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BRIEF REPORT

Serotype-specific and cross-reactive neutralizing antibody responses in cynomolgus monkeys after infection with multiple dengue virus serotypes

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Abstract Neutralizing antibody responses were examined in monkeys after dengue virus infections. In monkeys that had been infected once or twice with DENV-2, neutralizing antibody was cross-reactive with all four serotypes after secondary or tertiary infection with DENV-3. In monkeys that had been inoculated with DENV-1 and DENV-2 in the primary and secondary infections, neutralizing antibody titers did not increase after tertiary infection with DENV-3. These results indicate that antibody responses after secondary and tertiary infections with different serotypes are cross-reactive with all four serotypes, consistent with what has been observed in humans, and suggest that monkeys are useful for determining neutralizing antibody responses.

Keywords Dengue virus · Monkey · Neutralyzing antibody · Serotype cross-reactivity

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Dengue virus infections occur in most tropical and subtropical areas of the world. Dengue virus is transmitted by mosquitoes and causes dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1]. There are four serotypes of dengue virus: dengue virus serotypes 1, 2, 3 and 4 (DENV-1, DENV-2, DENV-3, and DENV-4) [2]. Four serotypes of dengue virus are circulating concurrently in most tropical and subtropical regions. This increases the possibility of sequential infections with two or more serotypes of dengue virus. Serotype specificity and cross-reactivity in neutralizing antibody responses after secondary and tertiary infections are complex.

It is important to define the serotype specificity and cross-reactivity in dengue virus infection, since neutralizing antibodies are considered to be the most important factor for protective immunity in humans. Studies using patients' sera are often obscure, because confirmation of time of infection or infection serotypes is usually difficult. In the present study, we analyzed serotype specificity and cross-reactivity of neutralizing antibodies induced after well-controlled primary, secondary and tertiary DENV infections in monkeys (*Macaca fascicularis*). This study

provides insight into the complexity of protection against the four serotypes of dengue virus.

DENV-1 strain 02-17, DENV-2 strain DHF0663, and DENV-3 strain DSS1403 were used. The 02-17 strain of DENV-1 was isolated from a traveler in Japan who came back from Indonesia. The DHF0663 strain of DENV-2 was isolated in Indonesia in 2001 from a case of DHF. The DSS1403 strain of DENV-3 was isolated in Indonesia during the 2001 DSS epidemic, propagated in C6/36, stored at −80°C, and used after the third passage in C6/36 cells. DENV-1, DENV-2 and DENV-3 were used after one, four and three passages in C6/36 cells, respectively, as described previously [3].

Eight female monkeys (*Macaca fascicularis*), aged 7 years and weighing 2,850-3,600 g, were used in the experiments. These monkeys were born and raised at the Tsukuba Primate Research Center, Tukuba, Japan. The monkeys were confirmed to be seronegative for antibodies to DENV. Monkeys were anaesthetized intramuscularly with ketamine HCl (5 mg/kg), and inoculated with dengue virus [3]. Monkeys were challenged intradermally with 0.5 ml of DENV-3 suspension containing 4.5×10^6 plaque-forming units (PFU)/ml.

Eight monkeys were separated into four groups. In group 1, KT1 and KT2 were inoculated with DENV-1 and DENV-2 in the primary and secondary infection, respectively, and then with DENV-3 in the tertiary infection. In group 2, KT4 and KT5 were inoculated two times with DENV-2 in the primary and secondary infections, and then with DENV-3 in the tertiary infection. In group 3, KT3 and KT6 were inoculated with DENV-2 and then with DENV-3 in the secondary infection. In group 4, KT7 and KT8 were infected with DENV-3 in the primary infection. Animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious, Diseases, Japan.

Clinical manifestations such as volume of food and water consumed and appearance of feces were observed each day. Before and after infection, and at the time of each blood sampling, hematologic and serum chemistry, rectal temperatures and body weight were recorded. Blood samples were collected on days 0, 3, 5, 7, 10 and at weeks 2 and 4 after inoculation with DENV-3. Serum was separated from blood and stored at −70°C until use.

Serum samples were tested for dengue-virus-specific IgM antibody by IgM-capture enzyme-linked immunosorbent assay (ELISA) (FOCUS, Cypress, California, USA) [3]. The assays were performed in duplicate. Serotype-specific and cross-reactive neutralizing antibody titers to DENV-1 DENV-2, DENV-3 and DENV-4 were measured by a standard PRNT50 method using LLC-MK2 cells as described previously [4]. Briefly, 4- to 10-fold serial dilutions of sera were incubated for one hour at 37°C with a working dilution of virus to give 40-60 PFU/ml in the final volume of virus-serum mixture. After incubation, 100 μL of virus-serum mixture was added to a LLC-MK2 cell monolayer grown in 6-well polystyrene plates for seven days. After incubation for one hour at 37°C in an atmosphere of 5% CO_2, 3 ml of Eagle's MEM containing 1% methylcellulose with 2% FCS was overlaid onto the cells, and the plate was incubated at 35°C in 5% CO_2 for 7 days. The cells were fixed with 1 ml of 3.7% formaldehyde for one hour and stained with methylene blue tetrahydrate solution, and the visualized plaques were then counted. The serum dilution that resulted in a 50% reduction in plaque count was calculated by the Reed and Muench method [5].

There were no notable differences in behavior or consumption of food in any of the eight monkeys after inoculation with DENV-3. Similarly, specific changes in hematologic and serum chemistry, rectal temperatures or body weight were not seen in any of the animals.

Levels of neutralizing antibody were examined at weeks 2 and 4 after inoculation with DENV-3 in the primary (KT7 and KT8), secondary (KT3 and KT6) or tertiary (KT1, KT2, KT4 and KT5) infection (Table 1). Neutralizing antibody in KT7 (group 4) was specific for DENV-3. The neutralizing antibody titer in KT8 (group 4) was highest against DENV-3, but these antibodies were also cross-reactive with DENV-2 and DENV-4 at much lower levels. These results indicate that the neutralizing antibody response is generally specific for the inoculated serotype or cross-reactive, with the highest titer to the inoculated serotype after primary infection.

High levels of neutralizing antibody to DENV-2 were present in KT3 and KT6 (group 3) before the second inoculation with DENV-3 because of primary infection with DENV-2. Neutralizing antibody titers against all four serotypes increased after secondary infection with DENV-3. Neutralizing antibody titers were high to DENV-2 and DENV-3, and cross-reactive to DENV-1 and DENV-4 at lower levels. These results indicate that the neutralizing antibody response is serotype cross-reactive after infection with two serotypes.

High levels of neutralizing antibody to DENV-2 were present in KT4 and KT5 (group 2) because of two inoculations with DENV-2. After inoculation with DENV-3, neutralizing antibody titers to all four serotypes increased in KT4, and in KT5, neutralizing antibodies to DENV-1, DENV-3 and DENV-4 increased. The titers to DENV-2 increased only 2-3-fold in KT4. The results indicate that the neutralizing antibody response is serotype cross-reactive after two inoculations with DENV-2 and a third inoculation with DENV-3.

Cross-reactive neutralizing antibodies to all four serotypes were present in KT1 and KT2 (group 1) before

Table 1 Fifty-percent plaque reduction neutralizing antibody titer ($PRNT_{50}$) after the third dengue virus infection in monkeys

Serotype used for inoculation			Designation of monkey	Challenge virus for assessing $PRNT_{50}$	Titer before the third inoculation	Titer after the third inoculation	
First inoculation	Second inoculation	Third inoculation				2 weeks	4 weeks
DENV-1	DENV-2	DENV-3	KT1 (group 1)	DENV-1	6129	4938	6813
				DENV-2	3518	4001	2973
				DENV-3	367	745	638
				DENV-4	44	33	26
DENV-1	DENV-2	DENV-3	KT2 (group 1)	DENV-1	24	78	38
				DENV-2	284	346	460
				DENV-3	23	204	179
				DENV-4	18	51	ND
DENV-2	DENV-2	DENV-3	KT4 (group 2)	DENV-1	<10	33	45
				DENV-2	1402	2888	4704
				DENV-3	15	529	522
				DENV-4	24	240	215
DENV2	DENV-2	DENV-3	KT5 (group 2)	DENV-1	<10	24	26
				DENV-2	1836	3441	2558
				DENV-3	11	376	422
				DENV-4	20	207	236
None	DENV-2	DENV-3	KT3 (group 3)	DENV-1	<10	96	53
				DENV-2	271	5832	2128
				DENV-3	<10	1374	1093
				DENV-4	<10	258	255
None	DENV-2	DENV-3	KT6 (group 3)	DENV-1	<10	113	25
				DENV-2	283	2066	1454
				DENV-3	<10	3293	1142
				DENV-4	14	612	251
None	None	DENV-3	KT7 (group 4)	DENV-1	<10	<10	<10
				DENV-2	<10	<10	13
				DENV-3	<10	3804	3410
				DENV-4	<10	<10	<10
None	None	DENV-3	KT8 (group 4)	DENV-1	<10	<10	<10
				DENV-2	<10	43	90
				DENV-3	<10	675	917
				DENV-4	<10	25	15

inoculation with DENV-3 because of two inoculations with DENV-1 and DENV-3. Neutralizing antibody titers did not increase in KT1. In KT2, the titers to DENV-3 increased, but those to DENV-1, DENV-2, and DENV-4 did not.

These results, in general, indicate that antibody responses after primary infections are specific for the infecting serotype and that those occurring after secondary and tertiary infections with different serotypes are cross-reactive with all four serotypes. These results are consistent with those observed in humans [6]. However, it should be noted that there is variability in responses among monkeys, and this variability is consistent with what is seen in humans.

IgM responses were also examined in the eight monkeys for 6 weeks after inoculation with DENV-3 (Table 2). Specific IgM was detected in KT7 and KT8 (group 4), which were inoculated with DENV-3 in the primary DENV infection, in KT3 and KT6 (group 3), which had been infected with DENV-2 in the primary infection and DENV-3 in the secondary infection, and in KT4 (group 2), which had been infected two times with DENV-2 in the primary and secondary infections and with DENV-3 in the tertiary infection. IgM response was not detected in KT5 (group 2), which had been infected two times with DENV-2 in the primary and secondary infection and with DENV-3 in the tertiary infection, and in KT1 and KT2 (group 1), which had

Table 2 IgM responses after the third dengue virus infection in monkeys

Designation of monkey	5 days	7 days	14 days	21 days	28 days
Group 1					
KT1	1.04[a]	0.95	0.93	1.06	1.01
KT2	1.18	1.53	1.87	1.73	1.59
Group 2					
KT4	1.05	1.23	9.21[b]	6.43	4.71
KT5	0.99	1.06	1.90	1.76	1.37
Group 3					
KT3	1.37	1.85	6.06	3.27	2.29
KT6	1.29	2.03	14.36	8.75	5.04
Group 4					
KT7	1.20	1.23	6.84	5.39	3.39
KT8	1.28	3.49	26.94	20.92	13.46

[a] Data are presented as IgM index calculated by the formula: Optical density of samples collected before inoculation/Optical density of samples collected on designated days

[b] The IgM response was considered positive when the IgM index was equal to or higher than 2.00, as indicated by underlining

been infected with DENV-1 and DENV-2 in the primary and secondary infections and with DENV-3 in the tertiary infection. Thus, IgM was detected in two monkeys that were infected with DENV-3 in the primary DENV infection, in two monkeys that had been infected with DENV-2 in the primary infection and with DENV-3 in the secondary infection, and in one of the two monkeys that had been infected two times with DENV-2 in the primary and secondary infections and with DENV-3 in the tertiary infection. An IgM response was not detected in other monkeys. The results are consistent with previous reports of human IgM responses that an IgM response is apparent in primary infection but not in all of the cases with secondary infection [7, 8].

In the present study, serotype specificity and cross-reactivity in the neutralizing antibody response were analyzed after primary, secondary and tertiary infections with dengue virus in monkeys (*Macaca fascicularis*). Neutralizing antibody responses after primary infection are similar to those reported using *Aotus nancymae* monkeys [9]. The responses in monkeys after primary and secondary infections were also consistent with those reported in humans. After inoculation with DENV-3, neutralizing antibody titers to all four serotypes increased in KT4 (group 2), and in KT5 (group 2), neutralizing antibody titers to DENV-1, DENV-3 and DENV-4 increased. These results indicate that the neutralizing antibody response is serotype cross-reactive after two inoculations with one serotype and a third inoculation with the other serotype.

Cross-reactive neutralizing antibodies to all four serotypes were present in KT1 and KT2 before inoculation with DENV-3. Neutralizing antibody titers did not increase in KT1. In KT2, the titers to DENV-3 increased, but those to DENV-1, DENV-2, and DENV-4 did not. These results suggest that DENV-3 infection was not established in KT1, and only slightly in KT2. This result is consistent with the general opinion that tertiary infection does not usually occur in humans [10]. In humans, analysis of neutralizing antibody responses in tertiary infection is difficult. Symptomatic tertiary DENV infection is rare, and determination of the order of infecting serotypes in tertiary infections in humans is difficult. Thus, the results after tertiary infection in KT1 and KT2 (group 1) in the present study are unique, and this type of study can be only performed in animal models.

In dengue virus infection in humans, it is usually difficult to determine the infecting serotypes in secondary and tertiary infection. The advantage of using monkeys is that the timing of infections and infecting serotypes can be controlled. The results obtained in the present study suggests that monkeys (*Macaca fascicularis*) are suitable animals for analyzing antibody responses to dengue virus and that detailed serotype-specific and cross-reactive responses can be analyzed in detail after multiple dengue virus infections.

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ORIGINAL PAPER

ULBP4/RAET1E is highly polymorphic in the Old World monkey

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Abstract Natural-killer group 2 member D (NKG2D) is an activating receptor that plays an important role in the immune response mediated by NK cells, $\gamma\delta^+$ T cells, and $CD8^+$ T cells. In humans, MHC class I chain-related genes and UL-16 binding protein (ULBP)/retinoic acid early transcript 1 (REAT1) gene family encode ligands for NKG2D. The rhesus and crab-eating macaques, which belong to the Old World monkeys, are widely used as non-human primate models in medical researches on the immunological process. In the present study, we investigated the polymorphisms of *ULBP4/RAET1E*, a member of the *ULBP/RAET1* family, and found 25 and 14 alleles from the rhesus and crab-eating macaques, respectively, of which diversities were far more extended than in humans. A phylogenetic study suggested that the allelic diversification of ULBP4/RAET1E predated the divergence of rhesus and crab-eating macaques.

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Keywords Rhesus macaque · Crab-eating macaque · ULBP4/RAET1E · NKG2D · Polymorphism

Introduction

Non-human primates, such as rhesus and crab-eating macaques, are important animal models for the study of infectious diseases, autoimmune diseases, and organ transplantation. These macaques are members of the Old World monkeys, and it has been reported that the genetic diversity in the rhesus macaque is quite unique, that is, more than 60% of the rhesus macaque-specific expansions are found in the protein coding sequences (Gibbs et al. 2007). To evaluate the results of immunological experiments in the macaque models, it is essential to characterize the genetic diversity of immune-related molecules which may control the individual differences in the immune response against foreign antigens and/or pathogens. It has been reported that the gene copy number in the major histocompatibility complex (MHC) loci in the rhesus and crab-eating macaques is higher than that in humans (Kulski et al. 2004; Gibbs et al. 2007; Otting et al. 2007). In addition, the extent of genetic diversity differed, in part, depending on the geographic areas, and we have reported that the diversity of MHC class I genes in the rhesus macaque is considerably different depending on habitat (Naruse et al. 2010).

Because the innate immune system is involved in the response to environmental pathogens, it is necessary to consider the function of natural killer (NK) cells in the experimental animal models. Natural-killer group 2 member D (NKG2D), a C-type lectin molecule, is an activating receptor expressed on the cell surface of NK, $\gamma\delta^+$, and $CD8^+$ T cells, which plays an important role in the immune response (Wu et al. 1999; Raulet 2003). In humans, MHC class I chain-related genes (MIC) and UL-16 binding protein (ULBP)/retinoic acid early transcript 1 (REAT1)

gene family are known to encode ligands for NKG2D (Bauer et al. 1999; Cosman et al. 2001; Chalupny et al. 2003; Bacon et al. 2004). These ligand molecules are usually stress-inducible, and their recognition by NKG2D can lead to the activation of NK cells, consequently killing virus-infected and tumor cells (Pende et al. 2002; Eagle et al. 2006; Pappworth et al. 2007; Ward et al. 2007).

The human *ULBP/RAET1* gene family is located on chromosome 6q24.2, which is composed of ten members including six functional genes, *ULBP1*, *2*, *3*, *4*, *5*, and *6*, corresponding to *RAET1I*, *H*, *N*, *E*, *G*, and *L*, respectively (Radosavljevic et al. 2001; Chalupny et al. 2003; Eagle et al. 2009a, b). In addition, several sequence polymorphisms in each *ULBP* gene have been identified (Romphruk et al. 2009; Antoun et al. 2010). Although it is evident that the cell surface expression of the ligand molecules on target cells is differentially regulated (Eagle et al. 2006), genetic polymorphisms in the coding regions might have a functional impact. We have previously investigated the genetic polymorphisms of *ULBP/RAET1* genes and have found that the *ULBP4/RAET1E* gene is the most polymorphic, with the allelic distribution differing among ethnic groups (Romphruk et al. 2009).

On the other hand, rhesus macaque *ULBP4/RAET1E* (GenBank: NW_001116520) is mapped on the long arm of chromosome 4 (i.e., positions from 31, 164, 822 to 31, 175, 032 of chromosome 4 in the rhesus genome; data obtained from the UCSC Genome Browser at http://genome.ucsc.edu/cgi-bin/hgGateway; Gibbs et al. 2007). However, its genetic polymorphisms are poorly characterized, although the MIC gene polymorphisms are well studied in the rhesus macaque (Seo et al. 1999, 2001; Doxiadis et al. 2007; Averdam et al. 2007). In the present study, we investigated the polymorphisms of *ULBP4/RAET1E* in rhesus and crab-eating macaques. This is the first report demonstrating the extreme diversity of the NKG2D ligand in the Old World monkey.

Materials and methods

Animals

A total of 38 rhesus macaques from seven lineages previously analyzed for the MHC polymorphisms (Naruse et al. 2010) and 24 crab-eating macaques from five lineages were the subjects. They were maintained in the breeding colonies in Japan. The founders of the rhesus macaque colonies were captured in Myanmar and Laos, whereas the founders of crab-eating macaque colonies were captured in Indonesia, Malaysia, and the Philippines. All care, including blood sampling of animals, were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85–23, revised 1985) and were subjected to prior approval by the local animal protection authority.

DNA extraction and sequencing analysis

Genomic DNAs from B lymphoblastoid cell lines of the rhesus macaque (Naruse et al. 2010) and from whole blood sample of the crab-eating macaque were extracted by using the QuickGene DNA kit (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. The genomic gene for *ULBP4/RAET1E* of rhesus and crab-eating macaques was amplified by polymerase chain reaction (PCR) with a primer pair designed for the region spanning from introns 1 to 3 of the rhesus gene (NC007861), ULBP4F (5′-TGGGCCTCTTCCCCTGTCC) and ULBP4R (5′-GTGGGAGGTGGGATGGG), using FastStart Taq DNA polymerase (Roche, Mannheim, Germany). The PCR condition was composed of the following steps: denaturation at 95°C for 4 min; 30 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 45 s; and additional extension at 72°C for 7 min. The PCR products of about 1,200 bp in length were cloned into pSTBlue-1 AccepTer vector (Novagen, WI, USA) according to the manufacturer's instructions and were transformed to Nova Blue Single™ competent cells (Merck4Biosciences Japan, Tokyo, Japan). Ten to 20 independent transformant colonies were picked up for each sample and subjected to sequencing on both strands by using a BigDye Terminator cycling system and an ABI 3730 automated sequence analyzer (Applied Biosystems, CA, USA).

Data analyses

Nucleotide sequences of *ULBP4/RAET1E* from cloned DNAs were aligned using the Genetyx software package (version 8.0, Genetyx Corp., Japan). If at least three clones from independent PCR or from different individuals showed identical sequences, the sequences were submitted to the DNA Data Bank of Japan (DDBJ). Neighbor-joining trees were constructed with Kimura's 2-parameter method for a phylogenetic analysis of *ULBP4/RAET1E* sequences spanning from exons 2 to 3 including intron 2 by using the Genetyx software. Bootstrap values were based on 5,000 replications. The *ULBP4/RAET1E* sequences from humans (GenBank accession number AY252119), chimpanzees (AY032638), and rhesus (NC007861) were included in the analysis as references.

Structure model analysis

A three-dimensional (3-D) structure model of rhesus ULBP4/RAET1E, with amino acid positions from 1 to 178, was created by a molecular visualization software RasTop2.2 (http://sourceforge.net/projects/rastop/), and the

human RAET1B in complex with NKG2D (Radaev et al. 2001) from the Molecular Modeling Database (MMCB No. 18231) was used as the reference. Polymorphic sites were mapped on the 3-D structure model of macaque RAET1E by using the Cn3D 4.1 program (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml).

Table 1 Identified alleles of the ULBP4 gene in rhesus and cynomolgus

Species	Allele name	Accession no.	Reference animal	Identical sequence
Rhesus macaque	*Mamu-ULBP4*1.1*	AB568525	R228, R367	
	*Mamu-ULBP4*1.2*	AB568533	R492, R396, R465	
	*Mamu-ULBP4*2*	AB568526	R283, R384, R328, R337	
	*Mamu-ULBP4*3*	AB568527	R346, R361, R396, R379, R408	
	*Mmau-ULBP4*4*	AB568528	R320, R490, R321, R465, R367, R446, R328, R234, R237, R314	
	*Mamu-ULBP4*5*	AB568529	R430, R453, R325, R477, R439, R360, R379, R446, R355	
	*Mamu-ULBP4*6*	AB568530	R437, R350,	
	*Mamu-ULBP4*7.1*	AB568531	R325, R384, R491, R333, R337	
	*Mamu-ULBP4*7.2*	AB568544	R477	
	*Mamu-ULBP4*8*	AB568532	R408, R454, R241, R342, R316	
	*Mamu-ULBP4*9.1*	AB568534	R312, R314	
	*Mamu-ULBP4*9.2*	AB568535	R333	
	*Mamu-ULBP4*10*	AB568536	R316	
	*Mamu-ULBP4*11*	AB568537	R241	
	*Mamu-ULBP4*12*	AB568538	R342	
	*Mamu-ULBP4*13*	AB568539	R491	
	*Mamu-ULBP4*14*	AB568540	R495	*Mafa-ULBP4*1.1*
	*Mamu-ULBP4*15*	AB568541	R350	
	*Mamu-ULBP4*16*	AB568542	R492	
	*Mamu-ULBP4*17*	AB568543	R495	
	*Mamu-ULBP4*18*	AB568545	R454	
	*Mamu-ULBP4*19*	AB568546	R321	
	*Mamu-ULBP4*20*	AB568547	R355	
	*Mamu-ULBP4*21*	AB571025	R437	
	*Mamu-ULBP4*22*	AB571026	R439	
Crab-eating macaque	*Mafa-ULBP4*1.1*	AB578934	M01, P01, P02, C001, C003, C004, C005, C006	*Mamu-ULBP4*14*
	*Mafa-ULBP4*1.2*	AB578935	M02, C004	
	*Mafa-ULBP4*2*	AB578936	P04, M06, C010, C011, C013	
	*Mafa-ULBP4*3*	AB578938	M03, C007	
	*Mafa-ULBP4*4*	AB578939	M03, C006	
	*Mafa-ULBP4*5*	AB578940	P04, P05, M05, M06, C012, C013	
	*Mafa-ULBP4*6*	AB578941	M05, C010, C011	
	*Mafa-ULBP4*7.1*	AB578942	M01, C002	
	*Mafa-ULBP4*7.2*	AB578943	P03, C008	
	*Mafa-ULBP4*8*	AB578944	P03, M04, C008, C009	
	*Mafa-ULBP4*9*	AB578945	P01, C001, C002	
	*Mafa-ULBP4*10*	AB578946	M04, C009	
	*Mafa-ULBP4*11*	AB578947	P02, C007	
	*Mafa-ULBP4*12*	AB578948	M02, C005	

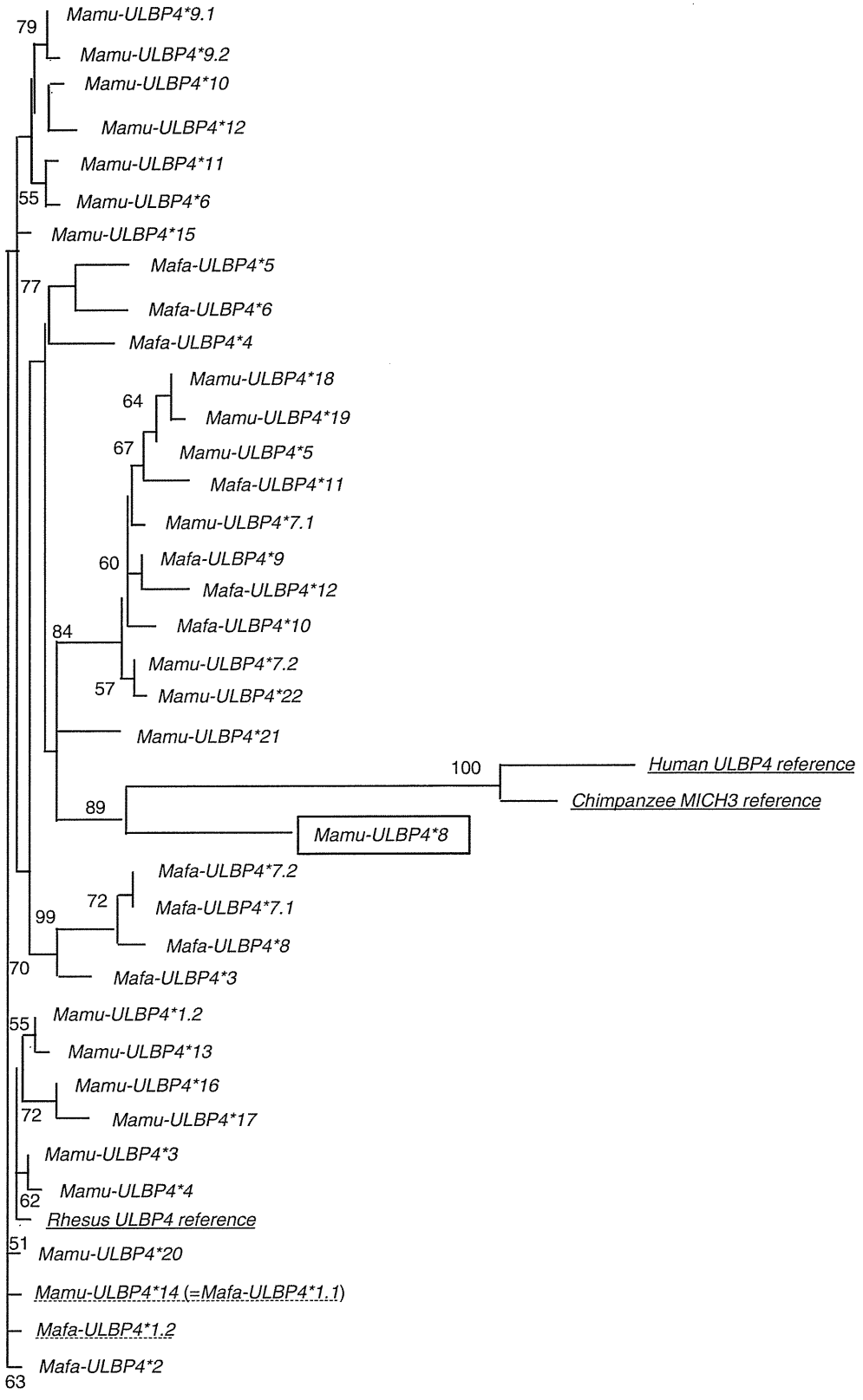

Fig. 1 Phylogenetic tree of *Mamu-* and *Mafa-ULBP4/RAET1E* alleles. A phylogenetic tree of *ULBP4/RAET1E* sequences spanning from exons 2 to 3, obtained in this study, was constructed by using the neighbor-joining method with bootstrap values of 5,000 replications. Values are indicated as percentages, and only those with more than 50% are shown. Sequences of human *ULBP4/RAET1E* (AY252119), chimpanzee *MICH3* (AY032638), and rhesus *ULBP4/RAET1E* (NC007861) were *underlined* and included in the analysis as reference sequences. Alleles represented with *broken underlines* had identical amino acid sequences predicted from the nucleotide sequences. The allele containing an in-frame termination codon was *boxed*

Results

ULBP4/RAET1E polymorphisms in the rhesus macaque

In the rhesus macaque genome (Gibbs et al. 2007), there are two paralogous genes for *ULBP4/RAET1E*, one of which appears to be functional, whereas the other is a pseudogene because it contains a large deletion containing the most part of exons 2, 3, and 4. Therefore, we designed primer pairs to amplify the region containing exons 2 and 3, which encode for α1 and α2 domains of ULBP4/RAET1E molecule, respectively, from the functional *ULBP4/RAET1E*. By using the primer pair, we obtained *ULBP4/RAET1E* sequences from 38 individuals of rhesus macaque. Because one or two sequences were obtained from each individual, the sequences were considered to be alleles of *ULBP4/RAET1E*. They were classified into 25 different alleles, designated as *Mamu-ULBP4*1.1* to *Mamu-ULBP4*22*, submitted to DDBJ, and given accession numbers (Table 1). The allele names with different numbers indicate that they are different in predicted amino acid sequences, whereas the alleles with the same deduced amino acid sequences but different nucleotide sequences, such as *Mamu-ULBP4*1.1* and *Mamu-ULBP4*1.2*, are designated as subtypes. None of the sequences obtained in this study was identical to the reference sequence, NC007861, which was previously deposited to the GenBank database as the rhesus *ULBP4/RAET1E*. On the other hand, when the sequences were aligned referring the human *ULBP4/RAET1E*, one rhesus allele (*Mamu-ULBP4*8*) was found to contain a nonsense mutation at codon 29, which would make the ULBP4/RAET1E molecule non-functional.

ULBP4/RAET1E polymorphisms in the crab-eating macaque

By using the primer pair designed for the rhesus *ULBP4/RAET1E*, we could amplify the *ULBP4/RAET1E* sequences from 24 individuals of the crab-eating macaque. Sequencing analysis revealed 14 different *ULBP4/RAET1E* alleles, and inheritance of each allele was confirmed by family studies. The identified allele sequences were submitted to DDBJ, given accession numbers, and designated as *Mafa-ULBP4*1.1* to *Mafa-ULBP4*12* (Table 1). The nucleotide sequences from exons 2 to 3 of *Mamu-ULBP4*14* were identical to those of *Mafa-ULBP4*1.1* and differed by only one nucleotide in intron 2 from those of *Mafa-ULBP4*1.2*. In addition, a neighbor-joining analysis performed by using nucleotide sequences spanning from exons 2 to 3 showed that the alleles of rhesus and crab-eating macaques were not separately clustered from each other (Fig. 1).

Comparative analysis of *ULBP4/RAET1E*

The alignment of *ULBP4/RAET1E* sequences from rhesus and crab-eating macaques with those from humans and chimpanzees showed that the macaque genes were homologous to the human gene by more than 90% and were equally diverged (Fig. 2). In addition, rhesus and crab-

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                                                                                50
                  Human :GHSLCFNFTI KSLSRPGQPW CEAQVFLNKN LFLQYNSDNN MVKPLGLLGK KVYATSTwge
              Chimpanzee :---------- -L-------- ---------- ---------- ---------- --N-------
           Rhesus macaque :A--------- --W------- ------M-K- -----D—S- ---------- --N-----Q-
      Crab-eating macaque :A--------- --W------- ------M--- -F---D—SN ---------- --N-----Q-

                                                                    100
                  Human :ltqtlgevgx dlrmllcdIK PQIKTSDPST LQVEMFCQRE AERCTGASWQ FATNGEKSLL
              Chimpanzee :---M------ ------L--- ------G--- ---------- ---------- --I-------
           Rhesus macaque :---K------ ------L-V- ------GP-- -----L---- ---------- -TI----C--
      Crab-eating macaque :---------- -LM---L-V- P-----G--- L----L---- ---------- -TI----C--

                                                          150
                  Human :FDAMNMTWTV INHEAskike twkkdrglek yfrklskgdc dhwlreflgh weampEPTV
              Chimpanzee :---------- ---------- ---------- ---------- ---------- --------Q
           Rhesus macaque :F----M---- ----AS---- ---K------ ------M--- N------L-- Q-A-----Q
      Crab-eating macaque :---------- -----S---- ---------- ------M--- N-WLR----- Q------TQ
```

Fig. 2 Alignment of deduced amino acid sequences of α1 and α2 domains of ULBP4/RAET1E. Amino acid sequences were deduced from the nucleotide sequences of *ULBP4/RAET1E* or *MICH3* from humans (AY252119), chimpanzees (AY032638), rhesus macaques (NC007861), and crab-eating macaques (AY032639). *Numbers* above the sequences represent the amino acid positions in mature protein. *Dashes* indicate identical sequences. Sequences for the predicted α helix structure were indicated by *small italicized characters*. Positions of polymorphic sites in the *human*, *rhesus macaque*, and *crab-eating macaque* were *underlined*

Table 2 Single nucleotide polymorphisms of ULBP4 gene among human and Old World monkeys

	Number of alleles	Exon 2		Intron 2	Exon 3	
		Polymorphism	Non-synonymous change (%)	Polymorphism	Polymorphism	Non-synonymous change (%)
Human	5	2	2 (100%)	3	3	3 (100%)
Rhesus macaque	25	9	5 (55.6%)	22	22	14 (63.6%)
Crab-eating macaque	14	17	9 (52.9%)	18	16	9 (56.3%)

eating macaques showed a higher degree of polymorphism in the analyzed region, namely, exon 2, intron 2, and exon 3, than in humans (Table 2). All polymorphisms found in exons of human *ULBP4/RAET1E* were non-synonymous, whereas a considerable part of the polymorphisms were synonymous in the Old World monkeys. On the other hand, the polymorphic sites in the rhesus macaque (positions 29, 46, 59, 64, 79, 88, 112, 121, 126, 135, 136, 144, 157, 158, 161, 168, 171, and 173) and the crab-eating macaque (positions 32, 39, 40, 59, 72, 73, 79, 91, 112, 136, 163, 164, 165, 171, 178, and 179) were shared at five positions (59, 79, 112, 136, and 171) by each other, whereas only one position (position 112) was shared with polymorphic sites in humans (positions 53, 99, 112, and 113) (Fig. 2). In addition, a termination at position 29 was found in a rhesus macaque allele *Mamu-ULBP4*8*; a single amino acid deletion caused by deletions of a total of three nucleotides was found in a crab-eating macaque allele *Mafa-ULBP4*6* [i.e., TGGCTCAGG sequences corresponding to codons 163–165 were changed to TGCTCA, which may be due to two different deletions at codons 163 (from TGG to TG) and 165 (from AGG to A)], whereas such polymorphisms were not observed in humans. These findings suggest that a selection pressure to generate and maintain the polymorphic sites might be considerably different between the lineages of humans and the Old World monkeys.

Discussion

It has been suggested that the ancestral gene for the ULBP/REAT molecule of placental mammals was originally diverged and duplicated in each species after an emigration from the MHC region (Kondo et al. 2010). In humans, MHC genes (*HLA* genes) are clustered and mapped on the short arm of chromosome 6, 6p21.3, whereas the *ULBP/RAET1* genes are located on the long arm of chromosome 6, 6q25.1. As for the *MHC* genes in the macaque, it was previously reported that rhesus macaque MHC, e.g., BAT1 gene, was localized to chromosome 6q24 by using fiber-fluorescence in situ hybridization (Huber et al. 2003) and cynomolgus (crab-eating) macaque MHC, e.g., Mafa-A and Mafa-B genes, was cytogenetically mapped to chromosome 6p13 (Liu et al. 2007), although the rhesus macaque MHC is mapped on the short arm of chromosome 4 in the draft genome sequence database of rhesus macaques (Gibbs et al. 2007); e.g., Mamu-A and BAT1 were mapped from positions 29, 517, 308 to 29, 520, 221 and from 31, 164, 822 to 31, 175, 032, respectively, on chromosome 4 (data were obtained from the UCSC Genome Browser at http://genome.ucsc.edu/cgi-bin/hgGateway). The discrepancy between the cytogenetic mapping and the assignment in draft genome sequence should be resolved in the future. On the other hand, it is interesting to note that each member of the *ULBP/RAET1* gene family, except for *ULBP6*, is completely or partially duplicated in the rhesus genome. As for the *ULBP4/RAET1E*, two related sequences, LOC695031 (NC007861) and LOC694265, have been identified as orthologs of human *ULBP4/RAET1E*. On the other hand, the configuration of *ULBP/RAET1* loci in the crab-eating macaque genome remained unknown. Because LOC694265 was a pseudogene lacking most part of the coding exons, we designed PCR primers by referring the NC007861 sequence. By using the designed primers, we could successfully amplify *ULBP4/RAET1E* alleles from both rhesus and crab-eating macaques.

In this study, we identified a total of 25 and 14 alleles from rhesus and crab-eating macaques, respectively. One of the rhesus macaque alleles had identical sequences to one of the crab-eating macaque alleles, and the phylogenetic analysis demonstrated that the *ULBP4/RAET1E* alleles were widely diverged. None of the alleles identified in this study were identical to the previously reported sequence NC007861, which was derived from an individual of Indian rhesus macaque. Given that we analyzed rhesus macaques of Burmese origin in this study, and allele distribution of MHC-related polymorphic genes are well known to be largely dependent on the habitat regions, the extent of diversity and variation in *ULBP4/RAET1E* may be further expanded.

It was demonstrated that the diversity of *ULBP4/RAET1E* in the Old World monkeys was much higher than that of human *ULBP4/RAET1E*. It is possible that the genes in the *ULBP/RAET1* locus, in particular, *ULBP4/RAET1E* and *ULBP/RAET1*s, might be highly polymorphic in the

Old World monkeys. We therefore investigated ten unrelated rhesus macaque subjects, in which we had detected 16 *ULBP4/RAET1E* alleles for polymorphisms in the adjacent *ULBP/RAET1* genes. We found one *ULBP1/RAET1I* allele, seven *ULBP2/RAET1H* alleles, and one *ULBP3/RAET1N* allele in these subjects. The observation suggested that *ULBP4/RAET1E* was highly polymorphic as compared to the adjacent *ULBP/RAET1* genes.

We revealed a high degree of polymorphism in the *ULBP4/RAET1E* of the rhesus and crab-eating macaques, although about half of the polymorphisms were synonymous changes (Table 2). Albeit the expression of the ULBP4/RAET1E molecule is known to be involved in the recognition of tumor cells by the NKG2D receptor (Cao et al. 2008; Kong et al. 2009), the functional significance of the polymorphisms in the extracellular domain of the ULBP4/RAET1E molecules remained unknown. To investigate a possible role of the polymorphisms, we have created a 3-D structure model of rhesus ULBP4/RAET1E molecule by using the structure data of human ULBP3/RAET1N in complex with NKG2D (Radaev et al. 2001) as the reference. As shown in Fig. 3, only one polymorphic site at 173 was on the surface of the α helix pointing to the NKG2D receptor, five sites at 59, 136, 144, 161, and 165 were positioned outside the α helix, and only two sites at 32 and 91 were mapped on the β sheet in the groove. The other polymorphic sites were on the β sheet outside of the groove or were not on the surface of the α helix. In addition, expression of *ULBP4/RAET1E* is predominantly found in the skin and tumor tissues and not induced by viral infection in normal cells (Chalupny et al. 2003; Eagle et al. 2006). These observations suggest that the polymorphisms are unlikely to be involved in the differential presentation of characteristic small molecules bound by the ULBP4/RAET1E molecules, as found in the presentation of antigenic peptides by the MHC molecules. Nevertheless, highly prevalent polymorphisms leading to amino acid replacements suggest that a selection pressure had operated on the configuration of diversity in *ULBP4/RAET1E*.

Of particular interest in this study was the rhesus macaque allele *Mamu-ULBP4*8*, which was supposed to contain a stop codon in the exon 2 coding sequence that would truncate the most part of the molecule. This is the first report of a non-functional *ULBP/RAET1* allele in primates; however, a similar situation was reported for another NKG2D ligand gene, *MIC*. For example, a specific human *MIC* haplotype linked to HLA-B*048 consists of non-functional *MIC* genes, in which *MICA* was deleted and *MICB* contained a termination codon (Ota et al. 2000); the non-functional *MIC* haplotype is widely distributed in the East Asian populations (Komatsu-Wakui et al. 2001). It is interesting to note that there are two distinct and polymorphic genes for *MIC* in the rhesus macaque, *MICA* (previously designated as *MIC1* and *MIC3*) and *MICB* (previous *MIC2*); however, they are not considered to be orthologous to the human *MICA* and *MICB* genes, respectively (Seo et al. 1999, 2001; Doxiadis et al. 2007; Averdam et al. 2007). Because members of the MIC and ULBP/RAET1 molecules are structurally related (Li et al. 2002), there is a functional redundancy in the recognition by NKG2D, and thus, the presence of a null allele had been allowed during the evolution of primates.

In the present study, we demonstrated the *ULBP4/RAET1E* allelic polymorphisms not only in the rhesus macaque but also in the crab-eating macaque. Although the localization of *ULBP4/RAET1E* in the crab-eating macaque

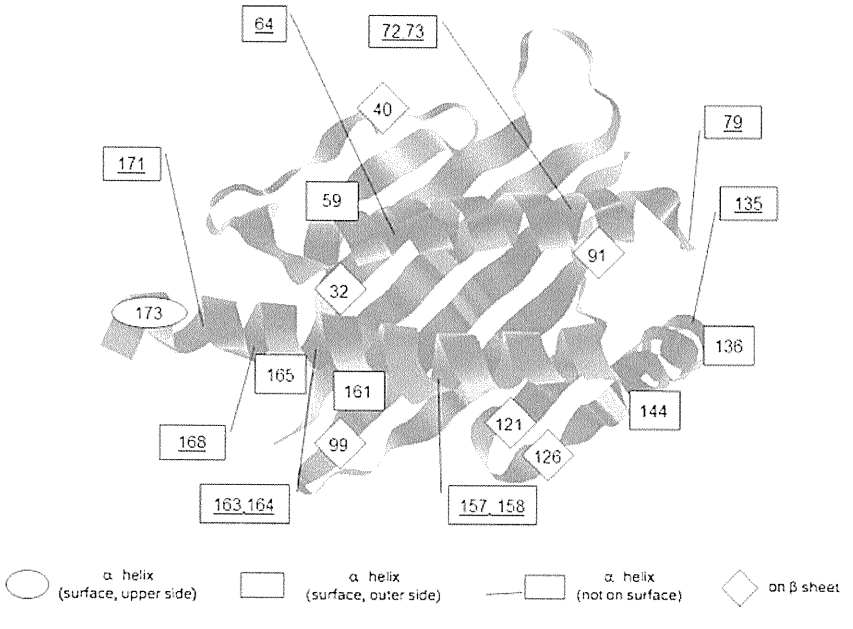

Fig. 3 Mapping of polymorphic sites on the structure model of the macaque ULBP4/RAET1E molecule. Polymorphic sites found in the Old World monkeys were mapped on the 3-D structure model of ULBP4/RAET1E. Residues on the upper and outer sides of the α helix structure were indicated by a *circle* and *squares*, respectively. Residues not found on the surface of the α helix were *underlined*, and those on the β sheet structure were represented by *rhombi*

genome is unknown, a homology search showed that a *Mafa-MICH3* gene (AY032639) was homologous to *Mafa-ULBP4/RAET1E* because the nucleotide sequences of *Mafa-ULBP4*1.1* showed a 96% homology to *Mafa-MICH3*. Similarly, nucleotide sequences of a chimpanzee gene, *Patr-MICH3* (AY032638), showed a 94% homology to the rhesus *ULBP4/RAET1E*. These findings strongly suggest that *MICH3* in the crab-eating macaque and chimpanzee is orthologous to *ULBP4/RAET1E* in the human and rhesus macaque.

In conclusion, we revealed a large diversity of *ULBP4/RAET1E* in two related species of the Old World monkey. Because there were extremely large polymorphisms in the extracellular domain of the ULBP4/RAET1E molecule in the Old World monkey, which was larger than that in the human, the functional impact of the polymorphisms and its significance in the evolution of primates should be investigated in future studies.

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Common marmoset (*Callithrix jacchus*) as a primate model of dengue virus infection: development of high levels of viraemia and demonstration of protective immunity

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Dengue virus (DENV) causes a wide range of illnesses in humans: dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Animal models that constantly develop high levels of viraemia are required for the development of protective and preventive measures. Common marmosets (*Callithrix jacchus*) demonstrated high levels of viraemia after inoculation with clinical isolates of four serotypes of DENV; in particular, over 10^6 genome copies ml^{-1} after inoculation with DENV-2. Non-structural protein 1 and DENV-specific IgM and IgG antibodies were consistently detected. The DENV-2 genome was detected in lymphoid organs including the lymph nodes, spleen and thymus, and also in non-lymphoid organs. DENV antigen was detected by immunohistochemistry in the liver and spleen from inoculated marmosets. Four marmosets were reinoculated with DENV-2 at 33 weeks after primary inoculation with DENV-2. The DENV-2 genome was not detected in any of these marmosets, indicating protection from a secondary infection. The results indicate that common marmosets are highly sensitive to DENV infection, and suggest that marmosets could be a reliable primate model for the evaluation of candidate vaccines.

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INTRODUCTION

Dengue virus (DENV) is an arthropod-borne flavivirus and is a serious cause of morbidity and mortality in the world. Approximately 2.5 billion people living in tropical and subtropical areas are at risk of DENV infection. Infection with any one of the four serotypes of DENV (DENV1–4) causes a range of symptoms: classical dengue fever (DF), and the sometimes fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DF is an acute febrile illness, characterized by headache, rash, retro-orbital pain, arthralgia and myalgia. DHF is characterized by capillary leakage, thrombocytopenia, haemorrhagic manifestations, hypotension and liver parenchyma necrosis. When profound plasma leakage results in shock, the disease is called DSS (Halstead, 2007; WHO, 2002). No vaccines or specific antiviral drugs are currently available.

Animal models of DENV could facilitate the development of vaccines and anti-dengue viral agents (Bente & Rico-Hesse, 2006). Mouse models have proved to be useful in therapeutics and vaccine efficacy studies (Balsitis *et al.*, 2010; Shrestha *et al.*, 2010). However, limitations of these models include the usage of immunocompromised mice and the development of only limited levels of viraemia in immunocompetent mice, even when high titres of DENV

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were inoculated. These aspects necessitate the development of immunocompetent animal models that are more relevant to DENV infection in humans, with the capability of supporting high levels of viral replication and antibody (Ab) response, and with additional advantages such as availability, ease of maintenance and handling. Non-human primates, rhesus monkeys, cynomolgus monkeys and owl monkeys, have been used in the evaluation of candidate vaccines (Koraka *et al.*, 2007); however, not all non-human primate models develop overt levels of viraemia (Ito *et al.*, 2010). It has been reported that disease severity is related to high levels of viraemia, and, reduction in viraemia is associated with reduced disease severity (Libraty *et al.*, 2002a). Neutralizing antibodies play a key role in protection and is an indicator of protective immunity as well as vaccine efficacy. A suitable animal model that consistently develops high levels of viraemia would provide a useful tool in addressing fundamental issues regarding vaccine development.

As viraemia is a critical parameter in assessing vaccine efficacy, establishment of models with close evolutionary proximity to humans would offer a valuable tool. In the present study, we attempt to establish a new animal model of DENV infection using common marmosets (*Callithrix jacchus*). Common marmosets are a small New World monkey that could be maintained with relative ease in captivity as compared with many other non-human primates. Subcutaneous inoculation of common marmosets with DENV consistently induces high levels of viraemia and Ab response. Marmosets that received a secondary inoculation with DENV-2 at 33 weeks after the primary inoculation did not develop detectable levels of viraemia. The results suggest that marmosets are a potentially reliable DENV infection model for the development of vaccine and anti-DENV therapeutics.

RESULTS

Infection of marmosets with four serotypes of DENV

Marmosets were subcutaneously inoculated with clinical isolates of DENV: 3.5×10^7 p.f.u. of DENV-1 (02-17/1 strain), 6.7×10^7 p.f.u. of DENV-2 (DHF0663 strain), 4.5×10^6 p.f.u. of DENV-3 (DSS1403 strain) and 1.5×10^6 p.f.u. of DENV-4 (05-40/1 strain). The strains DENV-1 02-17/1, DENV-2 DHF0663, DENV-3 DSS1403 and DENV-4 05-40/1 were clinical isolates that were propagated for less than four passages on C6/36 cells. Levels of dengue viral RNA (dengue vRNA) were assessed in plasma at various time points (Table 1, experiment 1). Dengue vRNA was detected in plasma samples from the marmosets on days 3 and 5 after inoculation. For each of the four animals (D1-1, D2-1, D3-1 and D4-1), the plasma levels of vRNA reached 5.0×10^5 copies ml^{-1} on day 5, 1.6×10^7 copies ml^{-1} on day 3, 5.5×10^4 copies ml^{-1} on day 5 and 2.5×10^4 copies ml^{-1} on day 3, respectively.

The level of non-structural protein 1 (NS1) in plasma samples was assessed by ELISA (Fig. 1a). NS1 was detected in plasma samples from D1-1, D2-1 and D3-1, and peaked on days 3–10 for D1-1, and on day 3 for D2-1 and D3-1. The results demonstrated that DENV-2 induced high levels of viraemia, and DENV-2 was, thus, used in the next series of experiments.

Infection of marmosets with four different doses of DENV-2

Four marmosets were inoculated with DENV-2 (DHF0663 strain) at two different doses; D2-2 and D2-3 with 4.4×10^7 p.f.u., and D2-4 and D2-5 with 1.8×10^5 p.f.u. Dengue vRNA was detected at levels of 10^6–10^7 copies ml^{-1} in plasma samples from all the marmosets on day 3 (Table 1, experiment 2). NS1 antigen was also detected in plasma samples from all the marmosets (Fig. 1d).

Four marmosets were then inoculated with lower doses of DENV-2 (DHF0663 strain); D2-6 and D2-7 with 1.8×10^4 p.f.u., and D2-8 and D2-9 with 1.8×10^3 p.f.u. Dengue vRNA was detected on days 2, 4 and 7 in all the four marmosets (Table 1, experiment 3). The plasma levels of vRNA reached the peak levels approximately 10^6 copies ml^{-1} in marmosets inoculated with 1.8×10^4 p.f.u. and approximately 5×10^5 copies ml^{-1} in those inoculated with 1.8×10^3 p.f.u. NS1 antigen was detected in plasma samples from all the marmosets (Fig. 1g). The results in experiments 2 and 3 indicate that marmosets inoculated with $\geqslant 1.8 \times 10^3$ p.f.u. developed viraemia at levels of more than 3.7×10^5 copies ml^{-1}.

Infection of marmosets with two other strains of DENV-2

Two other strains of DENV-2 were examined for the ability to induce viraemia. Marmosets D2-10 and D2-11 were inoculated with 1.2×10^5 p.f.u. of Jam/77/07 strain, and D2-12 and D2-13 with 1.9×10^5 p.f.u. of Mal/77/08 strain. The DENV-2 strains, Jam/77/07 and Mal/77/08, were clinical DENV strains that were propagated less than four passages on C6/36 cells. Dengue vRNA was detected in plasma samples from all four marmosets on days 2, 4 and 7, and the peak levels reached over 10^6 copies ml^{-1} on day 4 (Table 1, experiment 4). NS1 antigen was detected in plasma samples from all marmosets (Fig. 1j). The results in experiments 2, 3 and 4 indicate that marmosets developed similarly high levels of viraemia after inoculation with three different strains of DENV-2.

Antibody responses in marmosets after inoculation with DENV

DENV-specific IgM and IgG Ab responses were examined by ELISA after inoculation with DENV. In experiment 1, specific IgM Ab was first detected on day 5 for D1-1, D2-1

Table 1. Levels of dengue vRNA in plasma from marmosets inoculated with DENV

–, vRNA below the limit of detection using RT-PCR; NT, not tested.

Animal ID	Virus strain	Inoculated dose		Dengue vRNA copy numbers (copies ml^{-1})									
		p.f.u. per dose	copies per dose	Days after inoculation									
				0	2	3	4	5	7	8	10	14	21
Primary inoculation													
Experiment 1													
D1-1	02-17/1	3.5×10^7	4.6×10^8	–	NT	3.8×10^5	NT	5.0×10^5	–	NT	–	–	*
D2-1	DHF0663	6.7×10^7	8.2×10^8	–	NT	1.6×10^7	NT	1.0×10^5	–	NT	–	–	*
D3-1	DSS1403	4.5×10^6	2.5×10^8	–	NT	–	NT	5.5×10^4	–	NT	–	–	*
D4-1	05-40/1	1.5×10^6	1.1×10^9	–	NT	2.5×10^4	NT	–	–	NT	–	–	*
Experiment 2													
D2-2	DHF0663	4.4×10^7	5.4×10^8	–	NT	1.2×10^7	NT	NT	–	NT	NT	–	–
D2-3				–	NT	8.6×10^6	NT	NT	–	NT	NT	–	–
D2-4		1.8×10^5	2.2×10^6	–	NT	6.1×10^6	NT	NT	–	NT	NT	–	–
D2-5				–	NT	9.5×10^6	NT	NT	3.5×10^5	NT	NT	–	–
Experiment 3													
D2-6	DHF0663	1.8×10^4	2.2×10^5	–	3.4×10^5	NT	2.0×10^6	NT	5.1×10^4	NT	NT	–	*
D2-7				–	3.8×10^5	NT	9.4×10^5	NT	2.8×10^4	NT	NT	–	*
D2-8		1.8×10^3	2.2×10^4	–	2.2×10^4	NT	3.7×10^5	NT	1.4×10^4	NT	NT	–	*
D2-9				–	6.2×10^4	NT	6.9×10^5	NT	2.2×10^5	NT	NT	–	*
Experiment 4													
D2-10	Jam/77/07	1.2×10^5	1.4×10^7	–	1.0×10^6	NT	2.8×10^6	NT	4.6×10^4	NT	NT	–	–
D2-11				–	8.7×10^5	NT	2.0×10^6	NT	4.9×10^4	NT	NT	–	–
D2-12	Mal/77/08	1.9×10^5	7.1×10^6	–	4.8×10^6	NT	9.6×10^6	NT	5.8×10^3	NT	NT	–	–
D2-13				–	2.0×10^6	NT	7.0×10^6	NT	4.7×10^3	NT	NT	–	–
Experiment 5													
D2-14	DHF0663	6.7×10^7	8.2×10^8	–	8.3×10^6	5.9×10^6	*	*	*	*	*	*	*
D2-15				–	7.8×10^6	NT	3.7×10^6	3.5×10^5	*	*	*	*	*
D2-16				–	2.3×10^7	NT	6.5×10^6	NT	2.8×10^5	-	*	*	*
D2-17				–	3.1×10^7	NT	3.6×10^6	NT	6.0×10^4	NT	NT	–	–
Secondary inoculation													
Experiment 6													
D2-2	DHF0663	1.8×10^5	2.2×10^6	–	–	NT	–	NT	–	NT	NT	–†	–
D2-3				–	–	NT	–	NT	–	NT	NT	–†	–
D2-4				–	–	NT	–	NT	–	NT	NT	–†	–
D2-5				–	–	NT	–	NT	–	NT	NT	–†	–

*The marmosets were sacrificed and no samples were collected.
†Indicates day 15.

and D3-1, and, on day 7 for D4-1 (Fig. 1b). IgM Ab reached peak levels on day 10 for D1-1, D2-1 and D3-1. IgG Ab was first detected on day 10 for all four animals, and IgG Ab levels increased thereafter (Fig. 1c). Although DENV-4 inoculation induced only low levels of viraemia, both DENV-specific IgM and IgG Abs were induced. DENV-specific IgM and IgG were similarly detected in all the marmosets inoculated with DENV-2 in experiments 2, 3 and 4 (Fig. 1e, h, k for IgM, and Fig. 1f, i, l for IgG). These results, along with those shown in Table 1 and Fig. 1(b, c), indicate that DENV propagated and induced specific IgM and IgG antibodies in marmosets.

Detection of DENV in organs

The distribution of DENV in organs was examined. Four marmosets were inoculated with 6.7×10^7 p.f.u. of DENV-2 (DHF0663 strain). One animal each was euthanized and the organs collected on days 3, 5, 8 and 14. All marmosets demonstrated viraemia (Table 1, experiment 5). NS1 antigen was also detected in all four marmosets (Fig. 1m). IgM and IgG were first detected on day 5 and day 8, respectively (Fig. 1n, o). The results indicate that DENV-2 infection was established in these four marmosets.

Levels of DENV-2 vRNA were assessed in thymus, lung, liver, kidneys, pancreas, spleen, lymph nodes, urinary

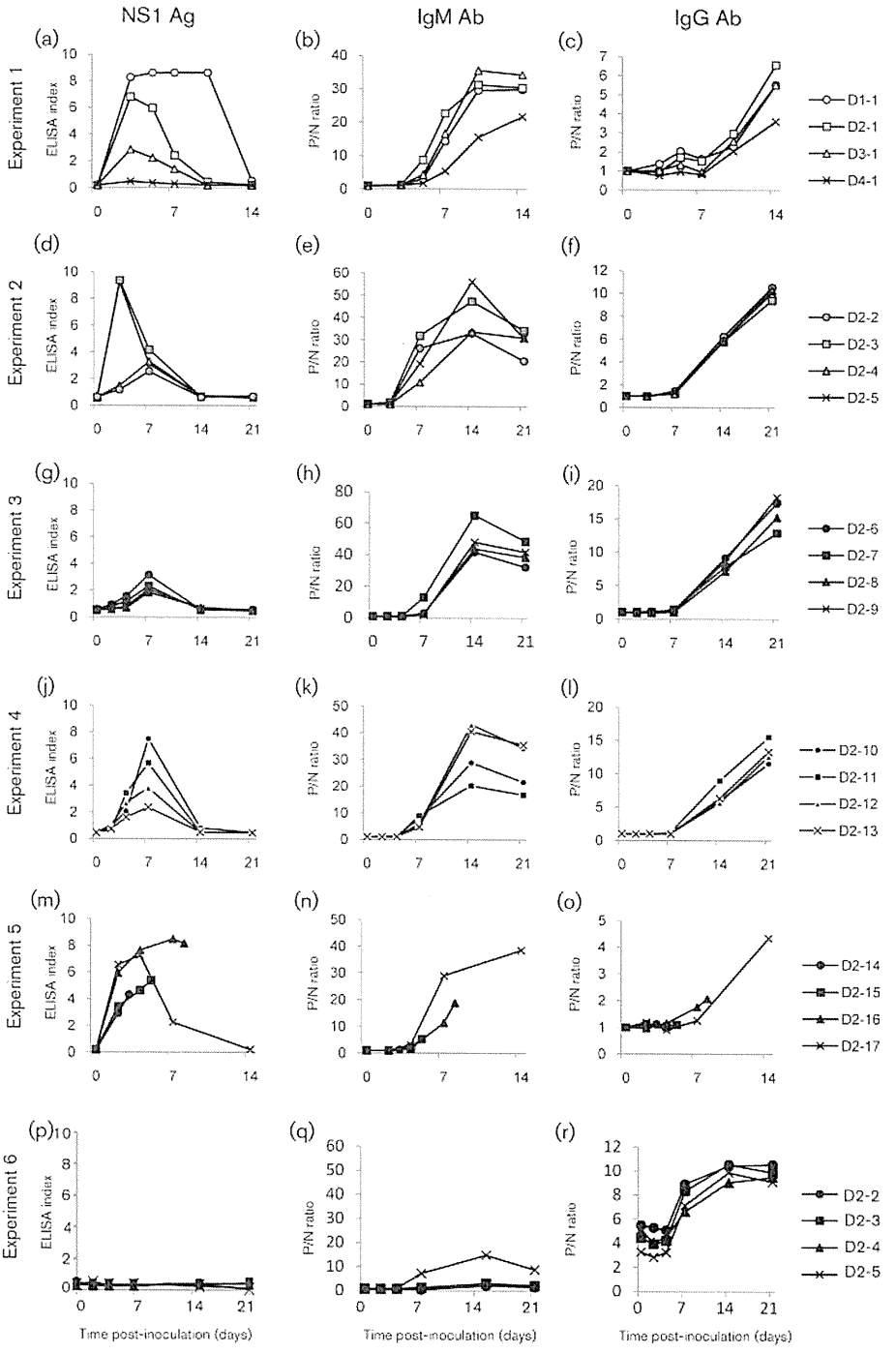

Fig. 1. Levels of DENV NS1 and DENV-specific IgM and IgG Abs in plasma samples from marmosets inoculated with DENV. ELISA index of NS1 antigen (a, d, g, j, m and p), P/N ratio of DENV-specific IgM Ab (b, e, h, k, n and q) and P/N ratio of DENV-specific IgG Ab (c, h, i, l, o and r) in plasma samples from marmosets inoculated with DENV in experiment 1 (a–c), experiment 2 (d–f), experiment 3 (g–i), experiment 4 (j–l), experiment 5 (m–o) and experiment 6 (p–r). Day 0 was defined as the day of virus inoculation.

bladder and bone marrow on days 3, 5, 8 and 14 after inoculation (Table 2). DENV-2 vRNA was detected in mandibular, axillary, mesenteric and inguinal lymph nodes from all animals. DENV-2 vRNA was detected in thymus on days 3, 5 and 8, and in spleen on days 5, 8 and 14. DENV-2 vRNA was also detected in the non-lymphoid

Table 2. Levels of dengue vRNA in organs from marmosets inoculated with DENV-2 DHF0663 strain

–, vRNA below the limit of detection using RT-PCR.

Organs	Dengue vRNA copy numbers in organs (copies μg^{-1} of total RNA)			
	Days after inoculation with DENV-2			
	3	5	8	14
Viraemia titres* (copies ml^{-1})	5.9×10^6	3.5×10^5	–	–
Lymph node				
Mandibular	–	1.4×10^4	9.2×10^4	–
Axillary	4.3×10^4	6.4×10^3	5.1×10^4	2.3×10^3
Mesenteric	–	2.9×10^4	4.2×10^4	8.8×10^3
Inguinal	8.8×10^3	3.0×10^4	4.9×10^4	2.9×10^3
Thymus	5.7×10^3	1.5×10^4	9.5×10^4	–
Spleen	–	2.8×10^4	1.4×10^5	3.0×10^4
Bone marrow	3.3×10^3	–	–	–
Lung	2.2×10^3	1.9×10^4	–	1.5×10^4
Liver	8.0×10^1	–	8.2×10^3	–
Kidney (right)	–	–	–	9.8×10^4
Kidney (left)	2.3×10^3	1.6×10^2	1.9×10^2	2.6×10^5
Pancreas	3.6×10^4	4.4×10^2	6.6×10^3	–
Urinary bladder	–	1.0×10^4	–	6.6×10^3

*Viraemia titres in serum samples at the day of sacrifice (copies ml^{-1}).

organs: lungs, liver, kidneys, pancreas and urinary bladder. The results suggest that DENV-2 propagated in the lymphoid organs and non-lymphoid organs.

The presence of DENV-infected cells was examined in the liver and spleen by immunohistochemistry. DENV antigen-positive cells were detected in Kupffer cells in the liver (Fig. 2a) and in lymphocytes and macrophages in the spleen (Fig. 2b). The results confirm that DENV infection was established in marmosets after inoculation with DENV.

Absence of dengue vRNA after a secondary inoculation with DENV-2

Four marmosets (D2-2, D2-3, D2-4 and D2-5) were reinoculated with 1.8×10^5 p.f.u. of DENV-2 at 33 weeks after primary inoculation with DENV-2. The levels of dengue vRNA were assessed on days 0, 2, 4, 7, 15 and 21 after the secondary inoculation (Table 1, experiment 6). Dengue vRNA was not detected in any of the marmosets on 2–21 days after inoculation. NS1 antigen was not detected in any of the four marmosets after secondary inoculation with DENV-2 (Fig. 1p).

Robust IgG responses were detected by ELISA in all the four marmosets (Fig. 1r). Neutralizing Ab was examined in the four marmosets (D2-2–D2-5) after the primary and secondary inoculations (Table 3, experiment 2). Neutralizing Ab was first detected on day 14 after the primary inoculation. Neutralizing Ab titres were at levels of 1 : 20–1 : 80 before the secondary inoculation. The titres increased from 1 : 160 to 1 : 640 on day 7 after the secondary inoculation (Table 3, experiment 6). The results indicate that primary inoculation with DENV-2 induced protective immunity to DENV-2.

DISCUSSION

Non-human primates and rodents have been used as animal models for the evaluation of candidate dengue vaccines. Although Ab responses and kinetics of viraemia in these models, in particular in macaques, were similar to those in human DENV infection, viraemia levels were modest and apparent clinical signs were absent (Ito *et al.*, 2010; Marchette *et al.*, 1973). There remains a need for the development of a reliable animal model that better recapitulates DENV infection in humans. Several aspects, including incubation period, duration of viraemia and Ab responses in experimentally infected marmosets in the present studies appear to recapitulate DENV infection in humans (Vaughn *et al.*, 1997).

DENV infection induces the NS1 antigen in blood (Alcon *et al.*, 2002; Flamand *et al.*, 1999; Libraty *et al.*, 2002b). As reported in human studies (Mackenzie *et al.*, 1996), the NS1 antigen was detected in DENV-infected marmosets, indicating DENV replication. Plasma viraemia levels reached a peak of approximately 10^6 copies ml^{-1} on day 4 followed by gradual decline, with inoculation as low as 10^3 p.f.u. of DENV. In addition, using a subset of samples,

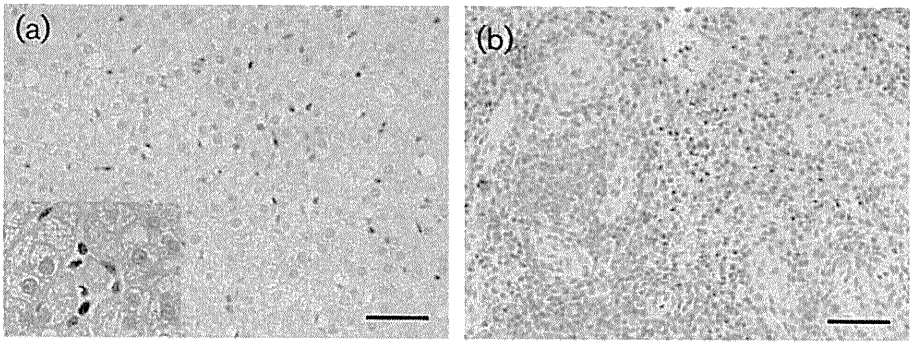

Fig. 2. Presence of DENV antigen in the liver and spleen of a marmoset inoculated with DENV. DENV antigen-positive cells were in the liver (a) and spleen (b) from marmoset D2-17. The inset (a) shows antigen-positive cells that correspond morphologically to the Kupffer cells. Kupffer cells were identified based on the morphological and localization characteristics of the cells; the cells were polygonal or spindal shaped, and were located in liver sinusoids. Bars, 50 μm.

peak viraemia titres were examined in three marmosets using the plaque titration method. Peak viraemia titres were constantly detected on days 3 and 4 after infection. Viraemia titres were 1.0×10^4 p.f.u. ml^{-1} (D2-6) and 1.0×10^3 p.f.u. ml^{-1} (D2-12) at 4 days post-inoculation, and 8.3×10^3 p.f.u. ml^{-1} (D2-14) at 3 days post-inoculation. Quantitative RT-PCR has proved to be useful in studies determining viraemia levels in other DENV animal models using rhesus macaque and mice (Bente *et al.*, 2005; Onlamoon *et al.*, 2010). Viraemia levels in marmosets determined by using quantitative real-time PCR (RT-PCR) and plaque assays were higher or at similar levels to those reported in other non-human primates (Bernardo *et al.*, 2008; Blair *et al.*, 2006; Goncalvez *et al.*, 2007; Guirakhoo *et al.*, 2004; Raviprakash *et al.*, 2008; Schiavetta *et al.*, 2003; Whitehead *et al.*, 2003). In the present study, all marmosets developed viraemia using inoculation doses from 10^7 to 10^3 p.f.u. per dose.

Although there was a tendency toward the development of differential viraemia patterns between the marmosets inoculated with 10^7–10^5 p.f.u. per dose and 10^4–10^3 p.f.u. per dose, further evaluations are needed to define the viraemia patterns between the two groups. The results indicate that a higher dose of viral inoculum does not necessarily lead to higher viraemia titres or longer viraemia durations. Overall, the results indicate that DENV infection was established after primary inoculation in marmosets. DENV viraemia was consistently detected in all of the experimentally infected marmosets using various DENV

Table 3. Neutralizing Ab titres to DENV-2 in marmosets after inoculation with DENV-2

NT, Indicates not tested.

Animal ID	Virus strain	Dose (p.f.u. per dose)	Neutralizing Ab titre							
			Days after inoculation with DENV-2							
			0	2	3	4	7	14	15	21
Primary inoculation										
Experiment 2										
D2-2	DHF0663	4.4×10^7	<20	NT	<20	NT	<20	80	NT	160
D2-3			<20	NT	<20	NT	<20	80	NT	80
D2-4		1.8×10^5	<20	NT	<20	NT	<20	160	NT	160
D2-5			<20	NT	<20	NT	<20	40	NT	80
Secondary inoculation										
Experiment 6										
D2-2	DHF0663	1.8×10^5	80	80	NT	40	640	NT	640	>1280
D2-3			40	80	NT	20	320	NT	640	640
D2-4			40	40	NT	80	160	NT	320	640
D2-5			20	20	NT	40	160	NT	320	320