All marmosets were inoculated on the backs with DENV as described previously [14]. Blood samples were collected on days 2, 4, 7, 14, and 21 for marmosets No. D2-6, D2-7, D2-8, D2-9, D2-10, D2-11, D2-12, and D2-13.

All the marmosets were observed for clinical signs at the time of blood collection. Inoculation with DENV and blood drawing were performed under anesthesia with 5 mg/kg of ketamine hydrochloride. Day 0 was defined as the day of virus inoculation.

Blood biochemistry

Blood cell counts were performed with a K-4500 (Sysmex, Kobe, Japan) automatic blood cell counting machine. Biochemical analyses were performed with an AU400 (Olympus, Tokyo, Japan) automatic analyzer, according to the manufacturer's protocol.

Body temperature and activity measurements

PhysioTel and Dataquest devices (Data Sciences International, St. Paul, MN, USA) were used for measuring body temperature and activity in DENV-inoculated marmosets. A small telemetric transmitter (TA10TA-F40; Data Sciences International) was implanted into the abdominal cavity of all 12 marmosets under anesthesia with 10 mg/kg of ketamine hydrochloride and 0.025 mg/kg of medetomidine. The animals were inoculated with DENV-2 at more than 10 days after the implantation procedure. Body temperature and activity signals were monitored every 10 minutes for 7 days before inoculation. The mean body temperature was calculated for each 12-hour light and dark period. The mean body temperature and standard deviation was calculated using data obtained 7 days before inoculation, and the threshold values of body temperature were defined as the mean plus two standard deviations. The activity was calculated as the sum of the activity counts during each 12-hour phase of light and dark. The mean and standard deviation were calculated using data collected 7 days before DENV inoculation, and the threshold value was defined as the mean minus two standard deviations.

Statistics

Results were expressed as mean, and lowest and highest value in each group (Table 1). The statistical significance of differences was determined by Student's t-test to compare the mean titers using the statistical package in Microsoft Excel (Microsoft Corp., Redmond, WA, USA). The difference was considered to be significant when the P-value was < 0.05 between the values of DENV-2 inoculated marmosets and the non-inoculated marmosets.

Table 1 Hematological and biochemical values in marmosets after inoculation with DENV-2

	Days after inoculation							
Parameters	0[1]	1	2[2]	3[3]	4[2]	7	14	21
DENV-2 inoculated group (N = 12)								
Platelet ($\times 10^4/\mu l$)	45 (11–66)[4]	n.d.[5]	40 (25–62)	49 (40–57)	38 (25–61)	40 (20–60)	53 (25–77)	55 (26–79)
WBC ($\times 10^4/\mu l$)	64 (29–88)	n.d.	51 (26–82)	50 (44–54)	41 (22–55)	44 (22–57)[6]	57 (40–86)	53 (22–77)
AST (IU/l)	86 (58–167)	n.d.	94 (56–156)	105 (73–196)	88 (63–133)	79 (42–133)	67 (43–132)	61 (42–92)
ALT (IU/l)	6 (0–21)	n.d.	11 (1–63)	3 (2–4)	6 (1–23)	6 (1–30)	10 (1–74)	4 (0–13)
LDH (IU/l)	159 (63–416)	n.d.	136 (63–296)	155 (106–205)	145 (61–244)	184 (73–381)	151 (67–432)	122 (55–279)
BUN (mg/dl)	22 (17–29)	n.d.	21 (15–26)	34 (24–51)	22 (14–29)	25 (15–33)	25 (17–32)	23 (13–32)
Uninoculated group (N = 4)								
Platelet ($\times 10^4/\mu l$)	47 (34–62)	53 (40–61)	n.d.	54 (40–63)	n.d.	60 (41–73)	59 (34–73)	58 (38–69)
WBC ($\times 10^4/\mu l$)	48 (42–54)	58 (52–70)	n.d.	54 (49–60)	n.d.	70 (53–90)	51 (41–58)	43 (32–54)
AST (IU/l)	77 (36–121)	107 (101–114)	n.d.	73 (68–81)	n.d.	46 (41–53)	44 (34–60)	40 (35–48)
ALT (IU/l)	9 (0–20)	4 (1–10)	n.d.	3 (0–8)	n.d.	1 (0–3)	2 (1–3)	1 (0–3)
LDH (IU/l)	221 (53–402)	147 (102–222)	n.d.	94 (65–137)	n.d.	76 (50–114)	80 (55–111)	102 (48–221)
BUN (mg/dl)	24 (21–28)	22 (20–25)	n.d.	22 (19–28)	n.d.	23 (17–27)	24 (17–28)	21 (18–24)

[1]Day 0 indicates before inoculation with DENV-2.
[2]Samples were obtained from eight DENV-2 infected marmosets.
[3]Samples were obtained from four DENV-2 infected marmosets.
[4]Numbers in parentheses indicate the lowest and the highest value in the group.
[5]Indicates test not done.
[6]Underline indicates $P < 0.05$ as compared to the uninoculated group.

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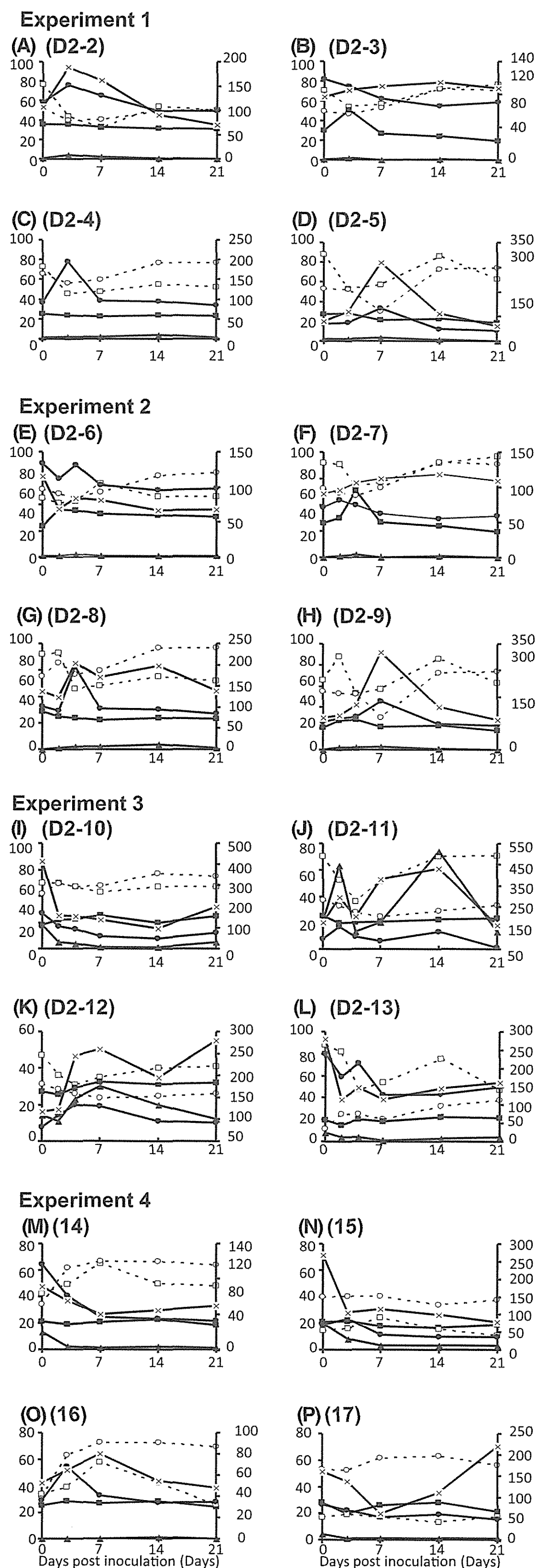

Results

Hematological changes after inoculation with DENV-2

Clinical laboratory data were collected from 12 marmosets inoculated with DENV-2 and 4 uninoculated marmosets. The duration of detectable viremia was 3 days after inoculation for marmosets D2-2, D2-3, and D2-4, and 7 days after inoculation for marmosets D2-5, D2-6, D2-7, D2-8, D2-9, D2-10, D2-11, D2-12, and D2-13. The decrease in platelet number was detected in 5 of the 12 marmosets inoculated with DENV-2 (Fig. 1A,C,D,F,H). The number of white blood cells decreased in 9 of the 12 marmosets inoculated with DENV-2 (Fig. 1A,B,D,F,H,J,L). The decrease in the number of white blood cells was statistically significant (P = 0.03) on day 7 after DENV-2 inoculation as compared to the uninoculated control group. The decrease in platelet counts after DENV-2 inoculation was not statistically significant as compared to the control group (Table 1).

Serum biochemical changes after inoculation with DENV-2

Aspartate aminotransferase (AST) levels increased in five marmosets inoculated with DENV-2 (Fig. 1-A,C,D,G,L). Additionally, alanine aminotransferase (ALT) levels increased in four marmosets inoculated with DENV-2. Lactate dehydrogenase (LDH) levels also increased in six marmosets inoculated with DENV-2 (Fig. 1A,D,G,H,J,K). Blood urea nitrogen (BUN) levels increased in two marmosets inoculated with DENV-2 (Fig. 1B,F). The increases in AST levels on days 7, 14, and 21 after inoculation with DENV-2

Fig. 1 Hematological and biochemical changes in each marmoset inoculated with DENV. Changes in platelet number (10^4 platelet/μl), white blood cell number ($10^2/\mu$l), AST levels (IU/l), ALT levels (IU/l), LDH levels (IU/l) and BUN level (mg/dl) in plasma from each marmoset. Data from DENV-2 infected marmosets are shown in Fig. 1A–L, and uninoculated marmosets are shown in Fig. 1M–P. Day 0 was defined as the day of virus inoculation. Marmosets D2-2, D2-3, D2-4, D2-5, D2-7, D2-8, D2-9, D2-11, and D2-12 exhibited decrease in number of white blood cells and platelets, and increase in biochemical parameters including GOT, LDH, and BUN. Open circles (○) indicate platelet number (× $10^4/\mu$l), open squares (□) indicate white blood cell number (× $10^2/\mu$l), closed circles (•) indicate AST levels (IU/l), closed triangles (▲) indicate ALT levels (IU/l), crosses (x) indicate LDH levels (IU/l), and closed squares (■) indicate BUN levels (mg/dl). The y-axis on the left indicates platelet numbers, white blood cell numbers, ALT levels, LDH levels, and BUN levels and that on the right indicates AST levels.

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was statistically significant as compared to the uninoculated control group ($P < 0.01$, $P = 0.03$, and $P < 0.01$ for days 7, 14 and 21, respectively). Additionally, increases in the levels of LDH on days 7 and 14 were statistically significant as compared to the uninoculated group ($P = 0.01$, $P = 0.04$ for days 7 and 14, respectively) (Table 1). The increases in ALT and BUN levels were not statistically significant as compared to the control group.

Clinical signs detected in marmosets after inoculation with DENV-2

The marmosets were observed for the development of clinical signs for 21 days. Body temperature and activity were measured after inoculation with DENV-2 (Fig. 2). Body temperature rose in 3 of the 4 marmosets (D2-2, D2-4, and D2-5) inoculated with the DENV-2 DHF0663 strain at doses of 4×10^7. Marmoset No. D2-5 remained febrile for 1 week. Along with the rise in body temperature, some animals showed a decrease in activity, defined as the total activity rate: on days 1–3 for D2-2, on days 1–4 and 7–9 for D2-3, and on days 3, 4, 7 and 8 for D2-5 (Fig. 2A–D). An increase in body temperature was detected in two animals (D2-7 and D2-9) and a decrease in activity in three of the animals (D2-7, D2-8 and D2-9, Fig. 2E–H), among those inoculated with the DENV-2 DHF0663 strain at doses of 2×10^4 or 2×10^3 PFU/dose. Additionally, body temperature rose in two marmosets (D2-11 and D2-12) and activity decreased in one marmoset (D2-10) inoculated with the Jam/77/07 strain at a dose of 1×10^5 PFU and the Mal/77/08 strain at a dose of 2×10^5 PFU (Fig. 2I–L). These results indicate that DENV infection induced fever and a decrease in activity in marmosets.

Discussion

In this study, hematological and biochemical changes and clinical signs were examined in the common marmoset (*Callithrix jacchus*) after inoculation with DENV-2. We have previously reported that after inoculation with various clinical isolates of DENV-2 strains, and a variety of input doses, high levels of viremia were consistently detected by quantitative RT-PCR in common marmosets [14]. In this study, some marmosets developed clinical signs, such as fever, and a decrease in activity upon inoculation with DENV-2. Hematological and biochemical changes, such as thrombocytopenia, leucopenia, elevation of liver enzymes, LDH, and BUN, were also detected in nearly half of the marmosets infected with three strains of DENV-2 (Fig. 1, Table 1). A decrease in white blood cell count and an increase in AST levels and LDH levels in DENV-2 inoculated marmosets were significant as compared to the uninoculated group (Table 1). The results indicate that the changes in hematological and biochemical parameters in marmosets inoculated with DENV were owing to DENV infection, and not as a result of anesthesia administration or blood sampling. Viremia titers peaked at day 3 after DENV inoculation for D2-2 (1×10^7 genome copies/ml, peak viremia), D2-3 (9×10^6 genome copies/ml), D2-4 (6×10^6 genome copies/ml), D2-5 (9×10^6 genome copies/ml), D2-12 (1×10^7 genome copies/ml), and D2-13 (7×10^6 genome copies/ml), and at day 4 after DENV inoculation for D2-6 (2×10^6 genome copies/ml), D2-7 (9×10^5 genome copies/ml), D2-8 (4×10^5 genome copies/ml), D2-9 (7×10^5 genome copies/ml), D2-10 (3×10^6 genome copies/ml), and D2-11 (2×10^6 genome copies/ml) [14]. Marmosets D2-6 and D2-10, however, did not exhibit hematological changes. In addition to viral factors, host factors including immunological factors and genetic factors are speculated to have important implications on disease severity [18–32]. Seroepidemiological studies consistently support the role of immunological factors during secondary heterotypic infection as a risk factor for severe dengue. It has been reported that higher viremia titers were detected in patients with secondary DENV-2 and severe dengue infection (DHF), as compared with patients with dengue fever [33]. Other investigators, however, reported no discrepancy in viremia between patients with secondary and primary infection [18, 34] or higher viremia in primary infection as compared with secondary infection [35]. Future manipulations of the model are expected to address the link between viremia and disease *in vivo*.

These clinical signs and hematological and biochemical changes are often present in human dengue patients [19, 20]. In the present study, all marmosets developed viremia using inoculation doses from 10^7 to 10^3 PFU/ dose, and biochemical and hematological changes occurred in both groups inoculated with 10^7–10^5 PFU/dose (Experiment 1) and 10^4–10^3 PFU/dose (Experiment 2). Further evaluations are, however, needed to define the biochemical and hematological changes between the two groups. The subcutaneous inoculation of marmosets with dengue virus was reasoned to mimic the route of natural mosquito infection. Infection of primates via DENV-2 infected mosquitoes may, however, reflect some aspects of natural infection because virus transmission is closely associated with mosquito saliva and is speculated to have a profound effect on host immune response and

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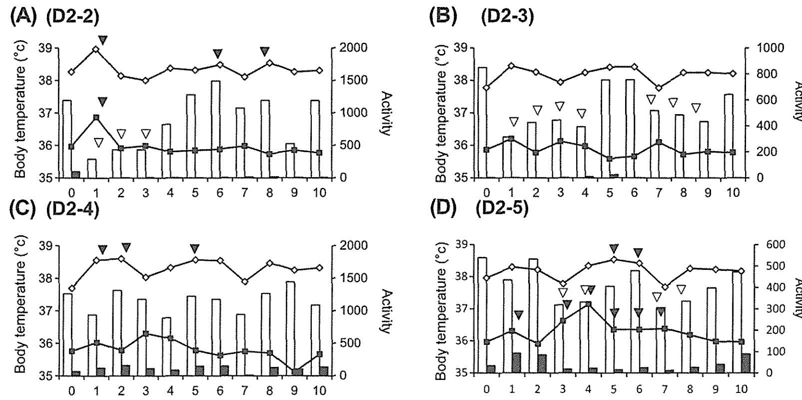

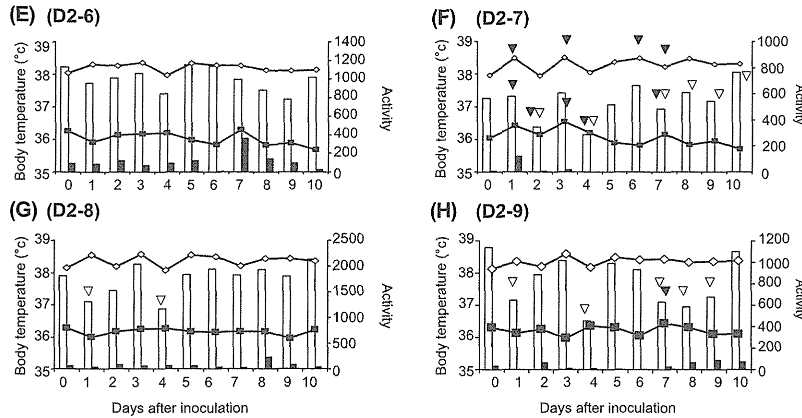

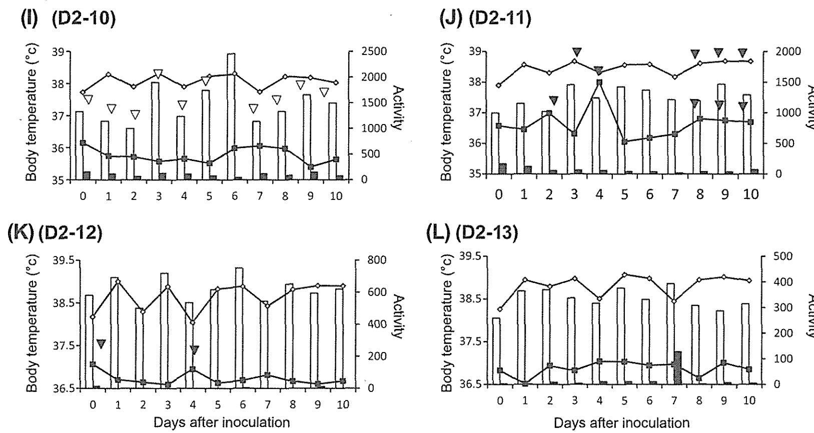

Fig. 2 Changes in body temperature and the activity rate for each marmoset. Daily changes of body temperature and the activity rate from day 0 to day 10 in D2-2 (A), D2-3 (B), D2-4 (C), D2-5 (D), D2-6 (E), D2-7 (F), D2-8 (G), D2-9 (H), D2-10 (I), D2-11 (J), D2-12 (K,) and D2-13 (L). Lines with open diamonds indicate average temperature during the light periods, and lines with closed squares during the dark periods. The mean body temperature and standard deviation were calculated using data obtained from 7 days prior to inoculation, and the threshold values of body temperature were defined as the mean plus two standard deviations. Closed arrow heads indicate that body temperature is above the threshold values. Open columns indicate the total activity rate in the light periods. Closed columns indicate the total activity rate in the dark periods. The mean activity rate and standard deviation was calculated using data obtained from 7 days prior to inoculation, and the threshold values of the activity rate were defined as the mean minus two standard deviations. Open arrow heads indicate that the total activity rate is below the threshold value. Day 0 was defined as the day of virus inoculation.

disease severity [36]. Although further studies are required to substantiate this hypothesis, the route of infection may have important implications on host immune response and disease severity. In contrast, injection of either attenuated or clinical strains of dengue virus into the skin is capable of inducing viremia, typical DENV infection signs, and symptoms in humans [37, 38]. Results of our studies using marmosets also found that subcutaneous inoculation induced viremia and clinical signs [14]. Thus, subcutaneous inoculation of DENV-2 (10^7–10^3 PFU/dose) resulted in certain clinical signs that reflect DENV infection in humans, including viremia, hematological, and serum biochemical changes. Clinical signs and changes in

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hematological and biochemical parameters were, however, not detected in all of the DENV-inoculated marmosets. Although studies including larger number of animals are required, the results suggest that discrepancies in clinical signs and changes in parameters may in part be due to host factors such as genetic variation among individual marmosets, as has been observed in humans [21, 24–32].

In summary, the results provide data on changes in hematological and biochemical parameters, and clinical signs in common marmosets inoculated with DENV. Additionally, measurable parameters such as high levels of viremia were consistently detected upon DENV infection [14]. The results indicate that marmosets develop changes in hematological and biochemical parameters and clinical signs upon infection with DENV-2 and suggest that marmosets may be useful for the development of therapeutics and vaccines, or for elucidation of disease pathogenesis.

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References

1 WHO: Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. Geneva, Switzerland: WHO, 2009.
2 Bente DA, Rico-Hesse R: Models of dengue virus infection. *Drug Discov Today Dis Models* 2006; **3**:97–103.
3 Koraka P, Benton S, van Amerongen G, Stittelaar KJ, Osterhaus AD: Efficacy of a live attenuated tetravalent candidate dengue vaccine in naïve and previously infected *cynomolgus* macaques. *Vaccine* 2007; **25**:5409–16.
4 Bernardo L, Izquierdo A, Prado I, Rosario D, Alvarez M, Santana E, Castro J, Martínez R, Rodríguez R, Morier L, Guillén G, Guzmán MG: Primary and secondary infections of *Macaca fascicularis* monkeys with Asian and American genotypes of dengue virus 2. *Clin Vaccine Immunol* 2008; **15**:439–46.
5 Blair PJ, Kochel TJ, Raviprakash K, Guevara C, Salazar M, Wu SJ, Olson JG, Porter KR: Evaluation of immunity and protective efficacy of a dengue-3 pre-membrane and envelope DNA vaccine in *Aotus nancymae* monkeys. *Vaccine* 2006; **24**:1427–32.
6 Simmons JS, St John JH, Reynolds FH: Experimental studies of dengue. *Philippine J Sci* 1931; **44**:1–252.
7 Rosen L: Experimental infection of New World monkeys with dengue and yellow fever viruses. *Am J Trop Med Hyg* 1958; **7**:406–10.
8 Whitehead RH, Chaicumpa V, Olson LC, Russell PK: Sequential dengue virus infections in the white-handed gibbon (Hylobates lar). *Am J Trop Med Hyg* 1970; **19**:94–102.
9 Halstead SB: *In vivo* enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J Infect Dis* 1979; **140**:527–33.
10 Onlamoon N, Noisakran S, Hsiao HM, Duncan A, Villinger F, Ansari AA, Perng GC: Dengue virus-induced hemorrhage in a nonhuman primate model. *Blood* 2010; **115**:1823–34.
11 Paul JR, Melnick JL, Sabin AB: Experimental attempts to transmit phlebotomus (Sandfly, Pappataci) and dengue fevers to chimpanzees. *Proc Soc Exp Biol Med May* 1948; **68**:193–8.
12 Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ: Monoclonal antibody-mediated enhancement of dengue virus infection *in vitro* and *in vivo* and strategies for prevention. *Proc Natl Acad Sci USA* 2007; **104**:9422–7.
13 Ito M, Mukai RZ, Takasaki T, Kotaki A, Kurane I: Antibody-dependent enhancement of dengue virus infection *in vitro* by undiluted sera from monkeys infected with heterotypic dengue virus. *Arch Virol* 2010; **155**:1617–27.
14 Omatsu T, Moi ML, Hirayama T, Takasaki T, Nakamura S, Tajima S, Ito M, Yoshida T, Saito A, Katakai Y, Akari H, Kurane I: Common marmoset (Callithrix jacchus) as a primate model of dengue virus infection: development of high levels of viraemia and demonstration of protective immunity. *J Gen Virol* 2011; **92**:2272–80.
15 Kurane I, Takasaki T, Yamada K: Trends in flavivirus infections in Japan. *Emerg Infect Dis* 2000; **6**:569–71.
16 Putvatana R, Yoksan S, Chayayodhin T, Bhamarapravati N, Halstead SB: Absence of dengue 2 infection enhancement in human sera containing Japanese encephalitis antibodies. *Am J Trop Med Hyg* 1984; **33**:288–94.
17 Moi ML, Lim CK, Kotaki A, Takasaki T, Kurane I: Detection of higher levels of dengue viremia using FcγR-expressing BHK-21 cells than FcγR-negative cells in secondary infection but not in

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primary infection. *J Infect Dis* 2011; **203**:1405–14.

18 Guilarde AO, Turchi MD, Siqueira JB Jr, Feres VC, Rocha B, Levi JE, Souza VA, Boas LS, Pannuti CS, Martelli CM: Dengue and dengue hemorrhagic fever among adults: clinical outcomes related to viremia, serotypes, and antibody response. *J Infect Dis* 2008; **197**:817–24.

19 Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Rothman AL, Ennis FA, Nisalak A: Dengue in the early febrile phase: viremia and antibody responses. *J Infect Dis* 1997; **176**:322–30.

20 Villar-Centeno LA, Díaz-Quijano FA, Martínez-Vega RA: Biochemical alterations as markers of dengue hemorrhagic fever. *Am J Trop Med Hyg* 2008; **78**:370–4.

21 Brinton MA, Kurane I, Mathew A, Zeng L, Shi PY, Rothman A, Ennis FA: Immune mediated and inherited defences against flaviviruses. *Clin Diagn Virol* 1998; **10**:129–39.

22 Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM: Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. *Lancet Infect Dis* 2010; **10**:712–22.

23 Ubol S, Halstead SB: How innate immune mechanisms contribute to antibody-enhanced viral infections. *Clin Vaccine Immunol* 2010; **17**: 1829–35.

24 Lan NT, Hirayama K: Host genetic susceptibility to severe dengue infection. *Trop Med Health* 2011; **4**(Suppl):73–81.

25 Loke H, Bethell D, Phuong CX, Day N, White N, Farrar J, Hill A: Susceptibility to dengue hemorrhagic fever in vietnam: evidence of an association with variation in the vitamin d receptor and Fc gamma receptor IIa genes. *Am J Trop Med Hyg* 2002; **67**(1):102–6.

26 Sakuntabhai A, Turbpaiboon C, Casadémont I, Chuansumrit A, Lowhnoo T, Kajaste-Rudnitski A, Kalayanarooj SM, Tangnararatchakit K, Tangthawornchaikul N, Vasanawathana S, Chaiyaratana W, Yenchitsomanus PT, Suriyaphol P, Avirutnan P, Chokephaibulkit K, Matsuda F, Yoksan S, Jacob Y, Lathrop GM, Malasit P, Desprès P, Julier C: A variant in the CD209 promoter is associated with severity of dengue disease. *Nat Genet* 2005; **37**:507–13.

27 Silva LK, Blanton RE, Parrado AR, Melo PS, Morato VG, Reis EA, Dias JP, Castro JM, Vasconcelos PF, Goddard KA, Barreto ML, Reis MG, Teixeira MG: Dengue hemorrhagic fever is associated with polymorphisms in JAK1. *Eur J Hum Genet* 2010; **18**:1221–7.

28 Soundravally R, Hoti SL: Immunopathogenesis of dengue hemorrhagic fever and shock syndrome: role of TAP and HPA gene polymorphism. *Hum Immunol* 2007; **68**:973–9.

29 Fernández-Mestre MT, Gendzekhadze K, Rivas-Vetencourt P, Layrisse Z: TNF-alpha-308A allele, a possible severity risk factor of hemorrhagic manifestation in dengue fever patients. *Tissue Antigens* 2004; **64**:469–72.

30 Perez AB, Sierra B, Garcia G, Aguirre E, Babel N, Alvarez M, Sanchez L, Valdes L, Volk HD, Guzman MG: Tumor necrosis factor-alpha, transforming growth factor-β1, and interleukin-10 gene polymorphisms: implication in protection or susceptibility to dengue hemorrhagic fever. *Hum Immunol* 2010; **71**:1135–40.

31 Nguyen TP, Kikuchi M, Vu TQ, Do QH, Tran TT, Vo DT, Ha MT, Vo VT, Cao TP, Tran VD, Oyama T, Morita K, Yasunami M, Hirayama K: Protective and enhancing HLA alleles, HLA-DRB1*0901 and HLA-A*24, for severe forms of dengue virus infection, dengue hemorrhagic fever and dengue shock syndrome. *PLoS Negl Trop Dis* 2008; **2**:e304.

32 García G, del Puerto F, Pérez AB, Sierra B, Aguirre E, Kikuchi M, Sánchez L, Hirayama K, Guzmán MG: Association of MICA and MICB alleles with symptomatic dengue infection. *Hum Immunol* 2011; **72**:904–7.

33 Wang WK, Chen HL, Yang CF, Hsieh SC, Juan CC, Chang SM, Yu CC, Lin LH, Huang JH, King CC: Slower rates of clearance of viral load and virus-containing immune complexes in patients with dengue hemorrhagic fever. *Clin Infect Dis* 2006; **43**:1023–30.

34 Gubler DJ, Suharyono W, Tan R, Abidin M, Sie A: Viraemia in patients with naturally acquired dengue infection. *Bull World Health Organ* 1981; **59**:623–30.

35 Thai KT, Phuong HL, Thanh Nga TT, Giao PT, Hung le Q, van Nam N, Binh TQ, Simmons C, Farrar J, Hien TT, van Doorn HR, de Jong MD, de Vries PJ: Clinical, epidemiological and virological features of Dengue virus infections in Vietnamese patients presenting to primary care facilities with acute undifferentiated fever. *J Infect* 2010; **60**:229–37.

36 Schneider BS, Soong L, Coffey LL, Stevenson HL, McGee CE, Higgs S: *Aedes aegypti* Saliva Alters Leukocyte Recruitment and Cytokine Signaling by Antigen-Presenting Cells during West Nile Virus Infection. *PLoS ONE* 2010; **5**:e11704.

37 Sabin AB: Research on dengue during world war II. *Am J Trop Med Hyg* 1952; **1**:30–50.

38 Kanesa-thasan N, Sun W, Kim-Ahn G, van Albert S, Putnak JR, King A, Raengsakulsrach B, Christ-Schmidt H, Gilson K, Zahradnik JM, Vaughn DW, Innis BL, Saluzzo JF, Hoke CH Jr: Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. *Vaccine* 2001; **19**:3179–88.

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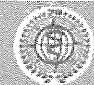

Dengue Virus Type-3 Infection in a Traveler Returning From Benin to Japan

Mugen Ujiie, MD, MTM,* Meng Ling Moi, BSc (Hons), MSc, PhD,† Taiichiro Kobayashi, MD,* Nozomi Takeshita, MD,* Yasuyuki Kato, MD, MPH,* Tomohiko Takasaki, MD, PhD,† and Shuzo Kanagawa, MD*

*Disease Control and Prevention Center, National Center for Global Health and Medicine, Tokyo, Japan; †Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan

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Dengue virus (DENV) infection is a major health threat for travelers. We describe here a case of dengue virus serotype-3 (DENV-3) infection in a traveler returning to Japan from the Republic of Benin. The isolated DENV-3 genotype 3 strain exhibited high sequence similarity to those from neighboring regions.

Dengue virus (DENV) is widely distributed in tropical and subtropical countries and is transmitted by *Aedes* mosquito. The global incidence of DENV infection has increased rapidly in recent years. In addition, disease prevalence has widely expanded geographically, leading to dengue emergence in nonendemic countries[1] or re-emergence elsewhere. Although DENV infection has been reported sporadically in travelers returning from Africa,[2–7] the extent of DENV transmission in Africa has not been clearly defined. There is limited availability of epidemiological and clinical data on dengue infection in Africa. Hence, improved clinical and molecular epidemiological data on DENV infection in travelers could contribute to better understanding of the clinical features associated with dengue infection from Africa, as well as the extent of disease prevalence in the region.

Although Japan has no endemic cases of dengue, the number of imported cases has increased steadily in recent years with some 245 cases reported in 2010.[8] Of these cases, three travelers from the African continent (two travelers from Tanzania and one from Benin) developed dengue fever (DF). In this study, we describe the clinical and molecular characteristic of a dengue virus serotype-3 (DENV-3) isolated from a traveler returning to Japan from the Republic of Benin in 2010.

Corresponding Author: Mugen Ujiie, MD, MTM, Disease Control and Prevention Center, National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. E-mail: infinite_u@hotmail.com

Case Report

A 28-year-old Japanese female presented to the emergency department of the National Center for Global Health and Medicine (NCGM) Hospital (August 6, 2010) one day after onset of high fever and headache. She had visited Cotonou, Dassa-Zoume, Parakou, Natitingou, and Porto-Novo in Benin between July 24 and August 3, 2010. She returned to Japan on August 4, 2010 and developed sudden fever the next day.

The patient visited our hospital complaining of headache, sore throat, nausea, diarrhea, bilateral myalgia of her thighs, and bilateral arthralgia over her knees, shoulders, and elbows. On examination, her body temperature was 39°C, blood pressure was 88/52 mmHg, and pulse was 92/minute. Systemic examinations revealed pharyngeal erythema, bilateral inguinal lymphadenopathy, and mild tenderness over her thighs and knees. Many mosquito bite marks were apparent on her lower limbs. A full blood count conducted on day 2 after onset of disease revealed the following: hemoglobin count (13.2 mg/dL), hematocrit concentration (39.2%), white blood cell count (6.76×10^9/L), and platelet count (227×10^9/L), all of which were within normal ranges.

Serum sample obtained on day 2 after onset of disease was negative for malaria antigen by a rapid malaria antigen diagnostic test (BinaxNOW, Inverness Medical, Ireland), and, was positive for dengue NS1 antigen by using a rapid DENV NS1 antigen immune-chromatographic test (SD BIOLINE, Korea). The patient was, therefore, admitted to our hospital for treatment, given intravenous infusions and observed for dengue warning signs. The patient's platelet count was

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at its lowest on day 7 after onset of disease (48×10^9/L) and her fever subsided on day 8 after onset. She was discharged after hospitalization for a total of 7 days.

DENV-3 genome was detected by real-time polymerase chain reaction (RT-PCR, Applied Biosystems, USA) and virus isolated using the *Aedes albopictus* mosquito cell line C6/36.[3] Although tests for anti-dengue IgM (Focus Diagnostics, USA), and IgG (Panbio, Australia) antibodies were negative on day 2 after onset of disease, tests using serum sample from day 8 after onset of disease was positive. Both day 2 and day 8 serum samples were positive for dengue NS1 antigen (Platelia, Bio-Rad, France). Serum samples were de-identified prior to being used in the experiments and thus, ethical approval was not required for this study. The nucleotide sequence of the envelope protein (E-protein) of the isolated virus (GenBank accession number AB690858) was compared to selected sequences of DENV-3. The isolated DENV-3 strain from Benin belonged to DENV-3, genotype III (Figure 1) and had the following characteristics: an E-protein sequence similarity of 99% to the DENV-3 D3/Hu/Côte d'Ivoire/NIID48/2008 strain, 99% to a DENV-3 strain isolated in Senegal in 2009, and 98% to a DENV-3 D3/Hu/Tanzania/NIID08/2010 strain isolated in Tanzania in 2010 (GenBank accession numbers: AB447989, GU189386, and AB549332, respectively).

Discussion

Sporadic cases or outbreaks of DENV infection have been reported in 34 countries in the African region. It is estimated that 2.4% of global dengue hemorrhagic fever (DHF) cases (100,000 cases) and up to 1 million cases of DF may occur in Africa.[2] Among travel-associated dengue cases in travelers returning to Europe, 2 to 8% had visited Africa.[2,5] In comparison, most of the travelers returning to Europe with dengue had traveled to Asia (54–61%) and Latin America (25–31%). Febrile illness was, however, more frequently reported in 41% of travelers to sub-Saharan Africa (2,559 patients) as compared to other regions (Southeast Asia, 33%, 1,218 patients; Caribbean and Central and South America, 18%, 1,044 patients).[9] Although dengue is frequently reported in travelers to Southeast Asia and South America as compared to Africa, the disease may be underreported in Africa due to limited awareness of the disease, and, limited availability of diagnostic tests and routine surveillance system.[2]

Imported cases of DENV type-3 infection from West Africa have been previously reported in European travelers.[2–6] The first possibility of DENV circulation in Benin was suggested by a seroprevalence study conducted in asymptomatic Germans working overseas from 1987 to 1993.[5,10] Although there have been four serologically confirmed cases of DENV infection in travelers returning from Benin to France in 2006 and 2010, the serotypes were not identified. As the isolated

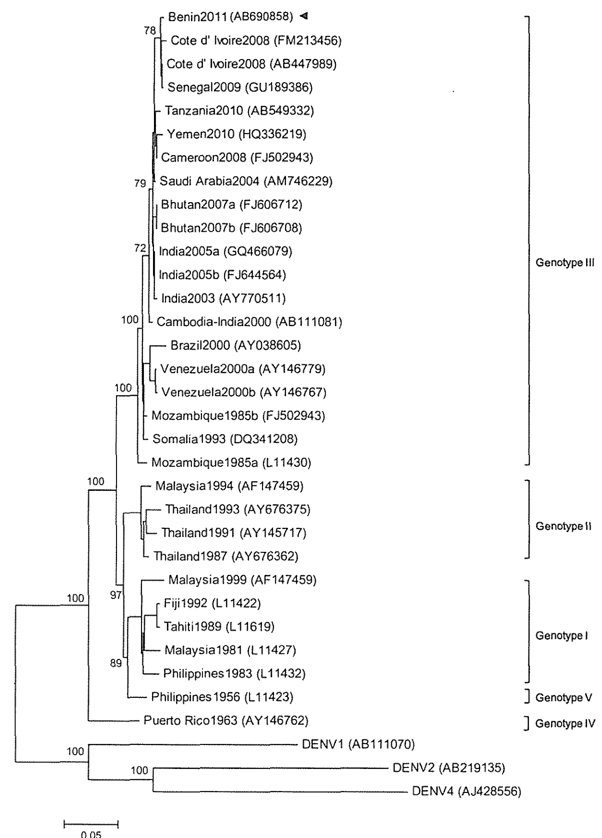

Figure 1 Phylogenetic tree based on the nucleotide sequence of the envelope (E) protein gene of DENV-3 strains. The tree was rooted to DENV-1, DENV-2, and DENV-4 outgroups. Multiple sequence alignments were performed. The tree was constructed using the neighbor joining method (Genetyx, Japan) and successful bootstrap replication is indicated at the nodes. The isolated strain from Benin (AB690858) was indicated with a closed triangle (▲). Scale bar (0.05) indicates nucleotide substitutions per site.

DENV-3 strain possesses high sequence similarity to DENV-3 strains in neighboring regions, the data suggests local transmission of the virus in the African continent. However, further epidemiological studies would be needed to identify DENV outbreaks and ascertain the virus strains causing local outbreaks.

Although close monitoring of febrile travelers provides data on DENV outbreaks in endemic regions, improved disease surveillance and a higher priority in dengue laboratory diagnosis in Africa is vital to reflect the true incidence of the disease. Identification of genotypes and strains along with disease prevalence in endemic areas is of importance because some DENV strains have been associated with increased disease severity and may possess higher epidemic potential.[3,4]

Currently, there are no effective drugs or vaccines against DENV infection. Transmission of DENV within Africa presents challenges for diagnosis and effective disease management of febrile travelers

returning from the continent. Additionally, there is a need for higher awareness toward the increasing risk of DENV infection in travelers among health care personnel in both endemic and non-endemic regions. Thus, rapid and accurate diagnosis of DENV is particularly important for travelers returning from West Africa in which other viral hemorrhagic fevers, including yellow fever and Lassa fever are endemic.

Acknowledgments

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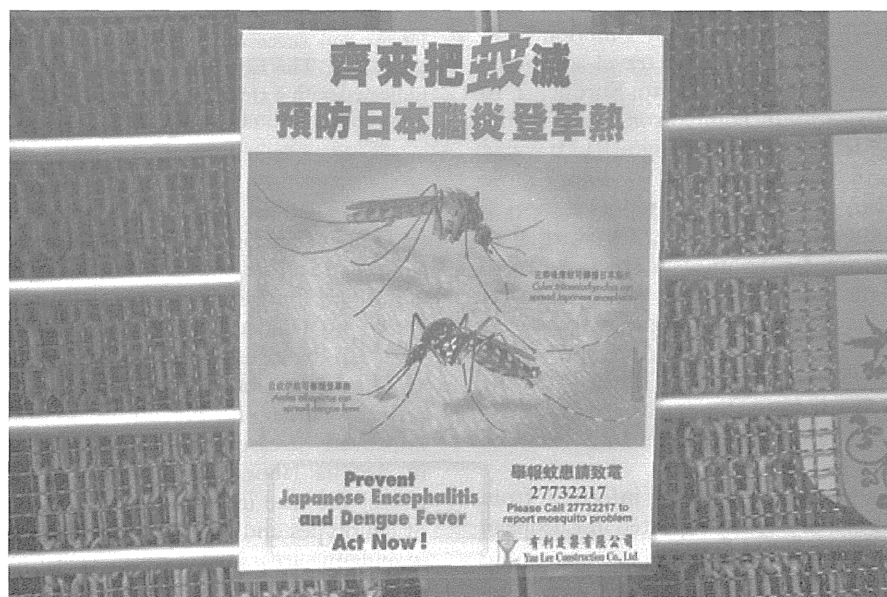
Declaration of Interests

The authors state that they have no conflicts of interest.

References

1. TDR/WHO. Dengue: guidelines for diagnosis, treatment, prevention and control. Geneva, Switzerland: TDR/WHO, 2009.
2. Amarasinghe A, Kuritsk JN, Letson GW, Margolis HS. Dengue virus infection in Africa. Emerg Infect Dis 2011; 17:1349–1354.
3. Moi ML, Takasaki T, Kotaki A, et al. Importation of dengue virus type 3 to Japan from Tanzania and Côte d'Ivoire. Emerg Infect Dis 2010; 16:1770–1772.
4. Franco L, Di Caro A, Carletti F, et al. Recent expansion of dengue virus serotype 3 in West Africa. Euro Surveill 2010; 15. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19490
5. Jelinek T, Mühlberger N, Harms G, et al. Epidemiology and clinical features of imported dengue fever in Europe: sentinel surveillance data from TropNetEurop. Clin Infect Dis 2002; 35:1047–1052.
6. Domingo C, Niedrig M, Gascón J, et al. Molecular surveillance of circulating dengue genotypes through European travelers. J Travel Med 2011; 18:183–190.
7. Schwartz E, Weld LH, Wilder-Smith A, et al. Seasonality, annual trends, and characteristics of dengue among ill returned travelers, 1997–2006. Emerg Infect Dis 2008; 14:1081–1088.
8. Infectious Disease Surveillance Center. Dengue fever, dengue hemorrhagic fever and chikungunya fever importation in Japan, as of May 2011. Infectious Agents Surveillance Report 32: 159-160, June 2011. Available at: http://idsc.nih.go.jp/iasr/32/376/tpc376.html.
9. Wilson ME, Weld LH, Boggild A, et al. Fever in returned travelers: results from the GeoSentinel Surveillance Network. Clin Infect Dis 2007; 44:1560–1568.
10. Eisenhut M, Schwarz TF, Hegenscheid B. Seroprevalence of dengue, chikungunya and Sindbis virus infections in German aid workers. Infection 1999; 27:82–85.



This is a warning sign at the entrance of the aviary located in Hong Kong Park, Hong Kong Island, Hong Kong. It stresses the risk of two arboviral diseases transmitted by mosquitoes: dengue fever is transmitted by *Aedes albopictus* and Japanese encephalitis is transmitted by *Culex tritaeniorhynchus*. These two diseases may affect travelers in this part of the world. *Photocredit: Eric Caumes (Location: Hong Kong Park, Hong Kong)*

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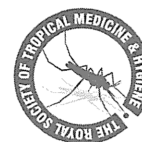

ORIGINAL ARTICLE

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Dengue virus infection-enhancing activity of undiluted sera obtained from patients with secondary dengue virus infection

Q1

Meng Ling Moi, Tomohiko Takasaki, Masayuki Saijo and Ichiro Kurane*

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Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan

*Corresponding author. Tel: +81 3 5285 1111; Fax: +81 3 5285-1356; E-mail: kurane@nih.go.jp (I. Kurane)

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Background: Dengue virus (DENV) infection-enhancing activity of pre-existing antibody has been suggested to be one of the factors responsible for the pathogenesis of the severe form of DENV infection, dengue haemorrhagic fever. Although studies of infection-enhancement activity using diluted serum sera are informative, infection-enhancement activity determined using undiluted sera may better reflect in vivo conditions.

Methods: We determined whether serum samples collected from dengue patients possessed the ability to enhance DENV infection without dilution using FcγR-expressing cells.

Results: Serum samples obtained at the early phase of secondary infection enhanced DENV infection without dilution. In contrast, sera obtained at the late stage of secondary infection demonstrated low levels or absence of infection-enhancing activity.

Conclusion: The results indicate that human sera obtained from patients with secondary infection possess the ability to enhance DENV infection without dilution, and suggest that antibody-dependent enhancement may occur in vivo during secondary DENV infection to infecting DENV serotypes.

Keywords: Dengue, Dengue haemorrhagic fever, Antibody-dependent enhancement, FcγR-expressing cells, Secondary infection

Introduction

Infection with any one of the four dengue virus serotypes (DENV1–4) causes a wide range of illnesses ranging from dengue fever to the most severe form of dengue infection, dengue haemorrhagic fever (DHF). Secondary DENV infection with serotypes different from that of primary infection tends to cause DHF.[1,2] Sub-neutralizing antibodies against DENV that are induced during primary infection is hypothesized to involve in the pathogenesis of DHF during secondary infection.[3] Virus-immune complex contributes to disease severity by a mechanism known as antibody-dependent enhancement (ADE). This leads to higher levels of infection in target cells, such as FcγR-expressing monocytes and macrophages.[4,5]

Presence of non-neutralizing, enhancing antibodies to DENV has been suggested as a possible risk factor contributing to the development of DHF during secondary DENV infection with other DENV serotypes.[6,7] The idea of the potential significance of ADE in vivo during secondary infection was supported by studies which demonstrated that high viraemia levels were associated with disease severity.[8,9] Although previous studies were clearly informative on the role of antibodies in DENV infection-enhancement, highly diluted serum samples (greater than $1{:}10^2$ dilutions) were used in the studies.[10,11] Undiluted serum samples obtained from pre-infection patients whom later developed severe dengue infection possessed higher ADE activity than those obtained from the group with less severe infection.[7]

To define the role of antibodies in infection-enhancement during secondary DENV infection in conditions that may better reflect those in vivo, we determined whether undiluted serum samples possess DENV infection-enhancing activities to infecting serotypes using FcγR-expressing baby hamster kidney (BHK) cells.

Materials and methods

Serum samples

Sixteen serum samples from 12 patients with secondary infection and six serum samples from three patients with primary infection were used in the study. Serum samples were heat inactivated at 56 °C for 30 minutes before use. The serum samples used had been tested for the presence of dengue viral RNA by reverse-transcriptase (RT) PCR, anti-DENV IgG and IgM by ELISA.[12–14] Dengue virus-specific IgM antibodies in serum samples were determined using IgM capture ELISA (Dengue Fever Virus IgM Capture ELISA, Focus Diagnostics, Cypress, CA, USA) according to the manufacturer's instructions. Dengue indirect IgG ELISA (Panbio Pty Ltd, Brisbane, Australia) was used for anti DENV-IgG antibodies according to the manufacturer's instructions. Early phase serum samples were obtained at 1–5 days after the onset of disease and were negative for anti-DENV IgM antibody by IgM-capture ELISA. Late phase serum samples were obtained at 5–14 days after the onset of disease, and were positive for anti-DENV IgM and anti-DENV IgG antibodies

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by ELISA. Serum samples P1a and P1b, samples P2a and P2b, and, samples P3a and P3b, were sequential serum samples obtained from three travellers with primary DENV infection (patients P1, P2, and P3). Serum samples S1a and S1b, S2a and S2b, S3a and S3b and, S4a and S4b were sequential serum samples obtained from four travellers of secondary DENV infection (patients S1, S2, S3 and, S4). Serum samples S5–S12 were obtained from eight patients with secondary DENV infection. Secondary infection was defined as serum samples with anti-DENV IgG antibody and absence of anti-DENV IgM antibody at the early stage of infection.[14] The definition of acute primary and secondary flavivirus infections was supported by DENV E/M protein-specific IgM/IgG OD ratios of ≥1.2 (primary infection) and <1.2 (secondary infection).[15] The institutional review board of National Institute of Diseases, Japan approved all legal, ethical and laboratory aspects of the study (approval reference no. 210). Patient data were analyzed anonymously.

Cell lines

BHK-21 cell line (Japan Health Science Research Resources Bank), and FcγR-expressing BHK cells were used for this study. BHK cells were cultured in Eagle's Minimum Essential Medium (EMEM), (Sigma-Aldrich, St. Louis, MO, USA), supplemented with heat-inactivated 10% FCS (fetal calf serum, Sigma) without any antibiotics at 37 °C in 5% CO_2. FcγR-expressing BHK cells were cultured in EMEM (Sigma), supplemented with heat-inactivated 10% FCS (Sigma) and 0.5 mg/ml neomycin (G418, PAA Laboratories GmbH, Pasching, Austria) at 37 °C in 5% CO_2.[14]

Virus

Dengue virus type 1 (DENV-1), 01-44-1HuNIID strain (GenBank accession no. AB111070), dengue virus type 2 (DENV-2) D2/Hu/OPD030NIID/2005 strain (GenBank accession no. AB219135), dengue virus type 3 (DENV-3) CH53962 strain, and dengue virus type 4 (DENV-4) TVP-360 strain were used. DENV-1 (01-44-1HuNIID strain) and DENV-2 (D2/Hu/OPD030NIID/2005 strain) were isolated from two imported dengue fever cases, and were established as laboratory strains for ADE assay and plaque reduction neutralization test (PRNT).[16] DENV-3 (CH53489 strain) and DENV-4 (TVP-360 strain) were laboratory established strains.[17] Viruses isolated from four individuals with DENV infection (patients S1, S2, S4, and S8) using BHK cells were used without further passage.

Antibody-dependent enhancement assay

Serum specimens were serially diluted 10-fold from 1:5 to 1:500 000 with EMEM supplemented with 10% FCS. DENV-antibody complex was prepared by mixing 50 μl of DENV-2 D2/Hu/OPD030NIID/2005 strain at titres of 100 PFU/ml with 50 μl of diluted serum samples. For infection with DENV-2 alone, the viral mixture was prepared by mixing 50 μl of DENV-2 at titres of 100 PFU/ml EMEM supplemented with 10% FCS.

For the infection-enhancement assay to four DENV serotypes, virus-antibody mixture was prepared by adding 50 μl of laboratory established DENV-1 (01-44-1HuNIID strain), DENV-2 (D2/Hu/OPD030NIID/2005 strain), DENV-3 (CH53489 strain) or DENV-4 (TVP-360 strain), or patient's own virus isolate at titres of 100 PFU/ml to 50 μl of undiluted or 1:5 diluted serum samples using FcγR-expressing BHK cells. For serum samples exhibiting infection-enhancing activities, the experiments were repeated using 50 μl of 1000 PFU/ml of virus. Previous studies by other investigators had shown that mosquito salivates between 0.2 to 3.2 log10 PFU and 0.5 to 5.3 log 10 genome copies of arboviruses.[18,19] All virus-antibody mixture was incubated at 37 °C for 60 minutes before infection assays.

Fold-enhancement values were calculated by the formula: (mean plaque count with addition of human serum using FcγR-expressing BHK cell line or BHK cell line)/(plaque count without addition of human serum using FcγR expressing BHK cell lines). The sum of the mean of the negative control plus a 3-times of the SD value obtained from at least four wells of negative control (negative control = mean plaque count without addition of human serum using FcγR expressing BHK cell lines or BHK cell line) was used as cut-off to differentiate ADE and non-ADE activity.[20] In non-DENV immune serum samples, the fold-enhancement values of FcγR expressing BHK cell lines ranges from 0.7–1.5.[17] Presence of ADE activity was defined as fold enhancement values with higher values as compared to fold enhancement cut-off value (fold enhancement cut-off value = mean plaque count without addition of human serum using FcγR expressing BHK cell lines + 3 SD)/(mean plaque count without addition of human serum using FcγR expressing BHK cell lines).

Plaque reduction neutralization test (PRNT)

For PRNT, heat-inactivated human serum samples were serially diluted 2-fold from 1:5 to 1:1280 with EMEM supplemented with 10% FCS. Virus-antibody mixture was prepared by mixing 50 μl of laboratory established DENV-1 (01-44-1HuNIID strain), DENV-2 (D2/Hu/OPD030NIID/2005 strain), DENV-3 (CH53489 strain) or DENV-4 (TVP-360 strain) at a titre of 1000 PFU/ml with 50 μl of serially diluted serum samples. For infection with DENV alone, 50 μl of virus at a titre of 1000 PFU/ml was mixed with 50 μL of EMEM supplemented with 10% FCS. $PRNT_{50}$ end points are expressed as the last serum dilution showing a 50% or greater reduction in plaque counts as compared to the number of plaques determined from wells of cells infected in the absence of human serum.[16–17]

Virus infection

After incubation at 37 °C for 60 minutes, 100 μl of virus-antibody mixture was inoculated onto BHK and FcγR-expressing BHK monolayers cultured in 12-well plates. The plates were then incubated for 60 minutes at 37 °C in 5% CO_2. After virus absorption, the cells were overlaid with maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industry, Osaka, Japan). The plates were incubated at 37 °C in 5% CO_2 for 5 days. After 5 days of incubation, the cells were fixed with 10% formalin for 60 minutes at room temperature and washed with water. The cells were then stained with methylene blue for 60 minutes at room temperature and washed with water.

Statistics

Data processing and Student's *t* test and correlation analysis were performed using Microsoft Excel® spreadsheet software

(Microsoft Corp., Redmond, WA, USA). For comparison analysis of days after onset of disease, only samples with detectable levels of viraemia were included.

Results

DENV infection-enhancing activity of serum samples at 1:5 dilution using sera obtained from patients with secondary DENV infection

Paired serum samples obtained from four individuals with secondary DENV infection were examined for DENV infection-enhancing activity. Serum samples S1a, S2a, S3a and S4a were determined to be negative for anti-DENV IgM antibody but positive for anti-DENV IgG antibody by ELISA. Although history of past infection was not available for the patients with secondary dengue infection, neutralizing activity against a single DENV serotype (against DENV-3 using serum sample S1a, against DENV-1 using S2a, and against DENV-3 using serum sample S4a) were detected in the serum samples. The results suggest that individuals S1, S2 and S4 may have been previously exposed to DENV-3, DENV-1 and DENV-3, respectively, in a prior infection. The serum samples (S1a, S2a, S3a and S4a) demonstrated enhancing activity to DENV at 1:5 dilution, with up to an 11.6-fold enhancement (Table 1; Figure 1A, C, E, G). During the late phase of disease, the serum samples exhibited infection-enhancing activity at higher serum dilutions (Figure 1B, D, F, H). Serum samples S1b, S2b, S3b and S4b collected from the respective individuals on disease days 5–14 demonstrated no infection-enhancing activity to homologous

Table 1. DENV infection-enhancing activity of serum samples obtained from patients with secondary and primary DENV infection.

(Sample name) infecting DENV serotype[a]	Neutralizing titre ($PRNT_{50}$)[b]				Fold enhancement values[c]			
	DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4
Secondary infection								
Early Phase[d]								
(S1a[e]) DENV-1	<10	<10	40	<10	1.6[f]	8.0[f]	1.2	3.5[f]
(S2a) DENV-2	10	<10	<10	<10	4.9[f]	11.6[f]	2.9[f]	5.4[f]
(S3a) DENV-3[g]	<10	<10	<10	<10	3.0[f]	6.3[f]	3.1[f]	5.3[f]
(S4a) DENV-4	<10	<10	40	<10	3.9[f]	6.1[f]	0.2	2.6[f]
Late Phase[h]								
(S1b[e]) DENV-1	80	80	160	40	0.2	1.0	<0.1	1.3
(S2b) DENV-2	320	80	<10	<10	4.8[f]	0.6	2.7[f]	6.2[f]
(S3b) DENV-3	80	40	1280	80	1.2	3.0[f]	<0.1	0.8
(S4b) DENV-4	1280	320	640	1280	0.6	2.7[f]	<0.1	<0.1
Primary Infection								
Early Phase[d]								
(P1a) DENV-1	<10	<10	<10	<10	1.1	1.1	1.0	1.0
(P2a) DENV-2	<10	<10	<10	<10	1.0	0.9	1.1	1.0
(P3a) DENV-3	<10	<10	<10	<10	1.0	1.0	1.0	0.9
Late Phase[h]								
(P1b) DENV-1	160	40	80	10	<0.1	0.2	0.1	1.2
(P2b) DENV-2	20	80	<10	<10	1.1	0.4	1.0	1.2
(P3b) DENV-3	<10	<10	320	<10	1.8[f]	1.0	0.2	2.3[f]

[a] Infecting serotypes were determined by RT-PCR
[b] Determined by using FcγR-negative BHK cells
[c] Calculated by the formula: (mean plaque count with addition of human serum, using FcγR expressing BHK cell lines)/(plaque count without addition of human serum using FcγR expressing BHK cell lines). Infection enhancement was tested using 1:5 diluted serum samples. Presence of infection-enhancing activity was defined by fold enhancement values greater than cut-off value (cut-off = mean plaque count without addition of human serum using FcγR expressing BHK cell lines + 3SD)/(mean plaque count without addition of human serum using FcγR expressing BHK cell lines)
[d] Anti-DENV IgM antibody negative serum samples collected on days 1–5 after onset of disease
[e] 'a' and 'b' indicate serial serum samples collected from a single patient
[f] Presence of infection-enhancing activity was defined as fold-enhancement value of greater than 1.4 for DENV-1, >1.3 for DENV-2, >1.3 for DENV-3 and >1.3 for DENV-4
[g] Neutralizing titres to all 4 DENV serotypes were <10. The serum sample was positive for anti-DENV IgG antibody and negative for anti-DENV IgM antibody at the early phase of the disease
[h] Anti-DENV IgM antibody positive serum samples collected on days 5–14 after onset of disease

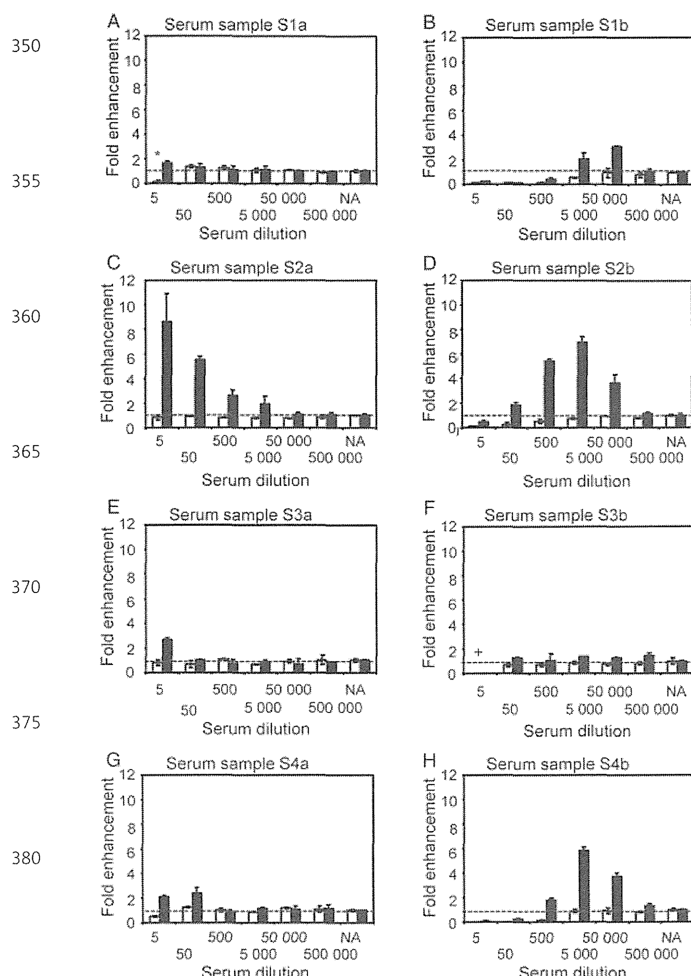

Figure 1. DENV infection-enhancing activity of serum samples obtained from patients with secondary infection. Serum samples were obtained from patients (S1, S2, S3 and S4) with secondary DENV infection at the early and late phase of the disease. Infection-enhancing activities (shown as fold-enhancement values) in the serum samples at dilutions of 1:5-1:500 000 to the infecting serotype (serum sample S1, S2, S3 and S4 against DENV-1, DENV-2, DENV-3 and DENV-4, respectively) were examined. Open bars indicate fold-enhancement values determined using FcγR-negative BHK cells. Solid bars indicate those determined using FcγR-expressing BHK cell line. NA indicates that infection was performed in the absence of serum samples. Asterisk (*) indicates test not done due to insufficient sample volume and plus (+) indicates that no plaques were detected.

DENV serotypes, while sample S1b demonstrated no ADE activity to heterologous serotypes. Serum sample from patient S2 exhibited infection-enhancing activity in the early phase of the disease to the infecting DENV-2 serotype but not in the late phase serum samples at low serum dilutions. As undiluted serum sample may better reflect in vivo conditions, the fold-enhancement to DENV-3 and DENV-4 of serum sample S2b was further tested using undiluted serum samples. Although serum sample S2b exhibited infection-enhancement activity at serum dilution of 1:5, neutralizing activity was prominent in FcγR-expresssing cells to DENV-3 and DENV-4, using undiluted serum samples (fold enhancement to DENV-3 and DENV-4 were <0.1). Similarly, serum samples S3b and S4b exhibited infection-enhancement activity at 1:5 serum dilution but neutralizing activity was greater as compared to infection-enhancing activity in FcγR-expresssing cells to DENV-2 when undiluted serum samples were used (fold enhancement S3b and S4b were <0.1). The results indicate that serum samples of early phase of infection possess infection-enhancement activity of 1:5 serum dilution and that the serum sample of late phase of infection possess no infection-enhancement activity.

Absence of DENV infection- enhancing activity of human serum samples at 1:5 dilution using sera from patients with primary DENV infection

Paired serum samples obtained at different time points from three individuals with primary DENV infection were examined for infection-enhancing activity. Serum samples P1a, P2a and P3a were negative for both anti-DENV IgM and IgG antibody by ELISA, and did not enhance DENV infection (Table 1; Figure 1A, C, E). Sequential serum samples P1b, P2b and P3b, which were collected on disease days 5-14 from the respective individuals were positive for IgG antibodies by ELISA exhibited neutralizing activity to the infecting serotype. Additionally, serum samples from patients with primary infection at the late phase of the disease exhibited infection-enhancing activity to the infecting serotype at higher serum dilutions (Figure 2A, C, E, G). With the exception for P3b, where there was a 1.8 and a 2.3-fold enhancement to DENV-1 and DENV-4, respectively, serum samples P1b and P2b demonstrated no infection-enhancing activity to all 4 DENV serotypes at 1:5 dilution (Table 1).

DENV infection-enhancing activity of undiluted serum samples to the infecting serotypes and to the DENV isolated from the respective patients

We examined whether the serum samples without dilution have the ability to enhance DENV infection without dilution. Four serum samples (S1a, S2a, S3a and S4a) were selected because they demonstrated ADE activity to the infecting DENV serotypes (Table 1). Eight serum samples (S5-S12) were also selected because of the presence of ADE activity to the infecting DENV serotypes at 1:5 dilution (Table 2). Six serum samples, S2a, S3a, S7, S9, S11, and S12 demonstrated infection-enhancing activity to laboratory established infecting serotypes without dilution. The fold-enhancement ranged from 2.0-fold to 11.0-fold.

DENV isolates recovered from patients S1, S2, S4, and S8 were also used in the ADE assays for serum samples obtained from the respective patients (serum samples S1a, S2a, S4a, and S8). Serum sample 2a, in which DENV-2 was isolated, enhanced the infection of a laboratory established DENV-2 strain D2/Hu/OPD030NIID/2005 strain and also enhanced the infection of the isolated DENV-2 (Table 2). Sample S8a demonstrated enhancing activity to the virus isolate from the patient (S8), but not to laboratory established DENV-1 strain (01-44-1HuNIID strain). The results indicate that some sera from DENV patients

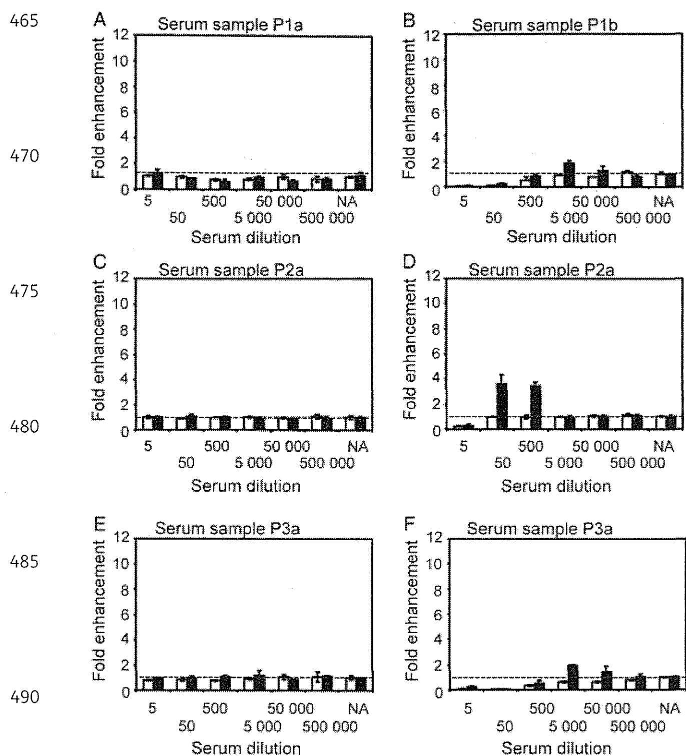

Figure 2. Dengue virus (DENV) infection-enhancing (ADE) activity of serum samples obtained from patients with primary infection. Serum samples were obtained from patients (P1, P2, P3) with primary DENV infection at the early and late phase of the disease. Infection-enhancement activities (shown as fold-enhancement values) in the serum samples at dilutions of 1:5–1:500 000 to the infecting serotype (serum sample P1, P2 and P3 against DENV-1, DENV-2, and DENV-3, respectively) were examined. Open bars indicate fold enhancement values determined using FcγR-negative BHK cells. Solid bars indicate those determined using FcγR-expressing BHK cell line. NA indicates that infection was performed in the absence of the serum sample.

Table 2. Dengue virus (DENV) infection-enhancing activity of undiluted serum samples from patients with secondary dengue infection to laboratory established strains and those isolated from the respective patients[a].

Infecting serotype[b], sample name	Fold enhancement[c]	
	Undiluted	1:5-dilution
DENV-1		
S1a	0.4	1.6[d]
S1a[a]	0.3	2.5[d]
S5	0.6	5.1[d]
S6	1.2	9.5[d]
S7	5.0[d]	10.2[d]
S8	1.3	7.5[d]
S8[a]	9.0[d]	12.5[d]
DENV-2		
S2a	3.0[d]	11.6[d]
S2a[a]	8.8[d]	9.2[d]
S9	7.0[d]	21.0[d]
S10	1.0	28.0[d]
DENV-3		
S3a	5.0[d]	3.1[d]
S11	11.0[d]	44.0[d]
DENV-4		
S4a	0.2	2.6[d]
S4a[a]	1.3	3.4[d]
S12	2.0[d]	7.3[d]

[a] DENV strains isolated from the respective patients (S1, S2, S4, S8) were used for determining the presence of infection-enhancing activity
[b] Determined by RT-PCR
[c] Calculated by the formula: (mean plaque count with addition of human serum using FcγR expressing BHK cell lines)/(plaque count without addition of human serum using FcγR expressing BHK cell lines). Infection enhancement was tested using 1:5 diluted serum samples
[d] Presence of infection-enhancing activity as defined by enhancement values greater than cut-off value (cut-off = mean plaque count without addition of human serum using FcγR expressing BHK cell lines + 3SD)/(mean plaque count without addition of human serum using FcγR expressing BHK cell lines)

possess the ability to enhance infection of the infecting DENV serotype without dilution.

Clinical characteristics of dengue patients

Laboratory findings of patients with primary dengue infection and secondary dengue infection are summarized in Table 3. The differences between the two groups with infection-enhancing activity and without enhancing activity using undiluted serum samples was not significant for each of the parameters of severe symptoms (severe thrombocytopenia, leucopenia and elevated levels of AST and ALT, $p > 0.05$). However, clinical signs and symptoms differ during the course of disease, resulting in differential diagnosis or outcome in disease severity.[15] All patients were diagnosed as DF patients at the point of sample collection.

The viraemia levels determined by BHK and FcγR-expressing BHK cells moderately correlated with fold-enhancement in undiluted serum samples (Pearson correlation, BHK $r = 0.71$ and FcγR-BHK $r = 0.82$ respectively). Viraemia levels were higher in patients in which undiluted serum samples exhibited infection-enhancing activity (BHK cells = $1.1\text{x}10^7$ PFU/ml, FcγR-expressing cells = $1.5\text{x}10^8$ PFU/ml, days after onset of disease = 3.0 ± 1.2 days) as compared to the viraemia levels in serum samples with no infection-enhancing activity (BHK cells = $2.1\text{x}10^5$ PFU/ml, FcγR-expressing BHK cells = $1.4\text{x}10^6$ PFU/ml, $P_{BHK} = 0.06$ and $P_{Fc\gamma R\text{-}BHK} = 0.03$, days after onset of disease = 3.8 ± 1.9 days, $P_{DAYS} = 0.52$, two-tailed t-test). The results suggest that higher magnitude of infection-enhancement activity may contribute to higher viraemia. Further studies using

Table 3. Clinical characteristics of the dengue patients.

Category	Age (Gender)	Days after onset of disease	Platelet count (platelets/μl)[a]	WBC count (cells/μl)	AST Levels (IU/L)	ALT Levels (IU/L)	Viremia (PFU/ml)[b] BHK(B)	FcγR (Fc)	Fold viremia (Fc/B)[c]	Fold infection-enhancement[d]
Primary Infection										
P1	53 (M)	2	100 000[a]	1 170[a]	46	30	8.0×10^{6}	8.0×10^{6}	1.0	1.1
P2	23 (M)	1	115 000[a]	2 410[a]	NA	NA	4.6×10^{6}	5.9×10^{6}	1.3	0.9
P3	20 (F)	1	131 000[a]	6 640	23	22	8.5×10^{5}	1.1×10^{6}	1.2	1.0
Mean ± SD	32 ± 15[e]	1.3 ± 0.6	115333 ± 15503	3407 ± 2868	35 ± 16	26 ± 6	4.5×10^{6}	5.0×10^{6}	1.2 ± 0.2	1.0 ± 0.1
Secondary Infection										
Infection-enhancing activity in undiluted serum sample										
S2	31 (M)	NA	NA	NA	NA	NA	9.0×10^{6}	3.6×10^{8}	40.0	8.8
S3	30 (M)	4	61 000[a]	1 800[a]	98[a]	37	8.0×10^{3}	1.2×10^{4}	1.6	5.0
S7	90 (M)	4	105 000[a]	4 950	169[a]	160[a]	ND	ND	ND	5.0
S8	NA (M)	3	83 000[a]	2 300[a]	66[a]	31	3.9×10^{7}	3.7×10^{8}	9.5	9.0
S9	41 (F)	1	153 000	3 430[a]	38	37	6.5×10^{6}	3.5×10^{7}	5.4	7.0
S11	NA	2	164 000	3 400[a]	43	26	ND	ND	ND	11.0
S12	21 (M)	3	158 000	2 900[a]	23	14	1.4×10^{5}	2.2×10^{6}	15.7	2.0
Mean ± SD	43 ± 27	2.8 ± 1.2	120667 ± 43684	3130 ± 1094	73 ± 54	51 ± 54	1.1×10^{7}	1.5×10^{8}	14.4 ± 15.2	6.8 ± 3.1
No infection-enhancing activity in undiluted serum sample										
S1	60 (M)	5	64 000[a]	2 280[a]	70[a]	50	1.1×10^{5}	8.0×10^{5}	7.3	0.3
S4	28 (M)	5	139 000[a]	3 050[a]	58[a]	49	3.1×10^{3}	3.0×10^{4}	9.7	1.3
S5	22 (M)	4	74 000[a]	2 400[a]	29	17	1.3×10^{5}	6.8×10^{5}	5.2	0.6
S6	26 (M)	4	113 000[a]	1 920[a]	43	24	ND	ND	ND	1.2
S10	32 (M)	1	153 000	2 300[a]	65[a]	34	6.0×10^{5}	4.0×10^{6}	6.7	1.0
Mean ± SD	35 ± 15	3.8 ± 1.6	108600 ± 39055	2390 ± 411	53 ± 17	35 ± 15	2.1×10^{5}	1.4×10^{6}	7.0 ± 2.0	0.9 ± 0.4

NA: not available; ND: not detectable

[a] Indicates thrombocytopenia (platelet count <150 000 platelets/μl), leukopenia (white blood cells count <4000 cells/μl), increased levels of AST (>50 IU/ml) and increased levels of ALT (>50 IU/ml). Laboratory findings were values of samples that were collected on the disease day shown

[b] Indicates viraemia as determined by a conventional plaque titration method using BHK cells (B) and FcγR-expressing BHK cells (Fc). The viraemia levels of serum samples P1-P3 and S1-S12 have been included in a previous study[20]

[c] Fold viraemia was determined using the formula: (viremia (PFU/ml) determined by FcγR-expressing cells/viremia determined by BHK cells)

[d] Fold infection-enhancement of serum to infecting serotype was determined using the formula: (mean plaque count with addition of human serum using FcγR expressing BHK cell lines)/(plaque count without addition of human serum using FcγR expressing BHK cell lines). Infection enhancement was tested using undiluted serum samples for patients S1-S12. For patients with primary DENV infection (P1-P3) 1:5 diluted serum samples were used

[e] Numbers indicate mean age (years) ± S.D

matched illness days and a larger number of samples are needed to further clarify the association between high viraemia titers, severe dengue and infection-enhancement activity levels.

Discussion

The main attribute to disease severity and protection is the immune system of the host.[21] The human immune response to dengue virus consists of cross-reactive neutralizing antibodies with both neutralizing and enhancing activity.[22,23] The association with disease severity of antibodies can be attributed to enhanced-infection of the target cells, the FcγR-positive cells, resulting in higher infected cell mass.[21] In the present study, human serum samples were examined for ADE activity at low (1:5) serum dilution or without dilution. Undiluted serum samples, which may better reflect in vivo conditions as compared to diluted serum samples, exhibited infection-enhancing activity to the infecting serotype during the early phase of the disease but not during later stages of the disease. Serum samples exhibiting high neutralizing activity in FcγR-negative cell lines exhibited no ADE in FcγR-expressing BHK cells using low serum dilutions (Table 1). Conversely, serum samples exhibiting low levels of neutralizing activity in BHK cells demonstrated infection-enhancing activity to heterologous DENV serotypes at low or no serum dilutions (Tables 1 and 2). The lowest serum dilution used in the neutralizing assay was a 1:10 dilution. As with some cases of secondary dengue infection, there remains a possibility that the neutralizing titer to DENV in sample S3a may be present but was below tested levels [17, 24, 25]. Protection would be determined by the balance between neutralizing and enhancing activity. Furthermore, the samples S1a–S4a were collected during the early phase of secondary infection during the onset of cross-reactive secondary antibody response. Thus, it is difficult to conclude if protection existed before secondary infection using these serum samples. Presence of ADE activity to the infecting serotype during the early phase but not during the late phase of secondary infection (Figure 1) suggests that antibodies possess ADE activity during the early phase of the disease. Our study extends those of previous studies in which serum samples from pre-exposure non-acute patients were examined.[7,24,25] Infection-enhancement activity to other serotypes was present in serum samples obtained from patients and animal models with prior-exposure to DENV in the pre-infection stage.[26] Presence of enhancing activity in undiluted serum samples implies that enhancing antibodies may be one of the factors contributing to disease severity during secondary infection.

As different strains of DENV may possess different infection enhancement properties,[27] the enhancing activities to the patient's own isolate was examined. Two serum samples (S1a and S4a) did not demonstrate enhancing activity to laboratory established DENV strains and to the strains isolated from the respective patients (patients S1 and S4). It is of interest that sample S8 demonstrated enhancing activity to the patient's own isolate, but not to the other established strain of the same serotype. Other investigators have previously reported that productive infection was modulated by different DENV strains.[27,28] Additionally, differential infection-enhancement activity between strains could be attributed to genetic difference between viruses which contributes to antigenic changes.[29,30] Discrepancies in the ability of American DENV-1 sera to neutralize American and Asian genotypes of DENV-2 were speculated to be attributed to modifications in the envelop protein structure of the Asian DENV-2 genotype.[21] These reports further support our results that anti-DENV antibodies may promote infection-enhancement during secondary infection with different levels of ADE activity to the infecting strain.

Taken together, the data in the present study support the hypothesis that antibodies with low or undetectable levels of DENV neutralizing activity enhance heterotypic DENV infection of FcγR-expressing cells and suggest that ADE may occur in vivo during secondary DENV infection. As infection-enhancement activity determined using undiluted serum samples may better reflect some aspects of infection in vivo, the results suggest the necessity for examining ADE activity in serum samples without dilution in vaccine development and pathogenesis studies.

Authors' contributions: MLM carried out the experiments; MLM and IK designed the study protocol; MLM, TT, MS and IK did the analysis and interpretation of these data; MLM and IK drafted the manuscript, MLM, TT, MS and IK critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. TT and IK are guarantors of the paper.

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Ethical approval: The Ethics Committee of the National Institute of Infectious Diseases, Japan approved this study protocol. All patient serum samples were de-identified prior to use in the experiments.

References

1 Graham RR, Juffrie M, Tan R. A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia. I. Studies in 1995–1996. Am J Trop Med Hyg 1999;61:412–9.

2 Sangkawibha N, Rojanasuphot S, Ahandrik S et al. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 1984;120:653–69.

3 Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science 1988; 239:476–81.

4 Kou Z, Quinn M, Chen H et al. Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells. J Med Virol 2008;80:134–46.

5 Littaua R, Kurane I, Ennis FA. Human IgG Fc Receptor II mediates antibody-dependent enhancement of dengue virus infection. J Immunol 1990;144:3183–86.

6 Kliks SC, Nimmanitya S, Nisalak A, Burke DS. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg 1988;38:411–9.

7 Kliks SC, Nisalak A, Brandt WE, Wahl L, Burke DS. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. Am J Trop Med Hyg 1989;40:444-51.

8 Vaughn DW, Green S, Kalayanarooj S et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 2000;181:2-9.

9 Wang WK, Chen HL, Yang CF et al. Slower rates of clearance of viral load and virus-containing immune complexes in patients with dengue hemorrhagic fever. Clin Infect Dis 2006;43:1023-30.

10 Moi ML, Lim CK, Takasaki T, Kurane I. Involvement of the Fcγ receptor IIA cytoplasmic domain in antibody-dependent enhancement of dengue virus infection. J Gen Virol 2010;91:103-11.

11 Rodenhuis-Zybert IA, van der Schaar HM, da Silva Voorham JM et al. Immature dengue virus: a veiled pathogen? Plos Pathogens 2010;6:e1000718.

12 Ito M, Takasaki T, Yamada K et al. Development and evaluation of fluorogenic TaqMan reverse transcriptase PCR assays for detection of dengue virus types 1 to 4. J Clin Microbiol 2004;42:5935-7.

13 Ito M, Yamada K, Takasaki T et al. Phylogenetic analysis of dengue viruses isolated from imported dengue patients: possible aid for determining the countries where infections occurred. J Travel Med 2007;14:233-44.

14 Moi ML, Lim CK, Kotaki A et al. Detection of higher levels of dengue viremia using FcγR-expressing BHK-21 cells than FcγR-negative cells in secondary Infection but not in primary infection. J Infect Dis 2011;203:1405-14.

15 WHO. Dengue guidelines for diagnosis, treatment, prevention and control. Geneva: World Health Organization; 2009. whqlibdoc.who.int/publications/2009/9789241547871_eng.pdf [accessed 6 August 2012]

16 Roehrig J. Guidelines for plaque reduction neutralizing testing of human antibodies to dengue viruses. Geneva: World Health Organization; 2007. whqlibdoc.who.int/hq/2007/who_ivb_07.07_eng.pdf [accessed 6 August 2012]

17 Moi ML, Lim CK, Chua KB et al. Dengue virus infection-enhancing activity in serum samples with neutralizing activity as determined by using FcγR-expressing cells. PLoS Negl Trop Dis 2012;6:e1536.

18 Vanlandingham DL, Schneider BS, Klingler K et al. Real-time reverse transcriptase-polymerase chain reaction quantification of West Nile virus transmitted by Culex pipiens quinquefasciatus. Am J Trop Med Hyg 2004;71:120-3.

19 Vazeille M, Mousson L, Martin E, Failloux AB. Orally co-Infected Aedes albopictus from La Reunion Island, Indian Ocean, can deliver both dengue and chikungunya infectious viral particles in their saliva. PLoS Negl Trop Dis 2010;4:e706.

20 Konishi E, Tabuchi Y, Yamanaka A. A simple assay system for infection-enhancing and -neutralizing antibodies to dengue type 2 virus using layers of semi-adherent K562 cells. J Virol Methods 2010;163:360-7.

21 Halstead SB. Dengue. London: Imperial College Press. Tropical Medicine, Science and Practice series, vol 5. 2008.

22 Beltramello M, Williams KL, Simmons CP et al. The human immune response to dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell Host Microbe 2010;8:271-83.

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

24 Guy B, Chanthavanich P, Gimenez S et al. Evaluation by flow cytometry of antibody-dependent enhancement (ADE) of dengue infection by sera from Thai children immunized with a live-attenuated tetravalent dengue vaccine. Vaccine 2004;22:3563-74.

25 Laoprasopwattana K, Libraty DH, Endy TP et al. Dengue virus (DV) enhancing antibody activity in preillness plasma does not predict subsequent disease severity or viremia in secondary DV infection. J Infect Dis 2005;192:510-9.

26 Ito M, Mukai RZ, Takasaki T et al. Antibody-dependent enhancement of dengue virus infection in vitro by undiluted sera from monkeys infected with heterotypic dengue virus. Arch Virol 2010;155:1617-24.

27 Diamond MS, Edgil D, Roberts TG et al. Infection of human cells by dengue virus is modulated by different cell types and viral strains. J Virol 2000;74:7814-23.

28 Morens DM, Halstead SB, Marchette NJ. Profiles of antibody-dependent enhancement of dengue virus type 2 infection. Microb Pathog 1987;3:231-7.

29 Kochel TJ, Watts DM, Halstead SB et al. Effect of dengue-1 antibodies on American dengue-2 viral infection and dengue haemorrhagic fever. Lancet 2002;360:310-2.

30 Rodrigo WW, Alcena DC, Kou Z et al. Difference between the abilities of human Fcgamma receptor-expressing CV-1 cells to neutralize American and Asian genotypes of dengue virus 2. Clin Vaccine Immunol 2009;16:285-7.

Immune-Related Gene Expression Profile in Laboratory Common Marmosets Assessed by an Accurate Quantitative Real-Time PCR Using Selected Reference Genes

Yoshiki Fujii[1,2], Kazutaka Kitaura[1,2], Takaji Matsutani[3], Kenji Shirai[1,2], Satsuki Suzuki[4], Tomohiko Takasaki[2], Kenichi Kumagai[1,5], Yoshie Kametani[6], Takashi Shiina[7], Shuji Takabayashi[8], Hideki Katoh[8,9], Yoshiki Hamada[5], Ichiro Kurane[2], Ryuji Suzuki[1]*

1 Department of Rheumatology and Clinical Immunology, Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, National Hospital Organization, Kanagawa, Japan, **2** Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan, **3** Laboratory of Immune Regulation, Wakayama Medical University, Osaka, Japan, **4** Section of Biological Science, Research Center for Odontology, Nippon Dental University, School of Life Dentistry, Tokyo, Japan, **5** Department of Oral and Maxillofacial Surgery, School of Dental Medicine, Tsurumi University, Kanagawa, Japan, **6** Department of Immunology, Division of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Kanagawa, Japan, **7** Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Kanagawa, Japan, **8** Experimental Animals Institute, Hamamatsu University School of Medicine, Shizuoka, Japan, **9** Laboratory of Animal Breeding and Genetics, Central Institute for Experimental Animals, Kawasaki, Japan

Abstract

The common marmoset (*Callithrix jacchus*) is considered a novel experimental animal model of non-human primates. However, due to antibody unavailability, immunological and pathological studies have not been adequately conducted in various disease models of common marmoset. Quantitative real-time PCR (qPCR) is a powerful tool to examine gene expression levels. Recent reports have shown that selection of internal reference housekeeping genes are required for accurate normalization of gene expression. To develop a reliable qPCR method in common marmoset, we used *geNorm* applets to evaluate the expression stability of eight candidate reference genes (*GAPDH, ACTB, rRNA, B2M, UBC, HPRT, SDHA* and *TBP*) in various tissues from laboratory common marmosets. *geNorm* analysis showed that *GAPDH, ACTB, SDHA* and *TBP* were generally ranked high in stability followed by *UBC*. In contrast, *HPRT, rRNA* and *B2M* exhibited lower expression stability than other genes in most tissues analyzed. Furthermore, by using the improved qPCR with selected reference genes, we analyzed the expression levels of CD antigens (CD3ε, CD4, CD8α and CD20) and cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12β, IL-13, IFN-γ and TNF-α) in peripheral blood leukocytes and compared them between common marmosets and humans. The expression levels of CD4 and IL-4 were lower in common marmosets than in humans whereas those of IL-10, IL-12β and IFN-γ were higher in the common marmoset. The ratio of Th1-related gene expression level to that of Th2-related genes was inverted in common marmosets. We confirmed the inverted ratio of CD4 to CD8 in common marmosets by flow cytometric analysis. Therefore, the difference in Th1/Th2 balance between common marmosets and humans may affect host defense and/or disease susceptibility, which should be carefully considered when using common marmoset as an experimental model for biomedical research.

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* E-mail: r-suzuki@sagamihara-hosp.gr.jp

Introduction

The common marmoset (*Callithrix jacchus*) is a New World monkey and is considered potentially useful as an experimental animal model in research fields such as drug toxicology [1,2], neuroscience [3,4], autoimmune diseases [5,6] and infectious diseases [7,8], because of its size, availability and high genetic similarity with humans [9,10]. Compared with mice, common marmosets are more useful as an *in vivo* model to study immune function [11]. However, essential tools and gene information for conducting studies using common marmosets are in short supply or unavailable. For example, monoclonal antibodies specific for common marmosets have been only partially established. Although DNA microarray research for common marmoset brain has been reported [12], sufficient studies have not been performed in other research fields.

Quantitative real-time polymerase chain reaction (qPCR) is the dominant quantitative technique for gene expression analysis due to its broad dynamic range, accuracy, sensitivity, specificity and

speed [13]. Thus, qPCR is very useful for investigating physiological and pathological status from a small amount of sample. Normalization to reference genes such as housekeeping genes is usually required for qPCR analysis. However, expression levels of reference genes may vary between tissues, cell types and experimental conditions. Therefore, the validation of suitable reference genes in each experiment is critical for the accurate evaluation of qPCR data. Recently, a set of guidelines for evaluating qPCR experiments was developed [14] and a strict method for the selection of reference genes suitable for normalization was proposed [15]. A freely available program, *geNorm* applet (http://medgen.ugent.be/~jvdesomp/genorm/), can determine gene stability ranking and the number of reference genes required for normalization in a given panel of samples [15].

To develop an accurate and reliable qPCR method for common marmosets, we examined the expression stabilities of candidate reference genes in various tissues of laboratory common marmosets using *geNorm* applet. Then, we compared expression levels of immune-related genes in peripheral blood leukocytes between common marmosets and humans. To the best of our knowledge, this is the first such study for the selection of reference genes in common marmosets. The present data will contribute to future studies of gene expression analysis by qPCR for common marmosets.

Materials and Methods

Ethics statement

The study was conducted in accordance with the Act on Welfare and Management of Animals of Japanese government. All animals were housed, cared for, and used according to the principles set forth in the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Research Council, 2011). All experiments using common marmosets were approved by the committee for animal experiments at the National Institute of Infectious Diseases (Approval Number: 610,007). For humans, whole blood was obtained from eight healthy volunteers (mean age $\pm$ sd: 35.7 ± 13.0 years old) after obtaining written informed consent. This study and the consent procedure were approved by the ethics committee of Tokai University School of Medicine (Approval Number: 10I-22).

Animals

Eight common marmosets (1.58 ± 0.29 years old) were obtained from CLEA Japan, Inc. (Tokyo, Japan) and maintained in specific pathogen-free conditions at the National Institute of Infectious Diseases (Tokyo, Japan). Common marmosets were housed solely or in pairs in a single cages 39 cm (W)×55 (D)×70 (H) in size on 12:12 h light/dark cycles. Room temperature and humidity were maintained at 26–27°C and 40–50%, respectively. Filtered drinking water was delivered by an automatic watering system and total 40–50 g/individual of commercial marmoset chow (CMS-1M, CLEA Japan) were given in a couple of times per day. Dietary supplements (sponge cakes, eggs, banana pudding, honeys, vitamin C and D3) were also given to improve their health status. Machinery noise and dogs' barks were avoided to reduce stress. The cages were equipped with resting perches and a nest box as environmental enrichment. The marmosets were routinely tested to assure the absence of pathogenic bacteria, viruses, and parasite eggs in the animal facilities and did not exhibited abnormal external appearances. Four common marmosets were euthanized by cardiac exsanguinations under anesthesia with Ketamine hydrochroride (50 mg/kg, IM) and Xylazine (3.0 mg/kg, IM). After sacrifice, various tissues removed, and whole blood was obtained from all eight common marmosets.

RNA isolation

Heparinized venous blood samples from common marmosets were obtained before sacrifice and incubated in erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). Following incubation on ice for 5 min, cells were centrifuged at $300\times g$ for 10 min at 4°C and washed with lysis buffer and then PBS. Leukocytes were lysed with QIAzol® Lysis Reagent (Qiagen, Hilden, Germany) and total RNA was extracted using an RNeasy® Plus Universal Mini Kit (Qiagen) according to the manufacturer's instructions. Tissue samples (spleen, mesenteric lymph node, jejunum, ileum, descending colon, cerebrum, cerebellum, brainstem, heart, lung, liver and kidney) were excised from each animal and immediately submerged in RNAlater® RNA Stabilization Reagent (Qiagen). Then total RNA was extracted using RNeasy® Plus Universal Mini Kit (Qiagen). RNA concentration and integrity were assessed using the Agilent RNA 6,000 Nano Kit (Agilent Technologies, Inc., CA, USA) in an Agilent 2100 Bioanalyzer. All RNA samples were confirmed to have no degradation and were of optimal quality for downstream qPCR applications.

Candidate reference genes

Based on a literature search, eight commonly used candidate internal control genes were selected for analysis: *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *ACTB* (actin, beta), *rRNA* (18S ribosomal RNA), *B2M* (beta-2-microglobulin), *UBC* (ubiquitin C), *HPRT* (hypoxanthine phosphoribosyltransferase 1), *SDHA* (succinate dehydrogenase complex, subunit A) and *TBP* (TATA-box binding protein). All genes chosen have independent cellular functions and are not thought to be co-regulated. The sequences of primers specific for each reference gene are shown in Table 1.

Quantitative real-time PCR

First-strand cDNA was synthesized using PrimeScript® RT reagent Kit (Takara Bio, Otsu, Japan) with attached random hexamers and oligo(dT) primers. Reactions were incubated at 37°C for 15 min followed by 85°C for 5 sec according to the manufacturer's instructions. Then each cDNA sample was diluted with RNase/DNase-free water to 25 ng/µL. The expression level of each gene was analyzed by qPCR using the Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR reactions consisted of 5 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad), 3.5 µL of RNase/DNase-free water, 0.5 µL of 5 µM primer mix, 1 µL of cDNA in a total volume of 10 µL. The primer sequences are shown in Tables 1 and 2. Cycling conditions were as follows: 30 sec at 95°C followed by 45 rounds of 95°C for 1 sec and 60°C for 5 sec. Melting curve analysis to determine the dissociation of PCR products was performed between 65°C and 95°C. Data were expressed as mean values of experiments performed in triplicate. Seven points of a 10-fold serial dilution of standard DNA was used for absolute quantification. Standard DNA was generated by cloning PCR products into pGEM-T Easy Vector (Promega, WI, USA). Sequences of the cloned plasmid were confirmed by DNA sequencing using the CEQ8000 Genetic Analysis System (Beckman Coulter). Quality and concentration of the plasmid DNA were validated using Agilent DNA 7,500 Kit in an Agilent 2100 Bioanalyzer.

Table 1. Sequences of qPCR primers for housekeeping genes.

Target gene	Species	5′-primer sequence -3′[a),b)]		Product size (bp)	PCR efficiency	Reference
		Forward	Reverse			
GAPDH	Cj	TCGGAGTCAACGGATTTGGTC	TTCCCGTTCTCAGCCTTGAC	181	0.920	DD279474
	Hs	---------------------	--------------------	181	0.921	AF261085
ACTB	Cj	GATGGTGGGCATGGGTCAGAA	AGCCACACGCAGCTCGTTGT	163	0.901	DD279463
	Hs	---------------------	---------------A----	163	0.883	NM_001101
HPRT	Cj	ATCCAAAGATGGTCAAGGTCG	GTATTCATTATAGTCAAGGGCATA	134	0.842	DD289567
	Hs	---------------------	------------------------	134	0.880	M31642
B2M	Cj	CTATTCAGCATGCTCCAAAGA	AAGACAAGTCTGAATGCTCCAC	168	0.928	AF084623
	Hs	----C----G-A---------	----------------------	168	0.950	AB021288
UBC	Cj	TCCCTTCTCGGCGGTTCTG	. TGCATTGTCAAGCGGCGAT	158	0.922	AB571242
	Hs	-------------A-----	TC----------T-A----	160	0.936	NM_021009
rRNA	Cj	CGACCATAAACGATGCCGAC	GGTGGTGCCCTTCCGTCAAT	145	0.918	AB571241
	Hs	--------------------	--------------------	145	0.940	M10098
SDHA	Cj	TGGGAACAAGAGGGCATCTG	CCACCACGGCATCAAATTCATG	86	0.934	XM_002745154
	Hs	--------------------	-------T--------------	86	0.948	BC001380
TBP	Cj	CCATGACTCCCGGAATCCCTAT	ATAGGCTGTGGGGTCAGTCCA	70	0.920	EU796973
	Hs	----------------------	---------------------	70	0.954	M55654

[a)]Hyphen indicates a nucleotide identical to human sequences.
[b)]Dot indicates a shift nucleotide to marmoset sequences.
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Analysis of gene expression stability

The expression stability of selected reference genes was evaluated using a publicly available program, *geNorm* applet [15]. *geNorm* calculates the stability of tested reference genes according to the similarity of their expression profiles by pairwise comparison and M value, where the gene with the highest value is the least stable one. It is possible to perform sequential elimination of the least stable gene in any given experimental group, thus resulting in the exclusion of all but the two most stable genes in each case.

Flow cytometry

Heparinized peripheral blood was collected from common marmosets and centrifuged in Lymphocepal (IBL Co. Takasaki, Japan) at 2,000 rpm for 30 min. Mononuclear cells were collected and re-suspended in RPMI1640 medium containing 10% fetal calf serum. Cells were stained with anti-common marmoset CD8 antibody (Mar8–10) [16] for 15 min at 4°C and washed with 1% (w/v) bovine serum albumin-containing PBS. Subsequently, cells were stained with phycoerythrin-labeled secondary antibody, peridinin chlorophyll protein cyanin5.5 (PerCPCy5.5)-conjugated anti-human CD3 (SP34-2) and Alexa488-conjugated anti-common marmoset CD4 (Mar4-33) antibodies [16]. Peripheral blood from healthy human volunteers was collected and mononuclear cells isolated by Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden) gradient centrifugation. The monoclonal antibodies used for cell staining were as follows: PerCPCy5.5-conjugated anti-human CD3 (SP34-2), allophycocyanin-conjugated anti-human CD4 (SK3), fluorescein isothiocyanate-conjugated anti-human CD8 (HIT8a) (BD PharMingen). Cells were analyzed by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Student's *t*-test was used for statistical analysis to assess significant differences in qPCR assays. A P value<0.05 was considered to be statistically significant.

Results

The expression levels of candidate reference genes in tissues

Eight housekeeping genes were chosen as reference genes: *GAPDH*, *ACTB*, *rRNA*, *B2M*, *UBC*, *HPRT*, *SDHA* and *TBP*. We determined the transcription levels of these eight genes in 13 tissues (leukocyte, spleen, lymph node, jejunum, ileum, colon, cerebrum, cerebellum, brainstem, heart, lung, liver and kidney) from four individual common marmosets by qPCR. The sequences of primers specific for each reference gene are shown in Table 1. The expression level of each gene in each tissue is shown as the copy number per µg of purified total RNA (Figure 1). The most abundant gene was *rRNA* while the rarest gene was *UBC* and the difference in expression level between the two genes was more than 100,000-fold. For several genes, the expression levels were highly different among tissues. For example, *B2M* expression in heart and brain segments (cerebrum, cerebellum and brainstem) was markedly lower than in other tissues. *HPRT* expression also showed a large variability among tissues. In addition, the expression levels of *rRNA*, *B2M* and *HPRT* varied among individuals; the mean values of standard deviation were 0.224, 0.235 and 0.303, respectively, while those of the other genes were below 0.2.