厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）
「我が国への侵入が危惧される蚊媒介性ウイルス感染症に対する総合的対策の確立に関する研究」（H23－新興－一般－010）

分担研究報告書（H23-25）

チクングニアウイルスのコモンマーモセットモデルにおける病理学的解析および夏期の日本旅行後デング熱を発症したドイツ人デング熱患者症例と実験室確認診断

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研究要旨

チクングニア熱が近年アフリカ東岸から南アジア，東南アジアにかけて大流行している．また温帯地域における初めての国内流行がイタリア（2007年），フランス（2010年）に7報告された．したがって媒介蚊の生息する日本国内へのチクングニアウイルス(CHIKV)の侵淫の可能性は否定できない．これまでに我々はコモンマーモセットを用いたCHIKV感染モデルの検討を行った．その結果，CHIKVの接種により高いウイルス血症と迅速な特異的抗体の上昇を観察した．そこで本研究においてCHIKV接種マーモセットに対する病理学的解析を行った．その結果CHIKV接種4～21日の長期に渡り脾臓および腋窩リンパ節にウイルス遺伝子が検出された．また肝臓および脾臓において特異的抗原が検出され，肝臓においては肝のシングルセルネクローシス，細胞浸潤，脾臓においては二次濾胞の形成およびstarry sky像が観察された．

日本（本州）旅行から帰国したドイツ人が、2013年9月9日にドイツ（ベルリン）の病院を受診。9月3日より、40度の熱、嘔気、続いて、斑状丘疹状皮疹が出現。入院9日前に、2週間の日本旅行（8月19～31日）から直行便にて帰国した。鑑別診断の結果、臨床像よりデング熱を疑った。発症後7日目の血清で、デングウイルスIgM及びIgG抗体価試験、デングウイルスNS1抗原及び迅速試験で全て陽性であったことから、患者はデングウイルス急性感染であることが示された。デングウイルスRNA遺伝子は陰性であった。日本からのデング熱の輸入症例は極めて珍しいことから、2013年12月（発症後110日目）に第2回目の血清を採取して検査した。 検体をドイツから国立感染症研究所に送付していただき、確認検査を実施した。その結果、デングウイルス非構造蛋白抗原陽性、抗デングウイルスIgM抗体陽性、IgG抗体陽性であった。また、中和抗体を測定した結果、デングウイルス2型に対して1、3、4型および日本脳炎ウイルスに対する中和抗体価より有意

に高く、デングウイルス２型感染であったと診断された。

A. 研究目的

近年チクングニアウイルス（Chikungunya virus: CHIKV）がアフリカ東岸からインド,東南アジアにかけて再興している．我が国においても毎年10数例の輸入症例が報告されている．CHIKVはトガウイルス科アルファウイルス属に分類される一本鎖の(+)RNA ウイルスであり，チクングニア熱の原因ウイルスである．CHIKVは蚊媒介性ウイルスであり,その媒介蚊はネッタイシマカ（*Aedes aegypti*）や日本にも広範囲に生息するヒトスジシマカ（*A. albopictus*）などのヤブカ属のカである．2005年のレユニオン島でのチクングニア熱の流行においては，呼吸器不全，心代償不全，髄膜脳炎，劇症肝炎,腎不全等の症状と219人の死者が報告された．したがってチクングニア熱の病態はいまだ不明である．近年コモンマーモセット（マーモセット）が新たな霊長類モデルとして注目されている．そこで我々はマーモセットを用いたCHIKV感染モデルの検討を行った．その結果，CHIKVの接種により高いウイルス血症と迅速な特異的抗体の上昇を観察した．そこで本研究の目的はCHIKV接種マーモセットに対する病理学的解析を行うことである．

また､デング熱もチクングニア熱ともにウイルス血症が高く輸入症例から国内発生の可能性がある。ドイツ Bernhard Nocht Institute for Tropical Medicine およびロベルトコッホ研究所からの情報提供を受け､日本からの輸出デング熱疑い症例の確認検査を実施した。

B. 研究方法

マーモセットモデル

ウイルスと培養細胞：感染実験にはCHIKV SL10571株を供試した．ウイルス分離およびウイルス中和試験にはサル腎由来のVero細胞を用いた（American Type Culture Collection).

動物：体重 300ｇ～379ｇのコモンマーモセット（*Callithrix jacchus*）を用いた．ウイルス学的解析は採取した組織よりウイルス遺伝子を抽出し，TaqMan リアルタイムRT-PCR 法により組織中のウイルス遺伝子量を検出した．採取した組織は病理学的解析を実施した。

輸出デング熱症例の検討（検査方法）

１）デングウイルスNS1抗原検出

Platelia Dengue NS1 Ag ELISA キット（BioRad社）を用いた。

２）抗デングウイルスIgM抗体

抗デングウイルスIgM抗体捕捉ELISAキット(Focus Diagnostics社）を用いた。

３）抗日本脳炎IgM抗体

抗日本脳炎ウイルス IgM 抗体捕捉 ELISA 法（in house）により、P/N 比＝（検体の OD 値）／（陰性コントロールの OD 値）を算出し、P/N比2.0以上を陽性と判定した。

４）抗デングウイルス、日本脳炎ウイルス中和抗体測定

BHK細胞、FcγR発現BHK細胞、Vero細胞を用いた 50%プラーク減少法により測定し、10 倍希釈よりの 2 倍階段希釈法によりEndpointを決定し抗体価を決定した。

C. 研究結果

● マーモセットモデル

組織中の CHIKV RNA 量の検討：脾臓，腋窩リンパ節においてウイルス RNA が検出された．脊髄，大腿筋，心筋，大脳，小脳，肺からはウイルスRNAは検出されなかった．また対照個体である#5017からはウイルスRNAは検出されなかった．

病理学的解析：マーモセットの肝臓において，細胞浸潤，肝のシングルセルネクローシスが認められ，類洞内への細胞浸潤,肝細胞，肝管上皮細胞およびkupper 細胞に特異的抗原が観察された．肝細胞，kupper細胞に特異的抗原が観察された．また脾臓においては二次濾胞の形成，二次濾胞においてstarry sky像

が観察された.

● 輸出デング熱症例の検討

日本からのデング熱の輸入症例は極めて珍しいことから、ドイツ Bernhard Nocht Institute for Tropical Medicine では、2013 年 12 月（発症後 110 日目）に第 2 回目の血清サンプルを採取し、デングウイルス IgM 及び IgG 抗体が有意に減少、デングウイルス NS1 抗原（ELISA 法、迅速試験）が陰性との結果が得られたことから、患者はデングウイルスに感染したと判断された。IgG 抗体が、20480 倍から 640 倍に有意に低下したという点が、通常デング熱感染では起こらない現象であるため、ワクチン接種歴を確認したところこの患者は 2009 年に、ケニヤに旅行する際に黄熱ワクチンを接種していたことが判明した。

デングウイルスおよび日本脳炎ウイルス IgM 捕捉 ELISA 法によりそれぞれの IgM 抗体を測定したところ、デングウイルスに対しても日本脳炎ウイルスに対しても陽性であったが、デングウイルスに対して高かった。また中和抗体価の測定結果では、デングウイルス 2 型に対して 640 倍、1 型および 3 型に対して 10 倍、4 型に対して 10 倍以下、日本脳炎ウイルスに対して 40 倍であった。また、FcgR 発現 BHK 細胞を用いた場合でもデングウイルス 2 型に対して有意な低下を認めず 320 倍であった。

D. 考察

CHIKV 接種 3, 7, 10, 21 日後に安楽殺を行いコモンマーモセットの各臓器を採取しウイルス学的および病理学的解析を行った. 接種後 4～21 日の長期に渡り脾臓および腋窩リンパ節にウイルス遺伝子が検出されたことから CHIKV の感染が成立した可能性が示唆された. また肝臓、脾臓に特異的抗原が検出され, 肝細胞に特異的抗原の観察, 肝のシングルセルネクローシス, 細胞浸潤が観察され, さらに脾臓においては二次濾胞の形成および二次濾胞内に starry sky 像が観察されたことから CHIKV の感染が成立した可能性が示唆された.

ドイツ Bernhard Nocht Institute for Tropical Medicine では、抗体測定に蛍光抗体法（IF）を用いている。この方法で IgG 抗体を測定すると感度が良いためかなりの交差反応を拾うことになる。急性期血清中の IgG 抗体価が 20,480 と非常に高値であったのは、2009 年に接種した黄熱ワクチンによる IgG 抗体が交差反応し抗体価を押し上げたものと考えられる。2013 年は FIFA ワールドカップがデング熱および黄熱の流行地であるブラジルで開催されることから黄熱ワクチン接種後のブラジルからのデング熱輸入症例の発生が危惧されているところである。血清診断において本症例と同様の現象がみられる可能性が高く十分な注意が必要である。

E. 結論

急速な輸送手段の発達とネッタイシマ蚊, ヒトスジシマ蚊の分布拡大, 熱帯雨林地域への人口拡張により世界の熱帯・亜熱帯地域, 特に東南アジアにおいてチクングニア熱およびデング熱は今後も流行が続くことが予想される. したがって日本においてもサーベイランス体制の充実、媒介蚊対策、将来のワクチン開発, 特異的治療法の開発は重要な課題である。

F. 健康危険情報

特記事項なし

G. 研究発表

研究成果の刊行に関する一覧表に記載した。

H. 知的財産権の出願・登録状況

特記事項なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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研究成果の刊行に関する一覧表(ガイドラインその他)

	タイトル	発行所	出版社名	発行年
濱田篤郎　監修	デング熱予防マニュアル	東京医科大学病院渡航者医療センター	(株)アイワエンタープライス	2013
濱田篤郎　監修	デング熱豆知識	東京医科大学病院渡航者医療センター		2013
濱田篤郎　監修	昼の吸血鬼に御用心（ポスター）	東京医科大学病院渡航者医療センター		2012
濱田篤郎、沢辺京子、駒形修、小川修平、高崎智彦	デング熱の予防対策（動画）	東京医科大学病院渡航者医療センター 国立感染症研究所		2014
高崎智彦、松井珠乃、モイ メンリン	デング熱対策ガイドライン	国立感染症研究所 ウイルス第一部 感染症疫学センター		2014

Ⅳ. 研究成果の刊行物・別刷

Tropical Medicine and Health Vol. 39 No. 4 Supplement, 2011, pp. 13-15
doi:10.2149/tmh.2011-S07

Review

Imported dengue fever/dengue hemorrhagic fever cases in Japan

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Published online 12 October, 2011

Abstract: Several dengue outbreaks occurred in Japan from 1942 to 1945. Dengue fever emerged in Nagasaki in August 1942 and soon spread to other cities such as Sasebo, Hiroshima, Kobe and Osaka, recurring every summer until 1945 and constituting the greatest outbreak in the temperate zone. Domestic outbreaks have not been reported in Japan since then. However, the number of imported dengue cases has increased year by year: 868 imported cases were reported in Japan between 1999 and 2010 according to the Infectious Diseases Control Law. Moreover, 406 imported cases were confirmed to be dengue virus infection among 768 dengue suspected cases received at NIID from 2003 to 2010. A total of 142 cases (35.6%), 103 cases (25.8%) and 62 cases (15.5%) were noted in the 20–29, 30–39 and 40–49 age groups, respectively. Infecting dengue virus serotypes were determined for 280 of the 406 cases. The number of cases infected with each of the 4 serotypes was 98 (35%) with type 1, 78 (28%) with type 3, 72 (26%) with type 2, and 32 (11%) with type 4. Sixty percent of dengue cases were imported from July to October, the summer vacation season in Japan.
Key words: dengue fever, imported case

Dengue virus infections are a major public health problem in tropical and subtropical countries around the world [1, 2]. In Japan, which is located in the temperate zone, there were dengue outbreaks from 1942 to 1945. Dengue fever emerged in Nagasaki in August 1942 and soon spread to other cities such as Sasebo, Hiroshima, Kobe and Osaka, recurring every summer until 1945 [3]. Endemic cases had been reported in Okinawa since 1893 before the outbreak [4]. Domestic outbreaks have not been reported in Japan since 1945, but there have been many imported dengue cases [5]. Dengue fever (DF), including dengue hemorrhagic fever (DHF), is one of the Category IV notifiable infectious diseases designated by the Infectious Diseases Control Law of Japan since 1999. In the present study, the demographic features of imported DF/DHF cases confirmed by laboratory tests were analyzed over the period from 2003 to 2010 at the Vector-Borne Virus Laboratory, Department of Virology 1, National Institute of Infectious Diseases (NIID), Japan.

We confirmed 406 cases of dengue virus infection from 2003 to 2010: 28 cases in 2003, 20 in 2004, 43 in 2005, 29 in 2006, 51 in 2007, 67 in 2008, 43 in 2009 and 124 in 2010 (Table 1). We received blood specimens from 768 suspected dengue cases for laboratory confirmation. The rate of confirmation was 52.8% among clinically suspected cases over the 8-year period.

Infecting dengue virus serotypes were determined for 280 cases by real-time reverse transcriptase polymerase chain reaction (TaqMan method) [6] (Table 2). The number of cases infected with each of the 4 serotypes was 98 (35%) with type 1, 78 (28%) with type 3, 72 (26%) with type 2, and 32 (11%) with type 4. Interestingly, there was no case infected with dengue virus type 4 in 2003 or 2006 and only one in 2007, but the number increased to 7 in 2008, 8 in 2009 and 10 in 2010. Age distribution was analyzed for the

Table 1. The number of DF/DHF cases confirmed in Department of Virology 1, National Institute of Infectious Diseases.

Year	Cases examined and confirmed in NIID		
	examined	confirmed	positive rate (%)
2003	41	28	68.3
2004	54	20	37.0
2005	71	43	60.6
2006	100	29	29.0
2007	104	51	49.0
2008	129	67	51.9
2009	86	44	51.2
2010	183	124	67.8
Total	768	406	52.9

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Table 2. Dengue virus types detected in dengue cases from 2003 to 2010

Dengue virus type	2003	2004	2005	2006	2007	2008	2009	2010	Total (%)
type 1	4	6	8	10	10	17	8	35	98 (35%)
type 2	8	4	5	1	5	9	13	27	72 (26%)
type 3	4	0	13	9	16	16	7	13	78 (28%)
type 4	0	2	4	0	1	7	8	10	32 (11%)
Total	16	12	30	20	32	49	36	85	280

Table 3. Age distribution of dengue cases

Age \ year	2003	2004	2005	2006	2007	2008	2009	2010	Total
0–9	0	1	2	1	2	1	0	2	9
10–19	2	2	1	5	3	3	1	13	30
20–29	9	5	10	10	24	22	14	48	142
30–39	11	9	16	4	9	14	16	24	103
40–49	2	1	7	6	8	9	7	22	62
50–59	0	2	3	1	4	9	3	8	30
≥60	1	0	4	1	1	8	1	7	23
unknown	3	0	0	1	0	1	2	0	7
Total	28	20	43	29	51	67	44	124	406

Table 4. Monthly distribution of dengue cases

month \ year	2003	2004	2005	2006	2007	2008	2009	2010	Total
Jan	1	1	3	1	0	2	5	3	16
Feb	2	0	2	0	3	7	4	4	22
Mar	2	0	0	3	4	2	0	8	19
Apr	2	0	1	5	5	1	2	9	25
May	2	0	0	1	3	6	3	5	20
Jun	2	0	3	2	3	4	0	5	19
Jul	5	3	2	4	7	7	2	12	42
Aug	5	9	7	2	10	10	7	25	75
Sep	2	6	11	3	5	12	9	25	73
Oct	3	1	5	2	6	9	10	19	55
Nov	1	0	7	1	3	6	0	6	24
Dec	1	0	2	5	2	1	2	3	16
Total	28	20	43	29	51	67	44	124	406

399 cases in which information on age was available (Table 3). A total of 142 cases (35.6%) were in the 20–29 year age-group, the most active for overseas travel, while 103 cases (25.8%) and 62 cases (15.5%) were noted in the 30–39 and 40–49 year age groups, respectively. Thus, 165 cases (41.4%) ranged in age between 30 and 49, an age-group that often traveled abroad for business. Monthly distribution was analyzed (Table 4), and cases were found to occur in all of the 12 months. However, 75 cases were diagnosed in August, 73 in September, 55 in October and 42 in July, indicating that 60% of dengue cases were imported from July to October. The period from July to September is the summer vacation season in Japan when many overseas travelers visit dengue endemic areas. According to Japanese Emigration and Immigration Management, about 40% of immigration was recorded from July to September.

Table 5.

Year	Number of reported cases
1999	9
2000	18
2001	50
2002	52
2003	32
2004	49
2005	74
2006	58
2007	89
2008	104
2009	88
2010	245
Total	868

Up to 100 million cases of dengue fever (DF) and 250,000 cases of dengue hemorrhagic fever (DHF) are estimated to occur annually in the world [7], and the epidemics have been expanding. Recently, dengue outbreaks occur every year in Taiwan [8], and dengue virus endemicity was confirmed for the first time in Nepal [9, 10]. Nearly 11 million Japanese visit tropical and subtropical areas annually and 2 million people visit Japan from these areas. The number of imported dengue cases has increased year by year, 245 imported cases being reported in 2010 and total 868 cases being reported between 1999 and 2010 according to the Infectious Diseases Control Law (Table 5). Dengue epidemics often occur in urban areas because the breeding sites of *Aedes aegipti* concentrate in residential areas. The urbanization of dengue endemic countries contributes to frequent dengue epidemics or outbreaks in tropical and subtropical regions. Japanese travelers trend to visit urban areas more frequently than rural areas. There is a need for closer surveillance of DF and DHF in Japan. Updated information on dengue should be made available for to the travelers to tropical and subtropical areas.

ACKNOWLEDGMENTS

This work was supported by grants for Research on Emerging and Re-emerging Infectious Diseases (H20-shinkou-ippan-015 and H23-shinkou-ippan-010) from the Ministry of Health, Labour and Welfare, Japan.

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Detection of Dengue Virus Genome in Urine by Real-Time Reverse Transcriptase PCR: a Laboratory Diagnostic Method Useful after Disappearance of the Genome in Serum

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The reemergence of dengue virus (DENV) infection has created a requirement for improved laboratory diagnostic procedures. In this study, DENV genome detection in urine was evaluated as a diagnostic method. The DENV genome was detected by real-time reverse transcriptase PCR (RT-PCR) in urine and serum of dengue patients. The detection rate of DENV genome in urine was 25% (2/8) on disease days 0 to 3 and 32% (7/22) on days 4 to 5. The rate was 50% or higher on days 6 to 16, 52% (11/21) on days 6 to 7, 78% (7/9) on days 8 to 9, 80% (4/5) on days 10 to 11, 50% (2/4) on days 12 to 13, and 60% (3/5) on days 14 to 16. The last positive urine sample was on day 16. The detection rates in serum were highest on days 0 to 3 and were greater than 50% on days 0 to 7. Detection rates decreased thereafter, and the last positive detection was on day 11. These results indicate that the time frames for positive detection differ between urine and serum samples, whereby detection rates of 50% or higher are evident between days 6 to 16 for urine samples and days 0 to 7 for serum samples. Nucleotide sequences of PCR products were identical between urine and serum samples. The detection of DENV genome in urine samples by real-time RT-PCR is useful to confirm DENV infection, particularly after viremia disappears.

Dengue virus (DENV) infections occur in most of the tropical and subtropical areas of the world. DENV infection with any of four serotypes leads to a broad spectrum of clinical symptoms and severity, including asymptomatic infection, dengue fever (DF), and fatal dengue hemorrhagic fever (DHF). DF/DHF is considered one of the most important reemerging infectious diseases (4). Physicians and pediatricians in countries in which these diseases are not endemic are often unfamiliar with the symptoms and unaware of the potential importation of patients with DF/DHF. As such, DF/DHF often may not be considered part of a differential diagnosis. Furthermore, laboratory diagnosis is hampered in areas where the disease is endemic because of the limited number of facilities with diagnostic capacity, and specimen collection in a proper time frame is not easy in areas were DF/DHF is endemic.

Several laboratory diagnostic techniques have been used for the confirmation of dengue virus infection: viral isolation, viral antigen detection, viral genome detection, and antibody (Ab) detection. IgM capture enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcriptase PCR (RT-PCR) are commonly used (6, 8, 16). NS-1 antigen detection tests have also recently become commercially available (10); however, they cannot determine specific viral types. The antibody/antigen detection of DENV provides less information than the other detection assays, and the virus can be successfully isolated only during limited stages of infection. For detailed analyses, the detection of the DENV genome in serum samples by RT-PCR is widely used. A fluorogenic probe-based assay, which has a number of advantages over conventional RT-PCR, has recently been developed. It has the advantages of reduced turnaround time and a much lower risk of contamination compared to that of conventional RT-PCR (3). However, it is usually difficult to detect viral genomes after the development of antibodies against DENVs and the onset of defervescense (14). The use of urine samples for laboratory diagnostic testing has some advantages over the use of serum samples, such as ease of use and noninvasiveness. Our group and others have previously reported the detection of DENV genome in urine samples for a limited number of patients (11, 12). In the present study, we attempted to determine the usefulness of urine samples in the laboratory diagnosis of DENV infection. In the present study, we evaluated the usefulness of urine samples in the laboratory diagnosis of DENV infection by comparing real-time RT-PCR from serially collected urine and serum samples from confirmed DENV cases. We also compared RT-PCR for urine and serum to IgG and IgM ELISAs for serum and virus isolation from urine.

MATERIALS AND METHODS

Sample collection. Serum and urine samples were collected from 53 dengue patients at clinics and hospitals in Japan from 2006 to 2008, and they were sent to the National Institute of Infectious Diseases (NIID) for laboratory diagnosis. The median age was 30 years with a range of 9 to 65 years. All patients had a history of visits to countries in which dengue is

Received 4 December 2011 Returned for modification 28 December 2011
Accepted 15 March 2012
Published ahead of print 21 March 2012
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Supplemental material for this article may be found at http://jcm.asm.org/.

doi:10.1128/JCM.06557-11

TABLE 1 Results of real time RT-PCR of urine and serum samples and levels of serum IgM and IgG

Patient no.	Disease day	Infecting serotype	RT-PCR result with[b]: Urine	RT-PCR result with[b]: Serum	Serum antibody[a] (index): IgM	Serum antibody[a] (index): IgG
1	7	D1	+	−	+ (9.0)	+ (2.0)
	8	D1	+	NT	NT	NT
	14	D1	+	−	+ (6.1)	+ (2.3)
	25	D1	−	−	+ (4.1)	+ (2.4)
2	5		−	−	+ (4.1)	− (0.4)
3	4	D1	−	+	+ (2.0)	− (0.3)
	14	D1	−	NT	NT	+ (3.5)
	2 M	D1	NT	NT	+ (2.7)	+ (1.9)
4	2	D1	NT	+	− (0.6)	− (0.3)
	7	D1	+	NT	+ (6.3)	+ (1.9)
5	4	D1	−	+	+ (1.2)	+ (2.7)
6	6	D1	−	+	+ (5.4)	+ (2.6)
	22	D1	NT	−	+ (5.9)	+ (3.0)
7	7	D1	NT	NT	+ (10.6)	+ (1.4)
	9	D1	+	NT	+ (17.0)	+ (1.5)
8	5	D1	NT	+	− (0.9)	+ (1.4)
	11	D1	+	NT	+ (5.4)	+ (3.9)
9	6	D1	+	+	+ (8.5)	+ (3.7)
10	1	D1	+	+	− (0.8)	− (0.2)
	5	D1	+	NT	+ (7.2)	+ (1.0)
	6	D1	+	NT	+ (7.5)	+ (1.8)
11	0	D1	NT	+	+ (1.1)	− (0.1)
	6		−	NT	NT	NT
	7	D1	+	+	+ (6.4)	+ (2.1)
	8	D1	NT	+	+ (8.1)	+ (2.5)
	10	D1	+	−	+ (11.3)	+ (3.2)
12	1	D2	NT	+	− (0.8)	+ (1.2)
	4	D2	NT	+	+ (1.2)	+ (1.8)
	6	D2	+	+	+ (3.2)	+ (2.2)
	11	D2	+	+	+ (4.1)	+ (3.5)
13	3	D2	+	+	+ (1.6)	+ (4.2)
	6	D2	−	+	+ (3.6)	+ (6.6)
14	4	D2	+	+	+ (1.0)	− (0.4)
15	5	D2	−	+	+ (2.5)	+ (3.6)
16	1	D2	NT	+	− (0.6)	+ (1.0)
	3	D2	NT	NT	− (0.9)	+ (1.3)
	4		−	NT	NT	NT
	5	D2	+	NT	− (0.9)	+ (1.1)
	9	D2	+	NT	NT	NT
	12	D2	NT	NT	+ (5.5)	+ (3.5)
17	4	D3	+	+	+ (2.8)	+ (3.1)
	14		−	−	+ (8.6)	+ (3.3)
18	3	D3	NT	+	− (0.5)	− (0.6)
	5	D3	NT	+	+ (1.8)	+ (1.0)
	8	D3	+	−	+ (6.5)	+ (2.2)
	14	D3	+	−	+ (5.4)	+ (2.9)
19	4	D3	−	+	+ (6.3)	+ (1.1)
20	4	D3	NT	+	+ (1.9)	− (0.2)
	6	D3	+	+	+ (8.4)	− (0.2)
21	5	D3	+	−	+ (6.8)	+ (2.5)
22	3	D3	−	+	− (0.9)	+ (3.1)
23	0	D3	NT	+	− (0.6)	NT
	1	D3	NT	+	− (0.7)	− (0.3)
	3	D3	−	+	+ (2.7)	+ (1.0)
24	9	D3	+	−	− (0.6)	NT
25	3	D3	NT	+	+ (1.1)	+ (3.74)
	5		NT	NT	+ (4.6)	+ (4.61)
	7	D3	+	NT	+ (7.6)	+ (4.7)
	12	D3	+	NT	+ (5.7)	+ (4.7)
26	4	D3	NT	+	+ (3.8)	+ (1.7)
	5	D3	+	+	+ (5.8)	+ (2.3)
	11		NT	NT	+ (9.6)	+ (6.2)
	13	D3	+	−	+ (10.6)	+ (6.1)
27	6	D3	NT	+	+ (2.4)	+ (3.4)
	7	D3	+	NT	+ (6.2)	+ (4.1)
	14		NT	NT	+ (9.0)	+ (7.0)
28	1	D3	−	+	− (0.6)	− (0.9)
	3		−	NT	+ (1.2)	+ (1.1)
	5		−	NT	+ (6.4)	+ (1.8)
29	5	D3	+	+	+ (6.2)	− (0.2)
	7		NT	NT	+ (12.0)	− (0.3)
30	1	D3	NT	+	+ (1.0)	− (0.1)
	16	D3	+	−	+ (8.9)	+ (2.2)
31	7	D3	+	+	+ (6.8)	+ (2.8)
32	7	D4	−	−	+ (6.8)	+ (2.9)
33	8	D4	−	+	+ (8.3)	+ (226.0)
	33		−	NT	+ (7.5)	+ (3.4)
34	3	D4	−	+	− (0.8)	+ (1.0)
	5	D4	−	+	+ (3.5)	+ (2.1)
	9	D4	+	−	+ (8.7)	NT
	11	D4	+	NT	NT	NT
35	1	D4	NT	+	+ (1.1)	+ (2.4)
	4		NT	NT	+ (1.5)	+ (1.5)
	6	D4	+	NT	NT	NT
	8	D4	+	NT	+ (6.0)	+ (3.0)
36	4	D4	−	+	− (0.8)	+ (1.1)
	7		NT	NT	+ (1.9)	+ (5.3)
37	5	D4	−	+	+ (3.1)	− (0.7)
	8		NT	NT	+ (4.3)	NT
38	5		−	NT	+ (5.1)	+ (1.9)
	12		−	NT	+ (11.6)	+ (3.0)

(Continued on following page)

TABLE 1 (Continued)

Patient no.	Disease day	Infecting serotype	RT-PCR result with[b]: Urine	RT-PCR result with[b]: Serum	Serum antibody[a] (index): IgM	Serum antibody[a] (index): IgG
39	4		NT	−	+ (3.0)	+ (1.0)
	9		−	−	+ (3.4)	+ (1.4)
40	7		−	−	+ (1.8)	+ (1.4)
	10		NT	NT	+ (3.6)	+ (2.0)
41	2		NT	−	+ (9.8)	+ (1.6)
	5		−	NT	NT	NT
42	4		NT	+	+ (4.4)	+ (2.1)
	6		−	NT	+ (11.4)	+ (2.7)
43	5		−	−	+ (5.6)	− (0.7)
44	5		−	−	+ (1.5)	+ (4.5)
45	7		−	−	+ (13.1)	+ (2.4)
46	4		NT	−	+ (11.8)	+ (5.1)
	5		−	−	+ (10.7)	+ (5.3)
	6		−	−	+ (10.3)	+ (5.0)
47	7		NT	−	+ (5.0)	− (0.7)
	10		NT	−	+ (5.3)	+ (1.4)
	12		−	NT	NT	NT
	18		NT	−	+ (8.9)	+ (2.9)
48	3		−	+	− (0.9)	− (0.4)
49	5		NT	−	+ (4.7)	+ (2.4)
	10	−	−	+ (6.0)	+ (4.0)	
50	21		−	−	+ (5.9)	+ (6.2)
51	7		−	+	+ (14.6)	+ (2.9)
52	10		−	−	+ (13.5)	− (0.5)
53	6		−	−	+ (3.7)	+ (1.5)

[a] IgM and IgG indexes were calculated by the following formula: index = optical density (OD) of samples/cutoff OD. A plus sign indicates positive IgM and IgG indices, and a minus sign indicates negative IgM and IgG indices.
[b] NT, not tested.

endemic before onset and had DENV genome detected by real-time RT-PCR or specific anti-dengue antibodies by ELISA.

Isolation of dengue viruses from urine samples. Vero cells were used to isolate DENV from urine samples. The urine samples were filtered through 0.45-μm filters (Mix GS; Millipore). Urine samples (0.1 ml) were inoculated onto Vero cell monolayers in a 6-well cell culture plate (Corning Inc., NY) and incubated for 1 h. The cells were washed twice with phosphate-buffered saline without potassium and with 2% fetal calf serum (FCS) minimum essential medium (MEM) and then cultured at 37°C in 5% CO_2 for 7 days. The presence of DENV in the culture fluids was checked by the real-time RT-PCR TaqMan method.

Real-time RT-PCR (TaqMan RT-PCR). Dengue viral genomes in serum and urine samples were examined by real-time RT-PCR (TaqMan RT-PCR) assay as previously reported (5). Primer and probe sequences are provided in Table S2 in the supplemental material. Briefly, RNA was extracted from 200 μl of samples using a High Pure viral RNA kit (Roche Applied Science, Mannheim, Germany) and eluted by 50 μl RNase-free distilled water. Five microliters of RNA extracted from each sample was mixed with 100 pmol of each primer and 15 pmol of each probe in a 25-μl reaction volume using a TaqMan RT-PCR Ready-Mix kit (PE Applied Biosystems). The samples were amplified in an ABI Prism 7000 sequence detection system (PE Applied Biosystems). The real-time RT-PCR assay consisted of a 30-min RT step at 48°C and 45 cycles of PCR steps (95°C for 15 s and 57°C for 60 s).

Detection of DENV-specific IgM and IgG antibodies. Anti-dengue virus IgM Abs in serum samples were detected by an IgM-capture ELISA kit (dengue virus IgM capture DxSelect; Focus Diagnosis, CA) according to the manufacturer's protocol. Anti-dengue virus IgG Abs were detected by ELISA using an IgG indirect ELISA kit (Dengue IgG indirect ELISA; Panbio Ltd., Queensland, Australia) according to the manufacturer's protocol. IgM and IgG Abs were determined to be positive when the IgM index and IgG index were equal to or greater than 1.1, respectively.

Sequence analysis of viral RNA. RNA genomes were sequenced for serum and urine samples from selected patients after both samples were determined to be positive by real-time RT-PCR. To determine whether DENV genome detected in the urine sample originated from DENV in sera, the sequences of the E gene were analyzed for comparison. The E gene was used for analysis because it is the most frequently analyzed gene among DENV genomes (1). Primers used for RT-PCR for sequence analysis are provided in Table S1 in the supplemental material. Multiple pairs of primers were used to amplify and sequence the DENV genome. RNA genomes might be partially damaged in urine samples.

Ethical aspects. This work was approved by the ethics committee of the NIID (application no. 98; 3 July 2006).

(Part of this paper was presented at the Second International Conference on Dengue and Dengue Haemorrhagic Fever International, Phuket, Thailand, October 2008.)

RESULTS

Detection of dengue viral genomes in urine samples. Seventy-seven urine samples from 53 confirmed dengue patients were examined for the presence of DENV genome by real-time RT-PCR (Table 1). The results of real-time RT-PCR are presented based on disease days (Fig. 1), and the data were further analyzed by the sum of data from 2 consecutive days (Table 2). Disease days were defined based on the time of fever onset. The onset day was defined as day 0. DENV genome was detected as early as day 1 in one urine sample; however, before day 6 the rate of detection was low: 25% on disease days 0 to 3 and 32% on disease days 4 to 5. The positive detection rate reached 50% on disease days 6 to 7 and remained at 50% or higher until days 14 to 16. Positive samples were detected as late as day 16.

Detection of viral genomes in serum samples. Seventy-eight serum samples from the 53 patients were also examined by real-time RT-PCR. The results are presented based on disease days (Fig. 2), and the data were analyzed by the sum of data from 2 consecutive days (Table 2). The positive detection rate was high on early disease days: 100% on days 0 to 1, 89% on days 2 to 3, and 68% on days 4 to 5. The positive rate decreased thereafter, and no positive samples were detected after disease day 11.

Comparison of real-time RT-PCR positive detection rates between urine and serum samples. The results shown in Table 1 and the figures indicate that the time courses of positive detection rates differed between urine and serum samples. Positive rates of 50% or more were detected on disease days 6 to 16 for urine samples and days 0 to 7 for serum samples.

This tendency was clearly demonstrated using serial samples from patients 11 and 34. For patient 34, urine samples were negative on disease days 3 and 5 but positive on days 9 and 11, whereas serum samples were positive on days 3 and 5 but negative on day 9.

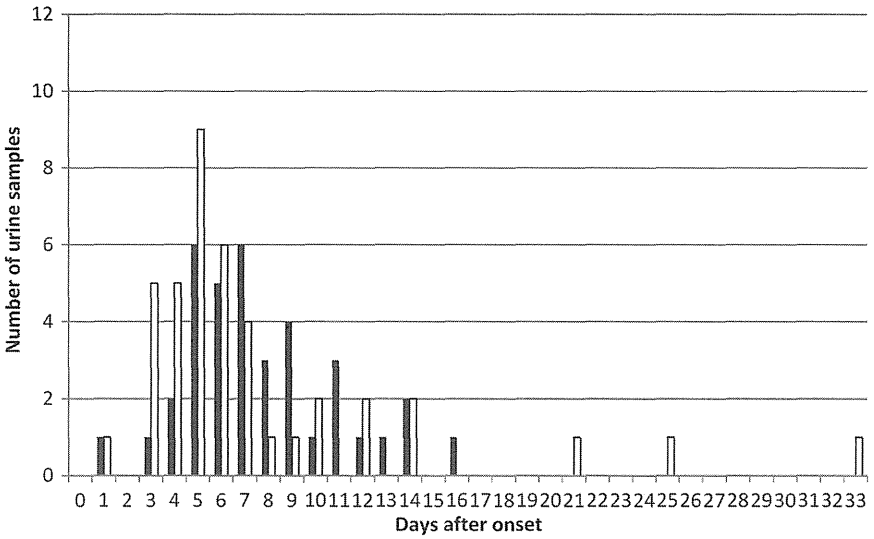

FIG 1 Number of DENV genome-positive and -negative urine samples determined by real-time RT-PCR based on disease days. Closed bar, positive; open bar, negative.

For patient 11, urine samples were negative on day 6 but positive on days 7 and 10, whereas serum samples were positive on days 0 and 7 but negative on day 10. The relative index between a positive rate of urine samples and days after onset is 0.087. The relative index between a positive rate of serum samples and days after onset is 0.97. The *P* value is 0.00068, indicating significant differences between serum and urine sample detection rates over time (Table 2).

Relationship of DENV genome detection in urine and serum samples and serum IgM and IgG antibody responses. IgM-positive rates were high (more than 87%) on and after days 4 to 5 (Table 2), and IgG-positive rates were high (more than 88%) on and after days 6 to 7. Real-time RT-PCR positive detection rates in serum declined once IgM and IgG were detected in serum samples, while real-time RT-PCR positive detection rates in urine samples remained at high levels, even after high IgM and IgG-positive rates in serum samples.

Comparison of nucleotide sequences of amplified DENV genomes between urine and serum samples. Nucleotide sequences of amplified DENV genomes were compared between urine and serum samples from the same patients. Partial nucleotide sequences were identical between urine and serum samples for each of the 6 cases examined. Nucleotide sequencing showed that all four DENV serotypes were represented among the 53 patients.

TABLE 2 Positive rate of real-time RT-PCR with urine and serum samples and serum IgM and IgG[a]

Disease days	No. (%) of samples positive by:				
	RT-PCR			Serum ELISA	
	Urine	Serum	Serum and urine	IgM	IgG
0–1	1/2 (50)	9/9 (100)	9/9 (100)	2/9 (22)	2/8 (25)
2–3	1/6 (17)	8/9 (89)	8/9 (89)	5/11 (45)	6/11 (55)
4–5	7/22 (32)	17/25 (68)	20/32 (63)	26/30 (87)	21/31 (68)
6–7	11/21 (52)	9/16 (56)	15/23 (65)	25/25 (100)	21/24 (88)
8–9	7/9 (78)	2/6 (33)	9/10 (90)	8/9 (89)	6/6 (100)
10–11	4/5 (80)	1/4 (25)	4/5 (80)	8/8 (100)	7/8 (88)
12–13	2/4 (50)	0/1 (0)	2/4 (50)	4/4 (100)	4/4 (100)
14–16	3/5 (60)	0/4 (0)	3/5 (60)	5/5 (100)	6/6 (100)
>16	0/3 (0)	0/4 (0)	0/5 (0)	6/6 (100)	6/6 (100)

[a] When samples of urine and blood were obtained from the same patient at the same time, the number of samples was considered to be 1.

DISCUSSION

The detection of DENV genome in serum samples by RT-PCR has been used widely for the confirmation of dengue virus infection. In the present study, the detection of DENV genome in urine samples was evaluated as an additional or alternative laboratory diagnostic test. Real-time RT-PCR was applied to the detection of DENV genome, and detection rates were compared between urine and serum samples. The nucleotide sequences of the PCR products on the E region were determined. The sequence results revealed that the nucleotide sequences of RT-PCR products were identical between urine and serum samples from respective patients, indicating that these represented the same infecting strain of DENV.

We previously reported a dengue case with the detection of DENV-1 genome in urine samples on disease days 7, 8, and 14, a time when the genome was not detected in serum samples (11), and another group has also reported the detection of DENV genome in urine samples (12). In those studies, the number of studied cases was limited to one and two cases, respectively. In the present study, we examined 77 urine and 78 serum samples from 53 confirmed dengue patients. The results indicate that real-time RT-PCR testing of urine samples is a useful diagnostic tool for DENV infection. It is of interest that DENV genome was detected at 50% or higher rates in urine samples even after disease day 7, when the positive detection rates of serum samples was lower than 50%. In particular, DENV genome was detected in 5 of 9 urine samples collected on disease days 12 to 16, when none of 5 serum samples was positive. For patients 30 and 34, DENV genome was detected in urine samples but not in serum samples after the onset of positive antibody detection in serum. The positive rates of the

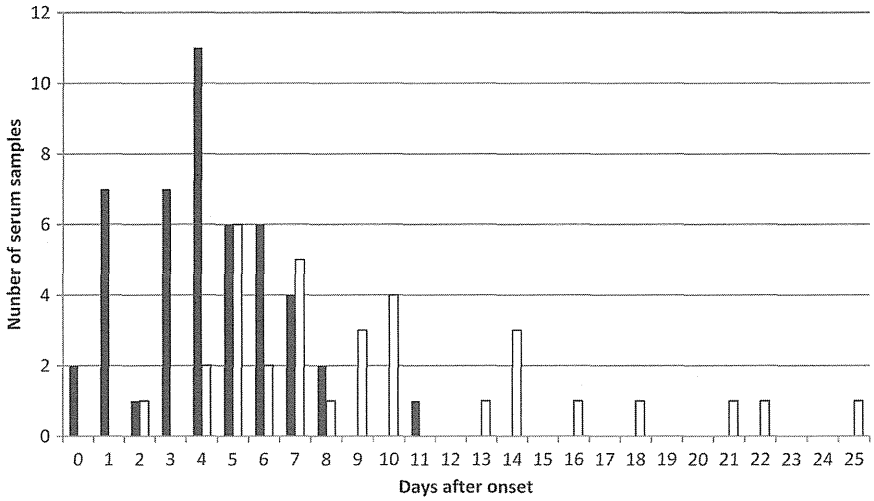

FIG 2 Number of DENV genome-positive and -negative serum samples determined by real-time RT-PCR based on disease days. Closed bar, positive; open bar, negative.

combined PCR results for urine and serum samples were calculated, and they were constantly at higher levels than the results for serum samples from days 6 to 16. The positive rates for urine samples started to increase on days 6 to 9. The positive rate was 67% (34 out of 51 cases) when only serum samples were tested, while it was 72% (38 out of 53 cases) when both serum and urine samples were tested. Furthermore, on day 6 and after, the positive rate was 41% (11 out of 27 cases) when serum samples were tested, while it was 56% (22 out of 39 cases) when both serum and urine samples were tested. The results suggest that real-time RT-PCR with urine samples is useful mainly as a supplemental test in the convalescent stages of DENV infection. Thus, it is recommended that both serum and urine samples be collected and examined in the convalescent stages of DENV infection. The RT-PCR method using urine samples has 3 advantages over serological assays: (i) infection can be determined using single samples, (ii) RT-PCR has high specificity, and (iii) serotypes can be determined. The serological diagnostic method needs 2 samples, at acute and convalescent stages, to confirm the latest infection. Serological assays often demonstrate cross-reactivity among flaviviruses, including DENV. The use of urine samples has advantages over the use of other samples for laboratory testing. Urine samples are easy to collect without invasive procedures, and they can be used for the dengue diagnosis of newborns, children, and patients with hemorrhagic symptoms. The isolation of infectious DENV was attempted from urine samples in which DENV genome was detected. Infectious DENV has not been isolated from any of the samples tested in this study. Further studies are needed to conclusively determine that there is no infectious DENV in urine samples. However, it is likely that DENV genome or noninfectious DENV virions, rather than infectious DENV, are present. The detection of dengue antibodies in urine samples has been reported (15). It is also possible that DENV-antibody complexes were detected by real-time RT-PCR in urine samples.

The detection of the genome of West Nile virus, another flavivirus, in urine samples from a patient with encephalitis has been reported; however, virus isolation from urine samples was also unsuccessful (13). Renal dysfunction is not common in dengue patients (9). The presence of DENV antigen in kidneys from dengue patients has been reported by several groups (2, 7). However, it has not clearly been determined whether DENV replication occurs in the kidney. Thus, the pathological mechanism corresponding to the presence of DENV genome in urine needs to be elucidated.

In conclusion, DENV genome was detected by real-time RT-PCR in urine samples in 24 of 53 confirmed dengue patients. It was possible to detect DENV genome in urine samples even after the appearance of antibodies in serum and the disappearance of viremia. Urine samples are readily obtainable, and the detection of DENV genome by real-time RT-PCR of urine samples is a useful laboratory diagnostic tool for DENV infection.

ACKNOWLEDGMENTS

This study was supported by grants (H20-shinkou-ippan-15 and H23-shinkou-ippan-10) for Research on Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labor and Welfare, Japan.

None of the authors have a commercial or other association that might pose a conflict or interest.

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J Med Primatol doi:10.1111/j.1600-0684.2012.00552.x

ORIGINAL ARTICLE

Changes in hematological and serum biochemical parameters in common marmosets (*Callithrix jacchus*) after inoculation with dengue virus

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Keywords
biochemical parameter – clinical sign – common marmoset – dengue virus – hematological parameter

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Accepted June 6, 2012.

Abstract

Background Marmosets are susceptible to dengue virus (DENV) infection. However, blood parameter data and clinical signs of DENV-infected marmosets are limited.
Methods Blood hematological and serum biochemical values were obtained from twelve DENV-inoculated and four mock-infected marmosets. Additionally, body temperature and activity level were determined.
Results Five DENV-inoculated marmosets demonstrated thrombocytopenia, nine demonstrated leucopenia, and five demonstrated an increase in the levels of AST, ALT, LDH, and BUN. Additionally, seven DENV-inoculated marmosets demonstrated clinical signs including fever and decreases in activity. None of the four mock-inoculated marmosets demonstrated changes in either hematological or biochemical parameters.
Conclusions Marmosets inoculated with DENV exhibited clinical signs and changes in hematological and biochemical parameters. The results suggest that blood parameter data and clinical signs could potentially be useful markers for understanding the progress of DENV infection in studies using marmosets.

Introduction

Dengue virus (DENV) is an arthropod-borne flavivirus and a serious cause of morbidity and mortality in tropical and subtropical regions of the world. Approximately 2.5 billion people are at risk of DENV infection. Infection with any of the four serotypes, DENV-1, DENV-2, DENV-3, and DENV-4, may cause asymptomatic infection, classical dengue fever (DF), or severe and sometimes fatal dengue infection: dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1]. The pathogenesis of DENV infection leading to DF and DHF/DSS has not been elucidated.

Animal models of DENV could facilitate an understanding of the progress of DENV infection [2]. Non-human primates, including rhesus monkeys, cynomolgus monkeys, and owl monkeys, have been

used in studies on pathophysiology of DENV infection and vaccine evaluation [3–12]. Because not all non-human primate models develop overt levels of viremia and clinical signs, a suitable animal model that consistently develops high levels of viremia and clinical signs would provide an effective tool in vaccine evaluation and elucidation of the pathogenesis of DENV infection [13]. We recently reported that common marmosets consistently develop viremia upon DENV infection [14]. In this study, we followed the clinical signs and hematological and biochemical changes in common marmosets after DENV infection. Subcutaneous inoculation of common marmosets with DENV-2 induced clinical signs, and hematological and biochemical changes in sera, although not in all of the infected marmosets. The results suggest that the hematological and biochemical changes in common marmosets after inoculation with DENV-2 could potentially be useful parameters for further studies on the pathogenesis of DENV infection and the development of treatments.

Materials and methods

Non-human primates

A total of 16 male marmosets were used. Marmosets were purchased from Clea Japan Inc. (Tokyo, Japan) and caged singly at 27 ± 2°C in 50 ± 10% humidity with a 12-h light–dark cycle (lighting from 7:00 to 19:00) at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Japan. All animal studies were conducted in accordance with 'Guides for animal experiments performed at National Institute of Infectious Diseases' approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases, Japan (Approval no. 608011, 609014), and 'National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare' approved by the National Institute of Biomedical Innovation, Japan (Approval no. 20-003, 21-013). Animals were fed twice a day with a standard marmoset diet (Clea New World Monkey Diet, CMS-1M, CLEA Japan) supplemented with fruits, eggs, and milk. Water was given *ad libitum*. The animals were in a healthy condition and confirmed to be negative for anti-DENV antibodies before inoculation with DENV. The animals were bred and housed in research facilities of Clea Japan Inc (Tokyo, Japan) prior to being kept at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Japan. The major flavivirus of concern in Japan includes Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). Both JEV and TBEV require vectors to cause infection, and control of vectors is carried out under stringent conditions in both animal facilities. TBEV infection is confined to the northern regions of Japan [15]. Anti-JEV antibodies are speculated to play a minimal role in antibody-dependent enhancement of DENV infection [16]. Anti-DENV antibodies were examined and confirmed to be negative in the marmosets used in this study. At appropriate times, animals were euthanized by cardiac exsanguinations under pentobarbitual anesthesia.

Virus

Three DENV type 2 (DENV-2) strains, DHF0663 (Genbank accession no. AB189122), D2/Hu/Jamaica/77/2007NIID (Jam/77/07, Genbank accession no. AB545873), and D2/Hu/Maldives/77/2008NIID (Mal/77/08, Genbank accession no. AB545874), were used for inoculation studies. The DENV-2 DHF0663 strain was isolated from a DHF case in Indonesia. The DENV-2 Jam/77/07 and Mal/77/08 strains were isolated from imported DF cases from Jamaica and Maldives, respectively [14]. The strains were selected on the basis that DHF0663 strain was isolated from a patient with severe dengue infection (DHF), and Jam/77/07 strain and Mal/77/08 strain were isolated from symptomatic dengue patients. The strains were also selected because they form well-defined plaques and cause cytopathic effects (CPE) upon inoculation on Vero and BHK cells. All DENV-2 strains were used within four passages on C6/36 cells. Culture supernatant from infected C6/36 cells was centrifuged at 800 *g* for 5 minutes to remove cell debris and then stored at −80°C until use. Virus titration was performed using Vero cells [17].

Infection of marmoset monkeys with DENV

Four marmosets were inoculated subcutaneously on the back with DENV-2: two (D2-2 and D2-3) with 4×10^7 PFU of the DENV-2 DHF0663 strain and two (D2-4 and D2-5) with 2×10^5 PFU of the DENV-2 DHF0663 strain. Blood samples were collected on days 3, 7, 14, and 21. Next, four marmosets were subcutaneously inoculated with lower doses of the DENV-2 DHF0663 strain: two of them (D2-6 and D2-7) with 2×10^4 PFU and two (D2-8 and D2-9) with 2×10^3 PFU. Additionally, four marmosets were subcutaneously inoculated with two other strains of DENV-2: two of them (D2-10 and D2-11) with 1×10^5 PFU of the Jam/77/07 strain and two (D2-12 and D2-13) with 2×10^5 PFU of the Mal/77/08 strain.

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