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Assessment of neutralizing antibodies elicited by a vaccine (Nakayama) strain of Japanese encephalitis virus in Taiwan

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SUMMARY

A total of 368 blood specimens were resampled from a serum collection containing 2914 blood samples which were collected by a random sampling in Taiwan in 1991. The plaque reduction neutralization test was applied to evaluate the neutralizing ability to two strains of Japanese encephalitis viruses, i.e. Nakayama (the present vaccine strain) and JE5 (a Taiwan isolate). The result revealed that antibodies against JE virus were present in each stratified age group. Antibody positive rates were both highest in the group older than 70 years although the lowest rates were located in different groups. In addition, the result showed that the immunogenicity potency of the antibody induced by the vaccine strain did not have a good coverage against JE5. The rate of neutralizing antibodies above the level of protective efficacy of the present vaccine was limited as low as 37·93%. Efficacy of the vaccine used at present was apparently not efficient. Consideration of a more promising vaccine may be necessary.

INTRODUCTION

Japanese encephalitis (JE) has been an important arthropod-borne infectious disease in Asia, especially Southeast Asian countries [1]. The aetiological agent, JE virus, belongs to the family Flaviviridae which was separated from the family Togaviridae in 1985 [2]. The virus has been well-known to distribute along with an ecosystem in which paddy cultivation and pig raising were co-existing [3, 4]. In Taiwan, *Culex annulus*, *Cx. tritaeniorhynchus* and *Cx. fuscocephala* have been documented to be involved in the virus transmission [5–7].

Infection rates of JE virus in epidemic regions usually vary, ranging from only a few cases to 20% of the population [8]. Clinically, some 50000 JE cases occurred in Asia annually [9]. Fatality rates of the infection were also divergent in different localities, statistically 20–50% [1, 10, 11]. Those who recovered from the infection with clinical symptoms often displayed sequelae with serious neurological impairments and/or mental retardation [12, 13].

It was noted that mass vaccination with formalin-inactivated Nakayama strain of JE virus can evidently reduce the incidence of the disease [8, 14, 15]. In Taiwan, the vaccination programme has focused on both infants and new school children since 1968 [10, 14, 16]. However, there were still about 200 reported cases annually in spite of the programme being implemented for more than 22 years [13, 14, 17]. Inefficiency of the neutralizing ability of antibodies induced by the vaccine was considered as one of the possibilities. To evaluate the presumption, plaque reduction neutralization test (PRNT) was used in this study because it has shown to be highly specific in

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neutralizing antibody assessment [18–20]. It was expected to demonstrate the continued occurrence of JE infections in Taiwan through this evaluation.

MATERIALS AND METHODS

Serum specimens

A total of 2914 blood specimens which were randomly sampled in 1991 from most areas (except Yi-Lan county) of Taiwan, were provided by the Department of Public Health, National Defense Medical Center, Taiwan. All specimens were stored at −20 °C before use for the neutralization test. Prior to testing, specimens were heat-inactivated at 56 °C for 30 min to remove possible non-specific reactivities. To run this study 368 specimens were resampled from this serum collection, which was preceded with stratification by both age and gender.

Cells and virus strains

The C6/36 clone of *Aedes albopictus* cells was used for virus propagation [15]. The cells were cultured in Dulbelco's minimum essential medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 7·5% (w/v) sodium bicarbonate, and 1% (w/v) antibiotics (penicillin and streptomycin) at 28 °C, 5% CO_2. For virus titration, baby hamster kidney (BHK-21) cells were grown in RPMI medium-1640 (Gibco) with the same supplements mentioned above at 37 °C, 5% CO_2. Nakayama (vaccine strain) and JE5 (a Taiwan strain isolated in 1985) of the JE virus were used for the neutralizing antibody test.

Virus preparation and titration

JE virus was propagated in C6/36 cells as previously described [15]. Briefly, the confluent cells were inoculated with 0·01 multiplicity of infection (MOI) of JE viruses for 60 min at 28 °C. The flask containing infected cells was then added with growth medium and was incubated at 28 °C with 5% content of CO_2 for 3 days. Viruses were harvested by collecting culture fluid and mixed with a final concentration of 20% (v/v) of FCS; 0·1 ml aliquots of the mixture were stored at −70 °C until use. For virus titration, BHK-21 monolayer in 24-well plates (Falcon) were inoculated with 0·1 ml of each tenfold serial dilutions of stocked JE virus diluted by phosphate-buffered saline (PBS, pH 7·5) containing 2% FCS. Virus adsorption onto cells was achieved by occasional shaking at 37 °C with 5% CO_2 for 60 min. Wells were then overlaid with 1% (w/v) low-melting agarose (Bio-Rad) in RPMI medium, and the plates were reincubated at 37 °C for 72 h. The inoculated cells were then fixed with 10% formalin (v/v in PBS and stained with crystal violet after removal of agarose. The virus titre was estimated by counting the plaque number.

Preliminary titration of virus prior to use in neutralization test

Prior to testing the serum samples, a preliminary neutralization test was done to define the exact virus dilution used. JE viruses (Nakayama and JE5) were first diluted to 1×10^3 PFU (plaque forming unit)/ml in virus diluent (growth medium supplemented with 20% FCS) as virus stock. Equal amounts (0·1 ml) of virus diluent and virus stock were mixed and incubated for 1 h at 37 °C with 5% CO_2. The diluted virus suspension was then added to BHK-21 cells in 24-well plates. Requirement for this analysis was to make 25 plaques/well; therefore, further adjustment was done when necessary.

Plaque reduction neutralization test (PRNT)

PRNT was performed according to the method described by Russell and colleagues [21]. Briefly, 0·1 ml of viral stock (1×10^3 PFU/ml) and 0·1 ml of diluted serum sample (1:10 and 1:20) were mixed in 96-well Falcon microplates, and incubated at 37 °C with 5% CO_2 for 1 h. The mixture was then transferred to BHK-21 cells monolayered on 24-well plates for virus inoculation. Each serum sample was tested in triplicate. The plates containing inoculated cells were incubated at 37 °C with 5% CO_2 for another 1 h to allow adsorption of viruses onto the cells. Subsequently, the wells were overlaid with 0·5 ml 1% soft-agar in BHK-21 growth medium and reincubated at 37 °C for 72–96 h. Antibody titre was estimated by counting the plaque numbers. A 70% reduction of plaque numbers comparing with the control was used as the cut-off value for positivity of neutralizing antibodies. The control specimen was prepared by inoculating the viruses without addition of the serum. Any specimen which showed plaque numbers equal to or over the cut-off value was denoted as negative.

Statistical analysis

Prior to estimating the neutralizing antibodies, rugged overall positive rates were adjusted with demographic data of Taiwan in 1991 to reflect the distribution of ages and genders. For statistical analysis, all tested specimens were divided into two groups based on the certainty of vaccination. The vaccinated group covered the age group of 15–19 years, whereas those who were older than 30 were included in the unvaccinated group. In turn, the 20–29 group was excluded because it may consist of specimens which were ambiguous regarding vaccination. Chi square test was used to examine the presence of neutralizing antibodies against the two virus strains tested.

RESULTS

Seroprevalence rates

The results showed JE antibodies present in all age groups. The pattern of antibody prevalence rates against Nakayama strain was similar to that against JE5 (Tables 1, 2). Both of them showed highest positive rates in the age group over 70 years old, whereas the lowest positive rates were not so clear-cut. Except for the group over 70 years old, the highest antibody positive rate against the Nakayama strain was in the 15–19 group (72·5%); whereas the lowest appeared in the group of 20–29 (41·07%) (Table 1). However, the lowest positive rate for JE5 virus was 40·0% in the age group of 15–19 (Table 1).

Cross neutralization of antibodies related to two virus strains

The programme of mass vaccination to prevent JE was commenced in Taiwan in 1968, suggesting that antibodies detected in this study must include those which were elicited either by natural infection or by vaccination. As mentioned above, antibodies presenting in persons less than 22 years old in 1990 was suggested to be specific to the Nakayama strain, whereas the others could be attributed to natural infection.

To make sure of the efficiency of the analysis, two groups including 15–19 and over 30 years old were reclassified for the assessment of neutralizing antibodies. The result showed that the antibody positive rate related to Nakayama strain was not significantly associated with the JE5 virus ($\chi^2 = 0{\cdot}18$; D.F. $= 1$; $P > 0{\cdot}05$) (Table 2). In fact, only 37·93% (11/29) of sera containing vaccine antibody can actually neutralize the JE5 virus. On the other hand, antibody positive rate induced by the JE5 virus was significantly associated with the vaccine strain ($\chi^2 = 86{\cdot}90$; D.F. $= 1$; $P < 0{\cdot}05$) (Table 3). The result showed that 81·05%

Table 1. *Distribution of antibodies against Nakayama strain and JE5 virus among age groups in Taiwan*, 1991*

Age	Examined	Positive (%)	
		Nakayama	JE5
15–19	40	29 (72·50)	16 (40·00)
20–29	56	23 (41·07)	24 (42·86)
30–39	72	41 (56·94)	54 (75·00)
40–49	71	42 (59·15)	41 (57·74)
50–59	48	30 (62·50)	32 (66·67)
60–69	45	29 (64·44)	32 (71·11)
> 70	38	33 (86·80)	33 (86·80)
Total	368	227 (61·69)	232 (63·39)

* Sera were collected from most areas of Taiwan, except for the I-Lan county.

Table 2. *Association of neutralizing antibodies against Nakayama strain and JE5 strain in the age group of 15–19 years old*

Antibody	Neutralization to JE5		Total	Protection rate (%)*
	+	−		
Nakayama				
+	11	18	29	37·93
−	5	6	11	

$\chi^2_{df=1} = 0{\cdot}18$; $P > 0{\cdot}05$.
* Protection rate (11/29) indicates the rate of neutralizing antibodies above the level of protective efficacy.

Table 3. *Association of neutralizing antibodies against Nakayama strain and JE5 strain in the age group over 30 years old*

Antibody	Neutralization to Nakayama strain		Total	Protection rate (%)*
	+	−		
JE5				
+	154	36	190	81·05
−	18	64	82	

$\chi^2_{df=1} = 86{\cdot}90$; $P < 0{\cdot}05$.
* Protection rate (154/190) indicates the rate of neutralizing antibodies above the level of protective efficacy.

(154/190) of antibody stimulated by the JE5 virus can actually neutralize the vaccine strain.

DISCUSSION

In most endemic regions, antibodies against JE virus are usually prevalent in local populations as long as the virus persists [8, 22]. The JE incidence has evidently decreased in the past years in Taiwan [23]. This phenomenom was believed to be associated with improved living conditions and changed agriculture pattern [9, 24]. The vaccination programme with the Nakayama strain which was initiated in 1968 could be another factor. However, sporadic JE cases remained to occur in Taiwan in spite of the high antibody prevalence rate [23]. Because some cases have been completely vaccinated before appearance of the disease [13], it is worthwhile to explore the protection of the vaccine in depth. As a result, the question of the efficiency of the present vaccine was then prompted. In other words, antibody which was induced by vaccination may not be able to prevent the virus infection later on. In turn, evaluation of the protection coverage of vaccine antibodies became necessary in order to assess the vaccination policy, including the efficacy and the fitness of the vaccine strain.

Although the serum collection used in this study was lacking the age group under 15 years old, there was a demarcation for the vaccination programme (23 years old group) based on the time of vaccination in Taiwan. Therefore, the presence of JE antibodies in age group younger than 23 years old could be attributed to vaccination. On the other hand, antibodies presented in those over 28 years old were most likely to be elicited by natural infection through mosquito bites since vaccination was only given to infants and first-grade school children.

Statistical analysis revealed that antibodies induced by the local isolate had complete ability to neutralize the Nakayama strain, but not vice versa. In other words, antibodies against the vaccine virus may not be able to protect from the natural infection, i.e. naturally infected by the local strain of the JE virus. The rate of neutralizing antibodies above the level of protective efficacy was as low as 37·93%. In contrast, the Nakayama strain was readily neutralized by antibodies from natural infection, resulting in a high positive rate in the seroassay using Nakayama strain. As a result, the surveillance of neutralizing antibodies for JE epidemiology could not reflect the real tendency of the vaccine protectivity. Biological evidence has proved that JE virus isolated from geographically distant regions or separate time period may possess different antigenic specificities [17, 25], reflecting genetic differences among virus isolates [24].

Therefore, those who possess neutralizing antibodies induced by the Nakayama vaccine may not be efficiently protected from infection by wild strain JE virus. As a result, we suggest that health authorities in Taiwan ought to reconsider the validity of Nakayama strain vaccine due to its uncertain neutralizing ability on the local isolate of the JE virus. In order to improve the efficiency of the vaccination program in Taiwan, perhaps a substitution of another strain such as Beijing-1 strain should be seriously considered [26].

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