd
	11	78E3	DMSC-24	±	++	+	++	99.5	100	93.9	98.8	IgG1	nd
	12	78F3	DMSC-28	++	++	+	++	99.6	100	96.3	96.0	IgG1	E
	13	78F5	DMSC-30	++	+++	+	+++	99.4	100	88.3	95.4	IgG1	nd
	14	78G2	DMSC-31	++	+++	++	+++	99.5	100	90.2	95.3	IgG1	E
	15	78H6	DMSC-33	++	++	++	++	91.9	97.4	85.2	97.0	IgG1	E
F	16	80C2	DMSC-34	++	++	++	++	100	100	90.8	96.5	IgG1	nd
	17	80E5	DMSC-36 (NIH 15)	++	++	++	++	99.5	99.6	92.6	96.0	IgG1	E
G	18	84A12	DMSC-37	++	++	++	++	91.1	100	87.3	94.9	IgG1	E
	19	84C10	DMSC-38	++	+++	++	+++	100	100	91.3	93.6	IgG1	E
	20	85C9	DMSC-39	+++	+++	++	++	100	98.9	80.2	93.0	IgG1	nd
	21	85H7	DMSC-40	+++	+++	+	++	96.9	100	85.8	91.3	IgG1	nd
H	22	87G5	DMSC-41	±	++	+	+	100	100	95.7	100	IgG1	E

E= envelope protein, nd = not determined

[0155]

In this study, we selected hybridoma clones showing strong neutralization to all DENV serotypes higher than 90% for CDR sequencing. Sequencing of CDR of heavy chains and light chains for 8 of 10 clones were completely done as shown Table 12.

[0156]

[Table 12-1]

Table 12- 1 The deduced amino acid sequences of CDRs in the V_H and V_L of 15 HuMAbs.

HuMAb clone	CDR1[a]	CDR2[a]	CDR3[a]
DMSC-4	DYGIQ (SEQ ID:No.55) TGTNSDVGGYNFVS (SEQ ID:No.154)	WINAGNGDTKYSQKLQA (SEQ ID:No.56) DVSNRPS (SEQ ID:No.155)	GLGYCSNTNCDSYYYYYMDV (SEQ ID:No.57) SSYTTSSTLV (SEQ ID:No.156)
DMSC-5	DHAMH (SEQ ID:No.58) TGADSDVGVYSLVS (SEQ ID:No.157)	GISWNSGGILYADSVKG (SEQ ID:No.59) EGTKRPS (SEQ ID:No.158)	DLGHCTTTTCSGSVY (SEQ ID:No.60) CSYAGVTTPGIV (SEQ ID:No.159)
DMSC-14	TYWMN (SEQ ID:No.61) TGADSDVGVYSLVS (SEQ ID:No.160)	NIKQDGTEKYYVDSVKG (SEQ ID:No.62) EGTKRPS (SEQ ID:No.161)	GNPPFYDISTGYQGAYFDY (SEQ ID:No.63) CSYAGVTTPGIV (SEQ ID:No.162)
DMSC-24	KYAMA (SEQ ID:No.64) SGSTSNIGNNYVY (SEQ ID:No.163)	AIGASGDSPYYADSVKG (SEQ ID:No.65) NNNQRPS (SEQ ID:No.164)	GEQWPGVL (SEQ ID:No.66) TAWDDYLSGPL (SEQ ID:No.165)

[a] The upper and lower sequences indicate the CDRs of heavy chain (V_H) and light (V_L) chains.

[0157]

[Table 12-2]

Table 12- 2 The deduced amino acid sequences of CDRs in the V_H and V_L of 15 HuMAbs.

DMSC -31	DDAYSWS (SEQ ID:No.70) TGTSSDVGGYNFVS (SEQ ID:No.169)	YIYHSGAAFYNPSLKS (SEQ ID:No.71) EVIKRPS (SEQ ID:No.170)	TGSRVLDP (SEQ ID:No.72) SSYGGNNNVV (SEQ ID:No.171)
DMSC -36	SFAMH (SEQ ID:No.73) SGSISNIGNNYVS (SEQ ID:No.172)	VISYDGSDKYYADSVKG (SEQ ID:No.74) DNNKRPS (SEQ ID:No.173)	GYNSPKSYFDY (SEQ ID:No.75) GTWDGSLSAL (SEQ ID:No.174)
DMSC -38	ASAMQ (SEQ ID:No.76) TGTSSDVGGYNSVS (SEQ ID:No.175)	LISGDGGTTYYADSVKG (SEQ ID:No.77) EVNKRPS (SEQ ID:No.176)	YTADPWALRDY (SEQ ID:No.78) NSYAGSKIV (SEQ ID:No.177)
DMSC -37	SYVIS (SEQ ID:No.79) TGTGSDVGGYNYVS (SEQ ID:No.178)	SIMPVFGSTQYAQKFQG (SEQ ID:No.80) DVSKRPS (SEQ ID:No.179)	TSASAGRPTYYFDH (SEQ ID:No.81) CSYVGSYTWV (SEQ ID:No.180)
DMSC -8	ASAMQ (SEQ ID:No.82) TGPSSDVGDYNYVS (SEQ ID:No.181)	LISGDGGTTYYADSVKG (SEQ ID:No.83) DVTERPS (SEQ ID:No.182)	GYNSGHYFDY (SEQ ID:No.84) SSYRINNTPI (SEQ ID:No.183)
DMSC -13	TYWMN (SEQ ID:No.85) TGADSDVGVYSLVS (SEQ ID:No.184)	NIKQDGTEKYYVDSVKG (SEQ ID:No.86) EGTKRPS (SEQ ID:No.185)	GNPPFYDISTGYQGAYFDY (SEQ ID:No.87) CSYAGVTTPGIV (SEQ ID:No.186)
DMSC -17	NYAMH (SEQ ID:No.88) TGTISDIGGHNDVS (SEQ ID:No.187)	VISYVVTTKYYADSVKG (SEQ ID:No.89) DVNKRPS (SEQ ID:No.188)	ELVSSEWDSDAEYFQY (SEQ ID:No.90) CSYAGRWV (SEQ ID:No.189)

* The upper and lower sequences indicate the CDRs of heavy chain (V_H) and light (V_L) chains.

[0158]

[Table 12-3]

Table12-3 The deduced amino acid sequences of CDRs in the V_H and V_L of 15 HuMAbs.

DMSC -30	TGDMCLS (SEQ ID:No.91) AGTSSDINDYNYVS (SEQ ID:No.190)	LIHRDDDTYYSTSLKT (SEQ ID:No.92) EVSKRPS (SEQ ID:No.191)	SLDYGDYVVGYAFDV (SEQ ID:No.93) NSFTSSSPSVL (SEQ ID:No.192)
DMSC -2	TETIS (SEQ ID:No.94) RASQSVSSNYLA (SEQ ID:No.193)	EIIPVFRTANYAQKFQG (SEQ ID:No.95) SAFNRAT (SEQ ID:No.194)	KNCTVNSCYAWFDP (SEQ ID:No.96) QQLRT (SEQ ID:No.195)
DMSC -1	SYSMN (SEQ ID:No.97) TGTSSDVGGYNYVS (SEQ ID:No.196)	SISTTSSFIAYADSVKG (SEQ ID:No.98) DVSKRPS (SEQ ID:No.197)	VYCSTTSCSPNYFDS (SEQ ID:No.99) CSYAGTYT (SEQ ID:No.198)

* The upper and lower sequences indicate the CDRs of heavy chain (V_H) and light (V_L) chains.

[0159]

The 8 infants patients used in this study, 3 patients were clinically diagnosed as DHF and 5 patients were diagnosed as DF. All were laboratory confirmed by Dengue IgG and IgM units. PCR serotype of Dengue infection showed negative results for 3 patients with DF. This might come from the small number of virus particles presence in the blood on the first visit day. A total of 43 hybridoma clones were obtained as producers of specific HuMAbs against DENV by the use of the PBMCs from 8 infant patients in Thailand. The 29 of 43 clones (67.4%) showed cross reaction to all Dengue serotypes by IF assay. Among them, 22 clones showed strong neutralization to all dengue serotypes higher than 80%. Further, 17 HuMAbs (DMSc-4, 5, 8, 13, 14, 17, 24, 28, 30, 31, 33, 34, 36, 37, 38, 40, 41) showed >85 neutralization to all DENV serotypes. At present, 12 of 17 clones (DMSc- 4, 5, 8, 13, 14, 17, 24, 30, 31, 36, 37, 38,) and 2 clones (DMSc-1,2) previously appeared in provisional patent were completely sequenced. The 22 HuMAbs could be candidate for development of therapeutic antibodies. Further, these HuMAbs also highly useful as probes to understand the complicate phenomenon how heterogeneous DENV serotypes showing neutralization as well as ADE with the same antibodies. Further studies for epitope mapping of these HuMAbs would be helpful to solve this phenomenon.

[0160]

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[0161]
Applicants specifically incorporate the entire contents of all cited references in this disclosure. Further, when an amount, concentration, or other value or parameter is given as either a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the invention be limited to the specific values recited when defining a range.

[0162]
Other embodiments of the present invention will be apparent to those skilled in the art from consideration of the present specification and practice of the present invention disclosed herein. It is intended that the present specification and examples be considered as exemplary only with a true scope and spirit of the invention being indicated by the following claims and equivalents thereof.

[Sequence Listing]
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[CLAIMS]

[Claim 1]
An anti dengue virus (DENV) monoclonal antibody or an antigen-binding fragment thereof, the monoclonal antibody or the antigen-binding fragment thereof comprising a neutralization activity against serotypes of DENV-1, DENV-2, DENV-3 and DENV-4, wherein the monoclonal antibody comprises a human monoclonal antibody or a humanized monoclonal antibody, and wherein the monoclonal antibody comprises heavy chain variable region (VH) CDR1, CDR2 and CDR3 sequences as set forth respectively in SEQ ID Nos. 13-15 and light chain variable region (VL) CDR1, CDR2 and CDR3 sequences as set forth respectively in SEQ ID Nos. 112-114.

[Claim 2]
An antiDENV human monoclonal antibody according to claim 1, wherein the human monoclonal antibody is produced by a hybridoma made by fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of DENV infection with a fusion partner cell capable of efficient cell fusion.

[Claim 3]
The antiDENV human monoclonal antibody according to claim 2, wherein the fusion partner cell is a SPYMEG cell.

[Claim 4]
The anti-DENV monoclonal antibody or antigen-binding fragment thereof according to claim 1 comprising an IgG, a Fab, a Fab', a F(ab')2, a scFv, or a dsFv.

[Claim 5]
An antidengue virus(DENV) monoclonal antibody or antigen-binding fragment thereof, comprising a heavy chain variable region and a light chain variable region of:
(a) a first amino acid sequence of a complementarity-determining region (CDR) of the heavy chain variable region comprising SEQ ID NO:13 of CDR1, SEQ ID NO:14 of CDR2, and SEQ ID NO:15 of CDR3; and
a second amino acid sequence of a CDR of the light chain variable region comprising SEQ ID NO:112 of CDR1, SEQ ID NO:113 of CDR2, and SEQ ID NO:114 of CDR3;
wherein the monoclonal antibody comprises a human monoclonal antibody or a humanized monoclonal antibody.

[Claim 6]
The antiDENV human monoclonal antibody according to claim 5 comprising an IgG, a Fab, a Fab', a F(ab')2, a scFv, or a dsFv.

[Claim 7]
A method for producing an antidengue virus (DENV) human monoclonal antibody comprising:
1) producing a hybridoma by fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of DENV infection with a fusion partner cell capable of efficient cell fusion;
2) obtaining an anti-DENV human monoclonal antibody from the hybridoma,
wherein the monoclonal antibody comprises heavy chain variable region (VH) CDR1, CDR2 and CDR3 sequences as set forth respectively in SEQ ID Nos. 13-15 and light chain variable region (VL) CDR1, CDR2 and CDR3 sequences as set forth respectively in SEQ ID Nos. 112-114.

[Claim 8]
A method for producing an antiDENV human monoclonal antibody according to claim 7, wherein the fusion partner cell is a SPYMEG cell.

[Claim 9]
A method for producing a hybridoma comprising fusing a peripheral blood mononuclear cell (PBMC) from a patient in an acute phase of dengue virus (DENV) infection with a fusion partner cell capable of efficient cell fusion wherein the hybridoma produces or is capable of producing a monoclonal antibody comprising heavy chain variable region (VH) CDR1, CDR2 and CDR3 sequences as set forth respectively in SEQ ID Nos. 13-15 and light chain variable region (VL) CDR1, CDR2 and CDR3 sequences as set forth respectively in SEQ ID Nos. 112-114.

[Claim 10]
The method for producing hybridoma according to claim 9, wherein the fusion partner cell is a SPYMEG cell.

[Claim 11]
The method for producing a hybridoma according to claim 9, wherein an antiDENV

human monoclonal antibody obtained from the hybridoma comprises a neutralization activity against serotypes of DENV-1, DENV-2, DENV-3 and DENV-4.