Arch Virol (2011) 156:1073–1077
DOI 10.1007/s00705-011-0959-2

BRIEF REPORT

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

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Received: 20 September 2010 / Accepted: 19 February 2011 / Published online: 16 March 2011

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.

Acknowledgments Animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious, Diseases, Japan. This work was supported by the Research on Emerging and Re-emerging Infectious Diseases from Ministry of Health, Labour and Welfare, Japan (H17-Shinkou-019 and H20-Shinkou-ippan-015), and the Research on Biological Resources and Animal Models for Drug Development from Ministry of Health, Labour and Welfare, Japan (H19-Seibutsushigen-ippan-003).

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