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Original Article

Analysis of cross-reactivity between flaviviruses with sera of patients with Japanese encephalitis showed the importance of neutralization tests for the diagnosis of Japanese encephalitis☆

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ABSTRACT

Japanese encephalitis (JE) is one of the most important viral encephalitis in Asia. JE is caused by the Japanese encephalitis virus (JEV), which belongs to the genus *Flavivirus*, family *Flaviviridae*. The diagnosis of JE is usually based on serological assays, and it has been reported that cross-reactivity between flaviviruses has complicated the interpretations of results from serological assays. Therefore, analysis of the cross-reactivity is an important subject for serological diagnosis of JE and other diseases caused by flaviviruses. In the present study, the cross-reactivity of the sera of patients with JE to other flaviviruses was analyzed using enzyme-linked immunosorbent assay (ELISA) and neutralization tests. Sixteen serum samples were collected from patients with JE and were tested for: i) IgM antibody against West Nile virus (WNV), dengue virus (DENV), zika virus (ZIKV), and tick-borne encephalitis virus (TBEV) using IgM-ELISA, ii) IgG antibody against DENV and TBEV using IgG-ELISA, and iii) neutralization tests with DENV 1–4, ZIKV, TBEV, and WNV. Out of the 16 samples tested using ELISA, 11 and 14 samples were positive for IgM and IgG, respectively, against at least one of the other flaviviruses. In neutralization tests, neutralizing potency against DENV, ZIKV, or TBEV was not detected in any samples. Although 13 samples showed neutralizing potency against WNV, their neutralizing antibody titers were equal to or less than one-eighth of those against JEV. These results show that neutralization tests are more specific than ELISA, indicating the importance of the neutralization tests in the diagnosis of JE.

1. Introduction

Japanese encephalitis (JE) is a serious public health concern in many Asian countries [1]. JE is caused by the Japanese encephalitis virus (JEV), which belongs to the genus *Flavivirus* of the family *Flaviviridae*, and is transmitted to humans primarily by *Culex* mosquitoes (principally *Culex tritaeniorhynchus*) bite [2]. Flaviviruses are divided into three groups based on their mode of transmission: mosquito-borne flaviviruses, tick-borne flaviviruses, and flaviviruses with no known vector [3]. The mosquito-borne flaviviruses include the West Nile virus (WNV), dengue virus (DENV), zika virus (ZIKV), as well as JEV. The tick-borne flaviviruses include the tick-borne encephalitis virus (TBEV). WNV and TBEV cause encephalitis in humans, namely, West Nile encephalitis (WNE) and tick-borne encephalitis (TBE), respectively.

The diagnosis of JE is usually based on serology because RNA of JEV is rarely detectable in cerebrospinal fluid or serum samples [4]. Antibodies raised against one flavivirus have been reported to show cross-reactivity with other flaviviruses. Hence, the results of serological tests involving flaviviruses need careful interpretation [5,6]. Therefore, a detailed analysis of cross-reactivity of sera from patients infected with flaviviruses is important for the serological diagnosis. In particular, the analysis of cross-reactivity between JEV, WNV, and TBEV is important for the differential diagnosis of JE, WNE, and TBE because WNE and TBE patients showed similar clinical symptoms and abnormal findings in cerebrospinal fluid

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(CSF) and magnetic resonance imaging (MRI) to those of JE patients [7–10].

Cross-reactivity between DENV and ZIKV has been reported in the sera of the patients confirmed with infection by either of these viruses using the laboratory tests [11,12]. However, cross-reactivity of the sera of patients with JE to other flaviviruses has not yet been well analyzed.

In the present study, we analyzed whether the sera of patients with JE showed cross-reactivity to other flaviviruses (DENV, ZIKV, WNV, and TBEV) using IgM- and IgG- ELISA, and neutralization tests.

2. Materials and methods

2.1. Cells

Vero cells (strain 9013) were cultured at 37 °C with 5% CO_2 in Eagle's minimal essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Aldrich, St. Louis, Missouri, USA) and 100 U/ml of Penicillin–Streptomycin (Life Technologies, Carlsbad, California, USA).

2.2. Virus strains

The flavivirus strains used in this study were described previously. Briefly, DENV-1 10-07 was isolated from a traveler returning from Indonesia to Japan in the year 2010 [13]. DENV-2 TLC-30 was isolated from a febrile patient in East Timor in the year 2005 [14]. DENV-3 00–40 was isolated from a traveler returning from Papua New Guinea to Japan in the year 2000 [13]. DENV-4 TVP360 is a laboratory strain that was adapted to cell culture [15]. ZIKV MR766 was isolated from a rhesus monkey in Uganda in the year 1947 [16]. WNV NY99-6922 was isolated from mosquitoes in New York in the year 1999 [17]. TBEV Sofjin was isolated from the brain of a patient with TBE patient in Russia in the year 1937 [18]. JEV Beijing-1 was isolated from a patient with JE in China in the year 1949 [19].

2.3. Serum samples of patients with JE

Sixteen serum samples collected from 10 patients with JE reported in Japan in the year 2016, were used in this study. These patients were diagnosed by detection of IgM antibody and neutralizing antibody against JEV using the JEV IgM-capture ELISA and neutralization tests, respectively, as described previously [20].

2.4. Ethics statement

All the tests were carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The present study was approved by the Ethical Committee for Biomedical Science at National Institute of Infectious Diseases (NIID) in Japan (number: 782, 783).

2.5. ELISA

Heat-inactivated serum samples were tested for IgM antibody against WNV, DENV, ZIKV, and TBEV by ELISA using West Nile Virus IgM Capture DxSelect (Focus Diagnostics, Cypress, California, USA), Dengue Virus IgM Capture DxSelect (Focus Diagnostics), ZIKV IgM μ-capture ELISA (NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany), and EIA TBE Virus IgM (TestLine Clinical Diagnostics s.r.o., Brno, Czech Republic), respectively. The samples were also tested for IgG antibody against DENV and TBEV by using Dengue ELISA IgG (VIRCELL, Granada, Spain) and EIA TBE Virus IgG (TestLine Clinical Diagnostics s.r.o., Brno, Czech Republic), respectively. All the tests were performed according to the manufacturer's instructions. The samples were interpreted to be negative if the ELISA index was <0.90, positive if the index was >1.10, and equivocal if the index was between 0.90 and 1.10.

2.6. Neutralization test

Neutralizing antibody titers against flaviviruses were measured using a 50% plaque reduction neutralization test ($PRNT_{50}$), as described previously with some modifications [19]. Heat-inactivated serum samples were serially diluted two-fold, mixed with each virus strain at a 1:1 ratio, and incubated at 37 °C for 60 min. The Vero cells cultured in 12-well plates were inoculated with these mixtures and then incubated at 37 °C for 60 min, followed by overlaying with EMEM containing 1% methylcellulose and 2% FBS and incubated for 4–6 days. $PRNT_{50}$ was calculated as the reciprocal of the highest dilution resulting in a 50% reduction in plaque formation relative to the non-serum control. In order to calculate the ratio of neutralizing antibody titers, a nominal value of 10 was assigned to the titer against WNV when its original value was below 20.

3. Results

3.1. IgM- and IgG-ELISA

The results of the IgM and IgG antibody indices against various flaviviruses determined by ELISA are summarized in Table 1.

Seven serum samples (sample IDs 1/1s, 3/1s, 4/1s, 5/1s, 5/2s, 6/1s, and 7/2s) were positive, while one sample (ID 8/3s) showed equivocal result for IgM antibody against WNV using IgM ELISA. Eight samples (IDs 1/1s, 3/1s, 4/1s, 5/1s, 5/2s, 6/1s, 6/2s, and 9/1s) were positive and two serum samples (IDs 2/1s and 7/2s) showed equivocal reactions for IgM antibody against DENV. All the samples showed negative results for IgM antibody against ZIKV. Only one sample (ID 4/1s) was positive for IgM antibody against TBEV (Table 1).

Twelve samples (IDs 1/1s, 2/1s, 3/1s, 4/1s, 6/1s, 6/2s, 7/2s, 8/1s, 8/2s, 8/3s, 9/1s, and 9/2s) were positive and two samples (IDs 5/1s and 5/2s) showed equivocal results for IgG antibody against DENV, and eight samples (IDs 1/1s, 2/1s, 6/1s, 6/2s, 7/2s, 8/2s, 8/3s, and 9/2s) were positive for IgG antibody against TBEV (Table 1).

3.2. Neutralization tests

$PRNT_{50}$ titers of all the analyzed serum samples were lower than 40 against DENV -1, −2, −3, and −4, and lower than 20 against ZIKV and TBEV. On the other hand, 12 samples (IDs 1/1s, 2/1s, 3/1s, 4/1s, 5/1s, 5/2s, 6/1s, 7/2s, 8/1s, 8/3s, 9/1s, and 9/2s) showed neutralizing capacity against WNV. However, the neutralizing antibody titers against WNV were equal to or less than one-eighth of that against JEV (Table 2).

4. Discussion

ELISA and neutralization tests are the representative serological assays for the diagnosis of flavivirus infections. The cross-reactivity between flaviviruses has not yet been fully analyzed with the sera of flavivirus infected patients, in particular, by using neutralization test. The possible reasons are as follows: 1) neutralization tests are time-consuming and laborious, 2) bio-safety level (BSL)-3 facilities are required for performing neutralization tests against some flaviviruses (for example, WNV and TBEV need to be handled under BSL3 conditions in Japan), 3) the sera of flavivirus infected patients are available only at a limited number of institutes. In the present

Table 1
Detection of IgM and IgG antibody against flaviviruses from the sera of patients with JE.

Sample ID[a]	Days after onset (days)	Indices in IgM-ELISA					Indices in IgG-ELISA	
		JEV[b]	WNV[c]	DENV[c]	ZIKV[c]	TBEV[c]	DENV[c]	TBEV[c]
1/1s	21	9.96	2.18	1.21	0.38	0.54	2.03	4.51
2/1s	32	9.48	0.52	0.95	0.39	0.25	1.89	1.43
3/1s	21	10.49	2.44	1.34	0.43	0.24	1.52	0.76
4/1s	6	10.28	1.92	7.57	0.47	3.45	1.23	0.26
5/1s	8	9.94	2.09	2.90	0.47	0.37	1.08	0.26
5/2s	24	9.65	1.76	2.22	0.46	0.30	0.98	0.20
6/1s	8	9.17	1.20	4.33	0.43	0.79	1.77	1.75
6/2s	20	7.88	0.60	3.05	0.42	0.57	2.32	4.51
7/1s	4	7.22	0.23	0.33	0.46	0.20	0.56	0.14
7/2s	19	9.76	1.21	0.91	0.44	0.39	2.65	5.30
8/1s	2	4.11	0.16	0.39	0.43	0.22	1.15	0.29
8/2s	9	8.22	0.30	0.44	0.44	0.21	1.96	1.37
8/3s	17	9.33	0.92	0.57	0.43	0.24	2.43	3.19
9/1s	10	10.08	0.76	2.67	0.45	0.26	1.27	0.88
9/2s	74	7.85	0.28	0.78	0.48	0.19	1.82	1.82
10/1s	7	9.41	0.46	0.80	0.50	0.21	0.68	0.15
Positive[d]		16	7	8	0	1	12	8
Equivocal[e]		0	1	2	0	0	2	0

[a] s, serum.
[b] JEV IgM-capture ELISA index was cited from Ref. [20]. The index values were calculated by the ratio of the sample's optical density to that of negative control serum. The samples are interpreted to be positive if the index is above 1.50.
[c] The interpretations of the ELISA index; < 0.90 negative; 0.90–1.10 equivocal; 1.10 < positive.
[d] The number of positive samples.
[e] The number of equivocal samples.

Table 2
$PRNT_{50}$ (50% plaque reduction neutralization test) titers against flaviviruses with the sera patients with JE.

Sample ID[a]	Days after onset (days)	$PRNT_{50}$ titers against								Ratio: JEV/WNV
		DENV-1 (10-07)	DENV-2 (TLC-30)	DENV-3 (00–40)	DENV-4 (TVP360)	ZIKV (PRVABC59)	TBEV (Sofjin)	WNV (NY99-6922)	JEV (Beijing-1)	
1/1s	21	<40	<40	<40	<40	<20	<20	40	2560	64
2/1s	32	<40	<40	<40	<40	<20	<20	40	1280	32
3/1s	21	<40	<40	<40	<40	<20	<20	20	10,240	512
4/1s	6	<40	<40	<40	<40	<20	<20	40	10,240	256
5/1s	8	<40	<40	<40	<40	<20	<20	20	20,480 <	10,240 <
5/2s	24	<40	<40	<40	<40	<20	<20	40	10,240	256
6/1s	8	<40	<40	<40	<40	<20	<20	20	1280	64
6/2s	20	<40	<40	<40	<40	<20	<20	<20[c]	1280	1280
7/1s	4	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	<20	<20[c]	80	8
7/2s	19	<40	<40	<40	<40	<20	<20	160	2560	16
8/1s	2	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]	<20	40	640	16
8/3s	17	<40	<40	<40	<40	<20	<20	640	20,480	32
9/1s	10	<40	<40	<40	<40	<20	<20	20	10,240	512
9/2s	74	<40	<40	<40	<40	<20	<20	20	2560	128
10/1s	7	<40	<40	<40	<40	<20	<20	<20[c]	1280	128

[a] s, serum.
[b] Not done.
[c] Nominal titer of 10 was assigned for the calculation of the ratio to the neutralizing antibody titers against JEV.

analysis, the cross-reactivity of the sera of patients with JE to other flaviviruses was analyzed by using both ELISA and neutralization tests.

Out of the 16 serum samples that were analyzed from the patients with JE using ELISA, 10 and 14 samples showed positive or equivocal reactions for IgM and IgG antibody against DENV, respectively (Table 1). In contrast, the neutralizing capacity against DENV was not detected in any of the analyzed serum samples (Table 2). In general, severe infections caused by DENV result in haemorrhagic diseases, and not in encephalitic diseases. Hence, DENV infections are not considered in the differential diagnosis of JE in many cases. Nevertheless, our results showed that neutralization tests are more specific than IgM and IgG ELISA for serological diagnosis of JE.

The clinical symptoms of TBE are similar to those of JE, although TBEV is transmitted by ticks, not by mosquitoes [10]. TBEV is endemic in the area ranging from northern China and Japan, through Russia, to parts of Northern Europe [21]. The first TBE patient in Japan occurred in Hokkaido, the northern island of Japan, in the year 1993 [22]. No other patient with TBE was reported for the next 20 years from Japan. However, cases of TBE were reported from Hokkaido during the year 2016 and 2017 [23,24]. In addition, rodents that were seropositive for TBEV were found in Shimane Prefecture, which falls in the western part of Japan, suggesting that TBEV might be circulating in nature in Honshu, the main island of Japan, and that the patients with TBE might not be diagnosed correctly because of the low awareness about the disease [25,26]. Therefore, TBE needs to be considered in the differential diagnosis

of patients with viral encephalitis not only in Hokkaido, but also in the other parts of Japan. Previous reports showed that serum from a TBE patient showed cross-reactivity to JEV [22]. In addition, in the present study, one and eight serum samples from the patients with JE showed positive reactions in the TBEV IgM- and IgG-ELISA, respectively. Therefore, neutralization tests are important for the correct diagnosis of JE in Japan.

Although no cases of WNE have been reported from Japan so far, outbreaks of WNV meningitis and encephalitis have been reported from many parts of the world, including the United States, European countries and Russia [27,28]. WNE needs to be taken into consideration if encephalitis patients have travelled to these regions before the onset of the disease because of the similarity of its symptoms with that of JE.

WNV belongs to the JEV serocomplex as well as JEV, and is closely related to JEV [5,6]. Li et al. reported that sera from patients infected with WNV showed cross-reactivity to JEV in the neutralization test, but the neutralizing antibody titers against JEV were lower than those against WNV [29]. In our analysis, although 12 out of the 15 serum samples of patients with JE showed cross-reactivity to WNV in the neutralization tests, the neutralizing antibody titers against WNV were lower than those against JEV (Table 2). These findings show that neutralization tests are important to differentiate between the infections caused by JEV and WNV.

Previous JEV vaccination might affect the neutralizing antibody titers against JEV in the patients with JE. In the present analysis, all the patients were not children, but adults (all the patients in the present analysis were over 40 years old), so, JEV vaccination histories were not clear. Therefore, it remains unclear whether previous JEV vaccination affects the immune responses against JEV or not. Neutralization tests with serum samples from patients with JE who have clear JEV vaccination histories are needed to address the question.

In conclusion, the sera of patients with JE showed cross-reactivity to WNV, DENV, and TBEV in IgM and/or IgG ELISA, but the cross-reactivity was not detected in the neutralization tests against DENV and TBEV. Although the sera of patients with JE showed cross reactivity to WNV in the neutralization test, the neutralizing antibody titers against WNV were equal to or less than one-eighth of those against JEV. Our analysis showed that neutralization tests are important for the correct diagnosis of JE.

Conflicts of interest

None.

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