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Homologous and Heterologous Neutralization Antibody Responses After Immunization With Japanese Encephalitis Vaccine Among Taiwan Children

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Because 21 immunized children (13%) among the 162 confirmed Japanese encephalitis (JE) cases during 1986–1991 occurred in Taiwan, we collected 320 serum samples from Taiwan children aged 15–31 and 27–44 months immediately before the 1st dose (n = 41) and 1–3 months after the 2nd dose (n = 78, 27 pairs), and immediately before (n = 58) and 1–3 months after the 3rd dose (n = 143, 44 pairs) to determine neutralization antibody (Nt Ab) against the Nakayama (N) and Beijing-1 (B) strains and two Taiwan wild type JE viruses (JEV): CC-27 and CH-1392. Our Nt results showed that (1) B vaccine stimulated a better homologous Ab response than N vaccine for Nt Ab seropositivity rate (NASR), produced a higher level of Nt titer after the primary immunization [2 doses = 100% vs. 91%, geometric mean titer (GMT) = 115 vs. 22], had a greater booster effect (3 doses: 100% vs. 95%; GMT = 320 vs. 33), and showed a better capability to neutralize two local Taiwan JEV strains, particularly only after 3 doses (ave. NASR for B vs. N = 90% vs. 10%; and GMT for B vs. N = 154 vs. 1); (2) the two wild type JEV strains had different plaque morphology and antigenic variation and the CC-27 strain was not neutralized as well as the CH-1392 strain after 3 doses of vaccine (BBB or NNN or NNB); and (3) 30% of the children had lost JEV Nt Ab one year after the 2nd dose of N vaccine and natural infection with JE virus did occur among those children after immunization. In conclusion, (1) three doses of mouse-brain vaccine are the minimum requirement to protect children against the local Taiwan JEV; (2) the best strain for a JE vaccine depends on level of Nt Ab it induced, the molecular epidemiology and antigenic variation of the JEV in each local area; and (3) future vaccine must produce better B- and T-cell memory.

KEY WORDS: Japanese encephalitis virus, vaccine memory, immunization, antigenic variation

INTRODUCTION

Japanese encephalitis (JE) virus, the most important cause of epidemic arboviral encephalitis in Asia, has a wide clinical spectrum, ranging from asymptomatic infection to permanent neurologic sequelae with a high case fatality rate of 30%–70% [Umenai et al., 1985; Monath, 1988]. A mouse-brain vaccine has successfully controlled JE viral infection among human populations in Japan, Korea, and Taiwan since 1968 [Oya, 1988]. The increased incidence rates of JE in India, Nepal, Sri Lanka and northern Thailand in recent years has raised public health attention and stimulated a search for effective prevention and control strategies [Vaughn and Hoke, 1992].

Hsu et al. [1971] conducted the first randomized control trial of JE vaccine in Taiwan in 1965 and demonstrated its 80% vaccine efficacy with 2-dose immunization. Therefore, the Department of Health in the Republic of China in Taiwan area implemented a nationwide immunization program for 2-year-old children, using the JE vaccine (Nakayama strain) produced by the National Institute of Preventive Medicine

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TABLE I. Strains of JE Vaccine Delivered in Various Taiwan Geographic Areas During 1990–1992

Year	Vaccine strains used in various areas			
	Taan	Hsintien	Tungkang	Lambay Islet
1991	Nakayama	Nakayama & Beijing-1	NR	NR
1992	Nakayama	Beijing-1	Beijing-1	Beijing-1

NR = no record on which strain of JE vaccine (Nakayama and/or Beijing-1) was used. The health personnel in Tungkang and Lambay Islet did not document the vaccine strain used in the first two doses of JE vaccine for each child that they delivered in 1990 and 1991 but they did document "the Beijing-1 vaccine" during immunization in 1992.

(NIPM) since 1968. The high JE vaccine coverage rates (73%–92% during 1970–1991) had decreased the disease incidence rate from 8/100,000 in 1967 to 0.17/100,000 in 1991 [Okuno et al., 1975; National Quarantine Service in R.O.C., 1992]. However, 21 immunized children were reported among 162 (13%) JE confirmed cases during 1986–1991 (NIPM, 1992, unpublished data). This aroused strong concern about the effectiveness of the Nakayama strain mouse-brain vaccine.

Because of the possibility that antigenic variation in different JE virus (JEV) strains may influence vaccine efficacy [Okuno et al., 1968; Kobayashi et al., 1984; Calisher et al., 1989; Chen et al., 1990], Japanese virologists prepared a JE vaccine made with Beijing-1 virus strain to induce a more immunogenic antibody with a wider neutralizing spectrum against other strains of JEV [Kitano et al., 1986]. However, neutralization capability of the JE vaccine in Taiwan for either the vaccine or local wild JEV strains has never been evaluated. To provide information needed to improve the JE immunization programs, we measured the levels and duration of neutralization antibody (Nt Ab) among Taiwan children after immunization with JE Nakayama (N) or Beijing-1 (B) vaccine and its protective efficacy from natural infection by cross-neutralization against wild type JEV.

MATERIALS AND METHODS

Japanese Encephalitis Vaccine

An inactivated, liquid, purified mouse-brain JE N vaccine produced by the NIPM in Taiwan [Hsu, 1969] was routinely used in local health centers until 1988. Since the lyophilized-powdered form of B vaccine (BIKEN) began to be imported from Japan in 1989, two strains of JE vaccine were randomly distributed in Taiwan areas (Table I). The potency test for each preparation of the two vaccines had been performed according to a standard method before human usage [Takaku et al., 1971; Chow et al., 1986]. The immunogenic component of antigen in these JE vaccines had not been documented. However, the optical density values of the total protein (wave length is 280 nm) in stock solution of the two vaccines were 1.05 for the N vaccine and 0.96 for the B vaccine.

Immunization Schedule and Records

All children aged 15–26 months in Taiwan are required to receive JE immunization during the period March through June of every year. Two shots of one-half dose (0.5 ml for N vaccine or 0.25 ml for B vaccine) are given on days 0 and 14 for the primary immunization. A full dose (1 ml for N vaccine or 0.5 ml for B vaccine) is administered one year after the 2nd dose as a booster immunization.

Each child's name, age, sex, dates at each immunization, and strain of JE vaccine were obtained from the immunization records at local health centers. The health personnel in Tungkang and Lambay Islet did not document the vaccine strain used in the first 2 doses during vaccination in 1990 and 1991, but they did document "the Beijing-1 vaccine" during immunization in 1992. For children in Taan and Hsintien, only those who had valid computerized immunization records on dose and vaccine strain given in 1991 and 1992 were recruited (Table I).

Serum Samples

We chose four local health centers located in northern (Taan and Hsintien, metropolitan areas) and southern Taiwan (Tungkang, a rural area) as well as an isolated island (Lambay Islet with high emigrant population) during 1991–1992 for evaluation. All parents of children gave informed consent to participate in the study. One to two milliliter blood samples were collected from each subject at various time points. The blood samples were centrifuged at 3,000 rpm for 10 mins and the serum was stored at −20°C for measuring Nt Ab.

Humoral immune responses after the primary and booster immunizations were evaluated simultaneously by taking serum samples from children before and after vaccination. Samples were collected at either of two time periods relative to immunization. Samples from group I were taken immediately before the first dose of JE vaccine (n = 41) and 1–3 months after the second dose (n = 78, 27 pairs). Samples from group II were taken immediately before (n = 58) and 1–3 months after (n = 143, 44 pairs) the third dose (Table II). Nt Ab tests against the B and N strains were done simultaneously. Serum samples from Lambay Islet in 1992 were tested against N strain alone for evaluating their heterologous neutralizing response because there was an insufficient volume of sera (n = 29). If enough serum was left after being tested against the N strain, serum samples from Lambay Islet were tested for Nt Ab against CC-27 strain of JEV. Only children with complete immunization records and confirmed serologic data were included in the analysis (Table III).

Viruses

The N strain, provided from the NIPM in Taiwan, was further passaged twice in BHK cells and three times in porcine kidney cells (obtained from the CDC,

TABLE II. Subjects and Bleeding Schedules in JE Vaccine Evaluation Study in Taiwan Areas During 1991–1992*

	No. of children participated in various study areas				
	Northern Taiwan		Southern Taiwan		
	(Urban)		(Rural)	(Isolated islet)	
Bleeding schedule	Taan[a]	Hsintien[b]	Tungkang[b]	Lambay Islet[c]	Total
1. Before 1st dose	NS	15	22	4	41
After 2nd dose	15	14	16	33	78
No. of pairs	0	8	16	3	27
2. Before 3rd dose	NS	15	23	20	58
After 3rd dose	25	26	19	73	143
No. of pairs	0	11	19	14	44

*NS = no serum samples were taken.
[a]Serum samples were obtained in October 1992.
[b]Both the first serum samples right before the 1st and the 3rd dose of JE immunization were taken from children in March 1992; the 2nd serum samples were obtained at one month after the 2nd and the 3rd dose.
[c]30 of 33 serum samples (2 months after the 2nd dose) and 28 of 73 serum samples (2 months after the 3rd dose) were taken in July 1991; the others were taken in July 1992. Serum samples taken right before the 1st and the 3rd dose were in April 1992.

TABLE III. Number of Subjects With Complete Immunization Records and Number of Serum Samples Tested Against Four Strains of JEV by Plaque Reduction Neutralization Test in Four Different Bleeding Groups

Time to take blood (sample size)	No. with complete immunization record	No. of sera tested against four JEV strains[a]			
		Vaccine strain		Taiwan wild type JEV	
		Nakayama	Beijing-1	CC-27	CH-1392
A. Before 1st dose (n = 41)	—[b]	40	39	13	13
B. After 2nd dose (n = 78)	70	70	67	40	36
C. Before 3rd dose (n = 58)	55[c]	48	38	17	22
D. After 3rd dose (n = 143)	116[c]	116	69	83	59

[a]All children serum samples tested for Nt antibody against Beijing-1 strain were tested against Nakayama strain simultaneously but the serum samples from Lambay Islet in 1992 were tested against Nakayama strain alone because of insufficient volume. Serum samples with enough volume were employed to test for Nt Ab against CC-27 or CH-1392 strain.
[b]Pre-immunization.
[c]23/55 serum samples taken from Tungkang area and 46/116 serum samples from Tungkang and Lambay Islet did not document on the strain of JE vaccine used in the first 2 doses.

USA) in the Virology Laboratory at National Taiwan University Hospital. The B strain, which was obtained originally from the National Institute of Health (NIH) in Japan in 1982, had been passaged 51 times in sheep embryo kidney cell and 38 times in adult mouse brain before it was grown once in C6/36 cells in our lab. Two local strains of wild type JEV, CC-27 and CH-1392, provided from the NIPM, were isolated in C6/36 cells from *Culex* mosquitoes collected in southern (1983) and central (1990) Taiwan, respectively (Table IV). All the infected culture supernatants were aliquoted to 0.1 ml/vial, stored at −70°C, and titrated by plaque assay in BHK-21 clone 15 cells (provided from the CDC, USA). These four strains of JEV, exhibiting variation in plaque size, morphology, and viral yields (plaque forming unit, PFU) in the same assay system, were used to evaluate Nt Ab (Fig. 1).

Plaque Reduction Neutralization Test (PRNT)

BHK-21 clone 15 cells were used for plaque assay and plaque reduction neutralization test (PRNT) [Russell and Nisalak, 1967]. Briefly, cell suspensions (1.0×10^5 ml/well) were cultured in a 24-well polystyrene plate (Costar, Cambridge, MA, USA) for 48 hr to form a monolayer. The diluted test serum and virus mixture was added, in duplicate, to BHK-21 monolayer and then was incubated at 37°C for 1.5 hr [Morens et al., 1985]. The formalin-fixed infected and uninfected cells were stained with 1% crystal violet and plaque numbers were counted. A Nt titer $\geq$ 10 and a 70% plaque reduction, compared to the virus control, was defined as "Nt Ab seropositive." All Nt Ab titers higher than 320 were recorded as 320 to calculate the geometric mean titer (GMT), which is equal to the antilog of the mean of the logarithms of the Nt Ab titers [Bahn, 1985]. In

TABLE IV. Sources and Passages of JE Viruses Used in Evaluation of JE Vaccine by Plaque Reduction Neutralization Test*

JEV	Year	Location	Source	Passage
A. Vaccine strain				
Nakayama	1935	Japan, Nakayama	Human brain	Att-2 BHK-3 PS[a]
Beijing-1	1949	China, Beijing	Human brain	51 SEKC-38amb-C6/36
B. Wild strain				
CC-27	1983	Southern Taiwan	*Culex tritaeniorhynchus*	3 C6/36
CH-1392	1990	Central Taiwan	*Culex tritaeniorhynchus*	3 C6/36

*Att = attenuated strain; BHK = baby hamster kidney cell; PS = porcine kidney cell; SEKC = sheep embryo kidney cell; amb = adult mouse brain.
[a]The Nakayama vaccine strain, provided from NIPM in Taiwan, had been attenuated in Japan and was further passaged twice in BHK cells and 3 times in PS cells in the Virology Laboratory at National Taiwan University Hospital.

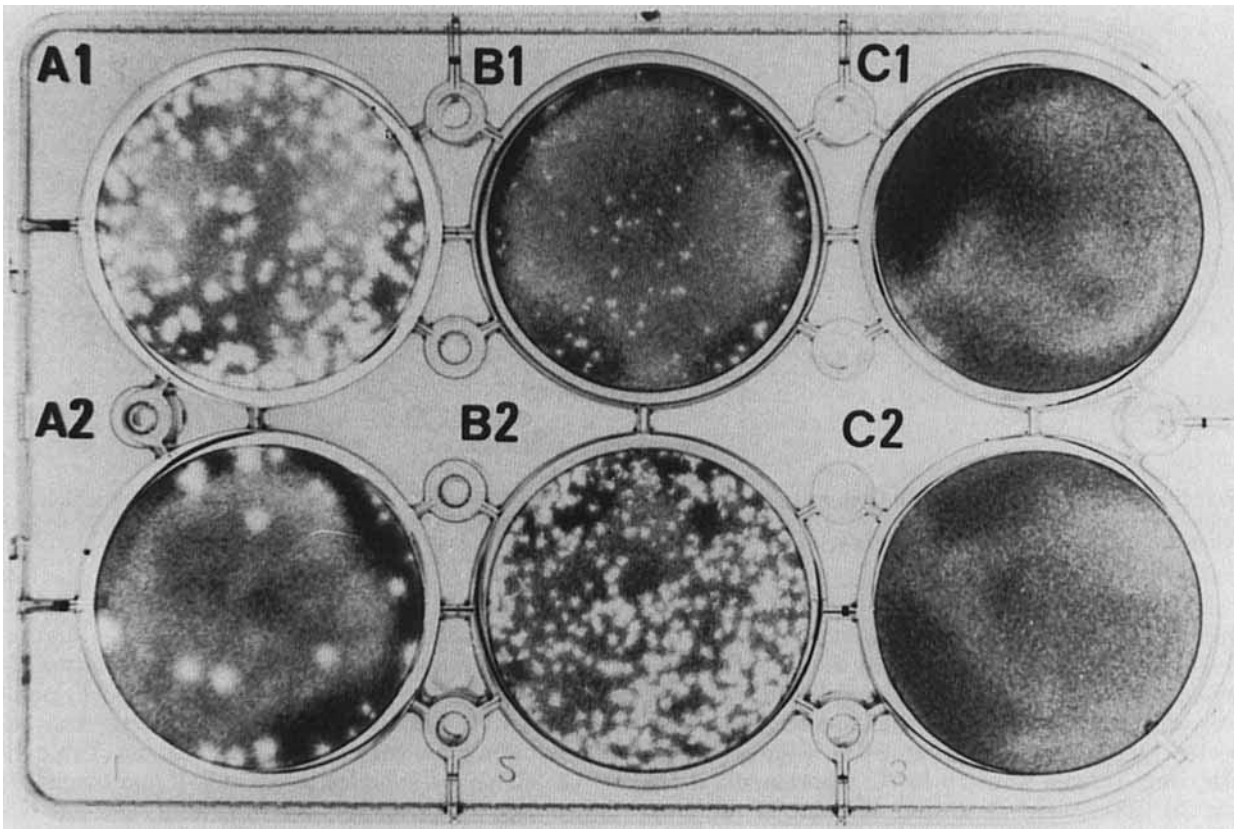

Fig. 1. Vaccine strains (Nakayama and Beijing-1) of JE virus had more homogeneous morphology and plaque size than Taiwan wild strain (CC-27 and CH-1392) in BHK-21, clone 15 cells. Nakayama strain had larger plaque size than Beijing-1 strain; CH-1392 strain had more opaque morphology and smaller size than CC-27 strain. **A:** Vaccine strain in a 10^{-6} dilution, 200 μl/well (**A1:** Beijing-1 strain with viral yield 8×10^8 PFU/ml; **A2:** Nakayama strain with viral yield 1×10^8 PFU/ml). **B:** Wild type strain in a 10^{-4} dilution, 200 μl/well (**B1:** CH-1392 strain with viral yield 3×10^6 PFU/ml; **B2:** CC-27 strain with viral yield 1×10^7 PFU/ml). **C:** Cell control (200 μl of diluent was added per well).

addition, the positive serum samples against the N (Nt titer $\geq$ 1,280) or against the B (Nt titer $\geq$ 2,560) strains, collected from mouse ascitic fluid and provided from the NIH in Japan, were employed as an internal positive control in each test. Three different titers of the subjects' serum samples (Nt titers $\leq$ 10, 80, and $\geq$ 320) collected from this study and two positive serum samples obtained from the NIH, Japan, were sent to the CDC, USA, for proficiency testing JE PRNT with a blinded code number. There was 100% agreement.

RESULTS

Pre-Immunization and Effects of JE Primary Immunization

The serum samples obtained before the first dose of JE vaccine were negative for Nt Ab against both of the vaccine strains [N and B, n = 40] or both of the Taiwan wild JEV strains (CC-27 and CH-1392, n = 13) in all subjects (n = 40). This indicates that none of the subjects had ever acquired JEV infection at the beginning of the study.

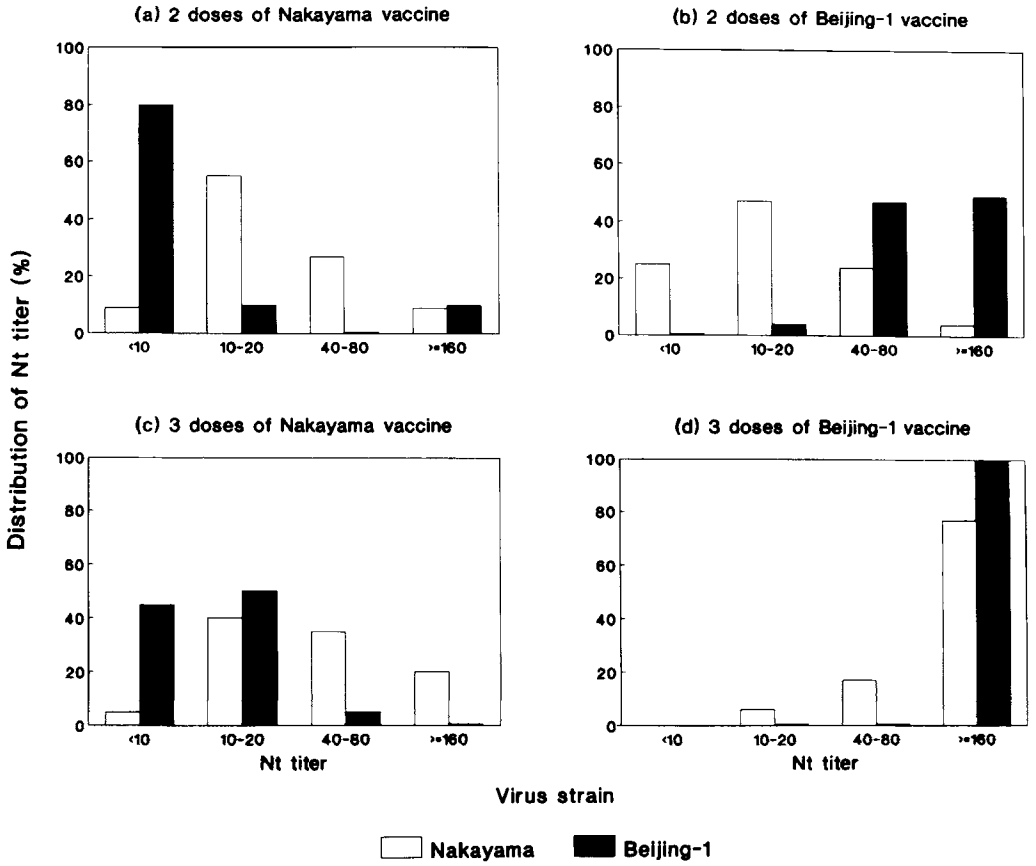

Fig. 2. Distribution of Nt titers against the homologous and the heterologous strains of JEV among Taiwan children whose serum samples were collected at 1–3 months after 2 doses of JE (**a**) Nakayama (serum 2N) and (**b**) Beijing-1 (serum 2B) vaccines; 1–3 months after 3 doses of JE (**c**) Nakayama and (**d**) Beijing-1 vaccines against the Nakayama (open bars) and the Beijing-1 virus (filled bars), respectively.

Children immunized with the N (serum 2N, n = 11) or B (serum 2B, n = 59) vaccine at 1–3 months after their second dose had 91% (10/11) and 100% (57/57) Nt Ab seropositivity rates against the homologous virus, respectively. Eighty-two percent (9/11) of the serum 2N Nt Ab titers ranged from 10 to 80, whereas all of the serum 2B titers were ≧ 20 and 49% (28/57) of them were higher than 160. However, the Nt Ab seropositivity rates to the heterologous JEV antigens, using serum 2N against the B strain and serum 2B against the N strain, were 20% (2/10) and 75% (44/59), respectively (Fig. 2; Table V). Nt titers against the heterologous virus strain were lower than Nt titers against the homologous virus strain for both serum 2N and serum 2B.

Effects of JE Booster Immunization

Of 58 subjects who had sufficient volume and complete immunization records, 48 serum samples were collected at one year after the second dose (e.g., immediately before the 3rd dose) and were tested for JEV Nt Ab. The Nt Ab seropositivity rates against the N and B strains in 25 children, living in Hsintien and immunized with 2 doses of B vaccine, were 88% (22/25, GMT = 16) and 100% (15/15, GMT = 79), respectively. The Nt Ab seropositivity rates against the N and B strains among the remaining 23 children living in Tungkang, for whom there was no documentation on the JEV strains, were 70% (16/23, GMT = 5) and 4% (1/23, GMT = 1), respectively, suggesting that they were immunized with 2 doses of N vaccine (Table V).

Of 143 serum samples taken at 1–3 months after the third dose of JE vaccine, 116 were analyzed. The Nt Ab seropositivity rates in the 46 Tungkang and Lambay Islet children, who were given an unknown vaccine strain in the primary immunization and bled in 1991 after the booster dose with B vaccine, were 100% (46/46, GMT = 85) against the N strain and 86% (25/29, GMT = 27) against the B strain (Table V). In contrast, all of the 50 children known to be immunized with 3 doses of B vaccine had a 100%, against either the N (n = 50) or B (n = 20) strain. The Nt titers in this group showed that 96% of them ranged from 40 to higher than 160 against the N virus (GMT = 182), and all of these children had Nt titers ≧ 320 against the B virus (Fig. 2d). The 20 children immunized with all 3 doses of N vaccine had 95% (19/20) and 55% (11/20) Nt Ab seropositivity rates against N and B virus, respectively, with Nt titers clustered around 10–80 (75%, GMT=33) against the N strain and 10–20 (50%, GMT = 4) against the B strain (Fig. 2c). These data suggest that

TABLE V. Neutralization Antibody Seropositivity Rates and Geometric Mean Titer (GMT) Against Nakayama, Beijing-1, CC-27 and CH-1392 Strains of JEV in Taiwan Children Sera Obtained at 1–3 Months After Second Dose, Right Before Third Dose, and at 1–3 Months After Third Dose, 1991–1992*

Time to take blood	No. of dose & strain received	Nt Ab against 4 JEV strains							
		Nakayama		Beijing-1		CC-27		CH-1392	
		% (95% CI) (n)	GMT	% (95% CI) (n)	GMT	% (95% CI) (n)	GMT	% (95% CI) (n)	GMT
A. 1–3 months after the 2nd dose	a. 2 Nakayama	91 (74–100) (10/11)	22	20 (10–45) (2/10)	2	20 (3–45) (2/10)	2	30 (2–58) (3/10)	22
	b. 2 Beijing-1	75 (64–86) (44/59)	7	100 (100) (57/57)	115	10 (0–21) (3/30)	1	35 (17–53) (9/26)	4
B. Right before the 3rd dose	a. No record[a] (may be 2 Nakayama)	70 (51–89) (16/23)	5	4 (0–12) (1/23)	1	0 (0) (0/8)	0	10 (0–29) (1/10)	1
	b. 2 Beijing-1	88 (75–100) (22/25)	16	100 (100) (15/15)	79	11 (0–31) (1/9)	1	67 (40–94) (8/12)	7
C. 1–3 months after the 3rd dose	a. (may be 2 Nakayama) + 1 Beijing-1[b]	100 (100) (46/46)	85	86 (73–99) (25/29)	27	84 (68–100) (16/19)	17	90 (77–100) (19/21)	46
	b. 3 Nakayama	95 (85–100) (19/20)	33	55 (33–77) (11/20)	4	0 (0) (0/20)	0	22 (3–41) (4/18)	2
	c. 3 Beijing-1	100 (100) (50/50)	182	100 (100) (20/20)	320	95 (89–100) (42/44)	123	100 (100) (20/20)	195

*GMT = $10^{\text{Sum of (log Nt titer)}/n}$; n = total No. of tested serum samples in the immunization group; 95% CI (confidence interval)

[a]No record was obtained on the primary immunization (2 doses).

[b]No record was obtained on the first 2 doses of JE vaccine strain but they did document "Beijing-1 vaccine" for the 3rd dose of booster immunization.

the children in Tungkang and Lambay Islet for whom no documentation of the vaccine used in the primary immunization was available, had been given with 2 doses of the N vaccine.

Of the 44 paired serum samples collected immediately before and 1–3 months after the 3rd vaccine dose, 30 pairs had both complete vaccination records and a sufficient serum volume remaining after the previous Nt Ab testing to be analyzed for the effectiveness of the booster immunization. Among the 18 paired serum samples collected from Tungkang children, the pre-booster serum samples against the N strain had Nt titers of 33% (n = 6) at < 10, 56% (n = 10) at 10–20 and 11% (n = 2) at 40–80. In contrast, the pre-booster serum samples against the B strain had Nt titer of 94% (n = 17) at < 10 and 6% (n = 1) at 10 (Fig. 3a). All 18 of these children were given a booster vaccine with one dose of B strain. The serum samples taken after the booster dose showed seroconversion and an increase in Nt titers against both the N [6%, (n = 1) at 10–20, 11% (n = 2) at 40–80 and 83% (n = 15) at ≥ 160] and the B strains [28%, (n = 5) at 10–20, 55% (n = 10) at 40–80 and 17% (n = 3) at ≥ 160] for all 18 children (Fig. 3a). Again, these data reconfirmed that Tungkang children had received the N vaccine in their primary immunization.

Of the remaining 12 paired serum samples, which were taken from Hsintien and Lambay Islet children in 1992, two pairs were seronegative against the N strain at one year after the first two vaccine doses with an unknown strain and both showed seroconversion after the 3rd dose of B strain when we tested against the same virus. Serum pairs from the other 10 children were Nt seropositive after the pre-booster vaccine doses, Nt titers of 67% (n = 8) at 10–20 and 16% (n = 2) at 40–80 against the N strain. All these Nt titers against the N virus, in either the seroconverted or seropositive before the booster dose (n =12), increased to 25% (n = 3) at 10–20 and 75% (n = 9) at ≥ 160 after the booster immunization. In addition, these 12 children were positive for Nt Ab against the B virus in the pre-booster serum samples [Nt titers were 33% at 10–20 (n = 4), 40–80 (n = 4), and ≥ 160 (n = 4), respectively], and showed a significant increase in Nt titers against the B virus after the booster immunization [8% (n = 1) at 10–20 and 92% (n = 11) at ≥ 160, $P = 0.007$, Wilcoxon rank sum test] (Fig. 3b).

Neutralization Antibody Responses Against Two Taiwan Wild Type JEV in Serum Samples From Children Immunized With JE Vaccine

The Nt Ab responses and GMTs against wild type JEV (CC-27 and CH-1392) varied in children receiving the different immunization protocols. Children immunized with 2 doses of either the N or B vaccine had similar neutralizing responses in both Nt seropositivity rate and GMTs against the CC-27 (CC) (N = 20% vs. B = 10%; GMT: 2 vs. 1) or against the CH-1392 (CH) JEV strains (N = 30% vs. B = 35%; GMT: 2 vs. 4). However, the Nt seropositivity rates and GMTs against

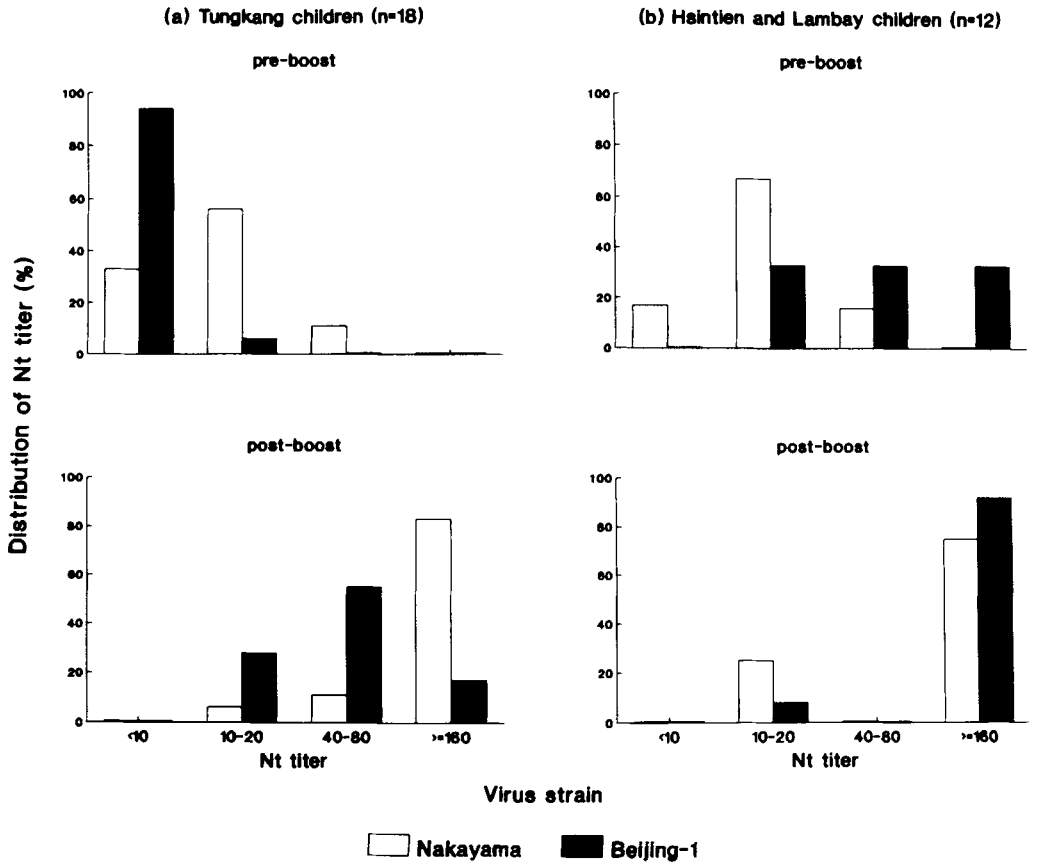

Fig. 3. Changing of Nt titers in paired children serum samples collected at right before and at 1–3 months after the booster immunization. **a:** 18 paired serum samples collected from Tungkang children who were boosted with one dose of Beijing-1 vaccine at one year after they had 2 doses of Nakayama vaccine for the primary immunization. **b:** 12 paired serum samples collected from Hsintien and Lambay Islet children who were immunized with 3 doses of Beijing-1 vaccine (i.e. boosted with the homologous strain of JEV).

both these two wild JEV strains were poor at one year after the primary immunization, either with N (CC vs. CH = 0% vs. 10%; GMT: 0 vs. 1) or B vaccine (CC vs. CH = 11% vs. 67%; GMT: 1 vs. 7) because of antibody waning. In addition, we found that 6 out of 15 Lambay Islet children who were Nt Ab negative after their 2nd dose became seropositive against the N strain immediately before the booster dose, probably because of a natural infection during 1991–1992. The serum samples of these 6 children also had increased Nt titers against the N and CC strains (GMT for N strain = 40, GMT for CC strain = 17) (data not shown).

Children who received a booster immunization had more cross-reactive neutralizing response with wild type JEV. Moreover, the children who were immunized with 3 doses of B vaccine had a greatest increase in Nt Ab seropositivity rates against the CC and CH strains (CC = 95% vs. CH = 100%; GMT: 123 vs. 195) compared to the children immunized with 3 doses of N vaccine (CC = 0% vs. CH = 22%; GMT: 0 vs. 2) and the children immunized with 2 doses of N and one dose of B vaccine (CC = 84% vs. CH = 90%; GMT: 17 vs. 46) (Table V).

DISCUSSION

This serologic evaluation of mouse-brain JE vaccine exhibited four significant findings: (1) B vaccine stimulated a better humoral immune response than Nakayama (N) vaccine in Nt Ab titers (GMT: 115 vs. 22) and Nt Ab seropositivity rate (100% vs. 91%) after the primary immunization; had a better booster effect (GMT: 320 vs. 33) and a greater capability to neutralize local Taiwan strains of JEV (GMT: 154 vs. 1). (2) Three doses of JE vaccine was the minimum requirement for generating higher and durable Nt Ab against heterologous JEV strains (Fig. 3). (3) There was an antigenic variation in local Taiwan JEV strains (CC and CH) because 3 doses of N or B vaccine elicited different Nt Ab seropositivity rates (N: CC = 0% vs. CH = 22%) and GMT (B: CC = 123 vs. CH = 195) against these two strains of JEV (Table V). (4) Original antigenic sin of JEV did exist. Two-dose immunization with either N or

B vaccine only conferred a narrow spectrum of neutralizing responses against two local Taiwan JEV strains (CC and CH) and Nt Ab waned very quickly during a one-year period. In addition, serum samples from children immunized with 3 doses of N vaccine neutralized these two Taiwan wild type JEVs only at the borderline level. Therefore, the strains of JEV used in vaccine and the booster immunization in the second year were important factors in maintaining protection in the community [Kanamitsu et al., 1970; Okuno et al., 1987; Poland et al., 1990]. Our results emphasize that local immunologic data are more important than vaccine efficacy in evaluation because the ecology of JE viral infection varies in different geographic areas over time and each encephalitis case has such a high probability of progression to a fatal outcome.

JEV strains vary in their neurovirulence, peripheral multiplication in mice, stability to heat and titers of hemagglutinin (A) [Huang, 1982]. Our neutralization results demonstrate that the antigenic variation among different strains of JEV appears not only at the level of inducing Nt Ab, but also in the capability to neutralize local strains of JEV. Although *in vitro* virus Nt Ab test may not measure protection *in vivo*, Kimoto et al. [1968] indicate that humoral immunity is very important in preventing JEV infections of neurons after a short period of viremia. We found that the higher the Nt Ab titers generated by the B vaccine, the more likely it is to neutralize the wild type JEV, implying more effective protection against human encephalitis by blocking at the early stages (attachment, penetration and uncoating) of the virus replication cycle. The higher neutralizing Ab in the B vaccine strain may be attributed to (1) the presentation of more cross-reactive epitopes, (2) the better T-helper cell ability to induce a B-cell response [Abbas et al., 1991], and (3) higher immunogenic effect after the interaction of adjuvant and B strain JEV [Ogata et al., 1970; Gregoriadis et al., 1989]. It means that the B-cell clone primed in the primary immunization has high affinity and specificity and that the humoral immunity generated after the booster immunization is synergistic and more durable. Therefore, while the protein content is standardized between N and B vaccine, the definition and detail structure of B- or T-cell epitopes that will give the best neutralization of different JEV strains remain to be elucidated [Colman et al., 1987]. Alternatively, if significant mutations in JEV immunogenicity emerge in some areas, it may lead to a large-scale encephalitis epidemic such as occurred in Korea in 1982 [Umenai et al., 1985]. Since viruses with changed antigenicity that may escape immune elimination, continued careful monitoring of viral antigen is always needed [Clements and Narayan, 1984; Wilson and Cox, 1990].

The major problem with a killed mouse-brain JE vaccine is the lack of long-term immunity (memory). Our results also demonstrated a quick decline in JEV Nt Ab titer by one year after the 2nd dose. Thus, the booster 3rd dose is very important for increasing "herd immunity" as well as in generating the capability to neutralize heterologous JEV strains (Table V). It is unclear why the JE vaccine is not able to elicit more durable immunologic memory after the 2nd dose as, like the Salk polio vaccine does [Zanetti et al., 1987]. The memory problem may be with the T or B cell only, or with both B- and T-memory cells, or with the regulation of B-cell activity by T cells. Whether the B-cell memory for JE vaccine antigens is due to a lower binding affinity for low dose antigen is unknown, but a higher JE viral antigen doses enhanced immunogenicity [Kanamitsu et al., 1970]. It is also very likely the T-suppressor cells induce low avidity memory B cells that do not mature efficiently [Okumura et al., 1976]. Alternatively, the lack of adhesion or activation molecules on memory T cells or without JE viral cryptic epitopes presented to T cells may lead to less durable JEV Nt Ab [Mackay, 1991; Gammon et al., 1991]. Furthermore, our unpublished data suggested that the currently used JE N vaccine also stimulated a lower titer of hemagglutination inhibition Ab [its seropositivity rates against the N strain were 17% (2/12) after 2-dose immunization and 20% (5/25) after 3-dose immunization] and resulted in a shorter duration (8 months) of Nt Ab even after the 4th dose of JE vaccine. The fact that in Japan the number of JE cases peaks in the over 40 age group (Igarashi at Nagasaki University, personal communication), and that 13% of reported JE cases during 1986–1991 in Taiwan had been immunized, indicated the urgent need to improve memory response of JE vaccine.

Because children who were immunized with the first two doses of N vaccine and boosted with B strain generated a higher seropositivity rate and greater levels of Nt Ab against the first encountered strain, the "original antigenic sin" which is demonstrated in other viruses also exists in JEV [Francis, 1953, 1955; Kanamitsu et al., 1970; Halstead et al., 1983]. In other words, the JEV strain used for the primary immunization provides higher immunogenicity and an epitope that has been conserved in many different JEV strains will give rise to even better immune response during repeated exposures through life. Since RNA fingerprint analysis and sequence data have documented different strains of JEV in China, India, and Sri Lanka, careful evaluation of the most appropriate strain for use in vaccine preparation must also rely on an understanding of JEV molecular epidemiology in the area [Banerjee and Ranadive, 1989; Chen et al., 1990].

Immunization is the most effective prevention measure against JE in Taiwan and elsewhere [Hoke et al., 1988; Poland et al., 1990]. Several investigators have developed a new generation of JE vaccines to express the relevant immunogens, particularly in envelope glycoprotein (E), a membrane protein (M), and its glycosylated precursor (PrM) [Haishi et al., 1989; Yasuda et al., 1990; Konishi et al., 1992]. Optimal vaccine design of JE requires: (1) an understanding of local viral strain genetic heterogeneity and immunotyping of the viral

infection, (2) identifying viral proteins containing peptides able to form antigenic complexes with prevalent class I and class II major histocompatibility complex alleles in the population for generating a higher level and wider spectrum of Nt Ab and cell-mediated immunity, and (3) elicitating the co-stimulatory activity of antigen presenting cells or adhesion molecules for a better memory response. In addition, we recommend developing a diagnostic test to differentiate natural infection from immunization for better evaluating vaccine efficacy.

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REFERENCES

Abbas AK, Lichtman AH, Pober JS (1991): B cell activation and antibody production. In Wonsiewicz MJ (ed): "Cellular and Molecular Immunology." W.B. Saunders Company, pp 187–203.

Bahn AK (1985): Description of a quantitative sample: comparison of a sample mean with an expected mean. In "Basic Medical Statistics." pp 127.

Banerjee K, Ranadive SN (1989): Oligonucleotide fingerprint analysis of Japanses encephalitis virus strains of different geographical origin. Indian Journal of Medical Research 89:201–216.

Calisher CH, Karabatsos N, Dalrymple J, Shope RE (1989): Antigenic relationship between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. Journal of General Virology 70:37–43.

Chen WR, Tesh RB, Rico-Hesse R (1990): Genetic variation of Japanese encephalitis virus in nature. Journal of General Virology 71:2915–2922.

Chow FY, Chang SN, Lu CH (1986): Comparison of plaque reduction and active mouse protection test for estimating the potency of Japanese encephalitis vaccine. Chinese Journal of Microbiology and Immunology 19(3):21–26.

Clements JE, Narayan O (1984): Immune selection of virus variants. In Notkins AL, Oldstone BA (eds): "Concepts in Viral Pathogenesis." New York: Springer-Verlag, pp 152–157.

Colman PM, Air GM, Webster RG, Varghese JN, Baker AT, Lentz MR, Tulloch PA, Laver WG (1987): How antibodies recognize virus proteins. Immunology Today 8(11):323–326.

Francis T (1953): Influenza: the newe acquayantance. Annals of Internal Medicine 39(2):203–221.

Francis T (1955): The current status of the control of influenza. Annals of Internal Medicine 43:534–538.

Gammon G, Sercarz EE, Benichou G (1991). The dominant self and the cryptic self: shaping the autoreactive T-cell repertoire. Immunology Today 12(6):193–195.

Gregoriadis G, Allison AC, Poste G (eds) (1989): "Immunologic Adjutants and Vaccines." Plenum Press.

Haishi S, Imai H, Hirai K, Igarashi A, Kato S (1989): Expression of envelope glycoprotein (E) of Japanese encephalitis virus by recombinant vaccinia virus. Acta Virologica (Praha) 33:497–503.

Halstead SB, Rojanasuphot S, Sangkawibha N (1983): Original antigenic sin in dengue. American Journal of Tropical Medicine and Hygiene 32(1):154–156.

Hoke CH, Nisalak A, Sangawhipa N, Jatanasen S, Laorakapongse T, Innis BL, Kotchasenee SO, Gingrich JB, Latendresse J, Fukai K, Burke DS (1988): Protection against Japanese encephalitis by inactivated vaccine. New England Journal of Medicine 319(10):608–614.

Hsu ST (1969): Production of Japanese encephalitis vaccine in Taiwan. Japan Journal of Tropical Medicine 10:214–217.

Hsu TC, Chow LP, Wei HY, Chen CL, Hsu ST, Huang CT, Kitaoka M, Sunaga H (1971): A completed field trial for an evaluation of the effectiveness of mouse-brain Japanese encephalitis vaccine. In Hammon WMcD, Kitaoka M, Downn WG (eds): "Immunization for Japanese Encephalitis." Baltimore: Williams & Wilkins, pp 258–265.

Huang CH (1982): Studies of Japanese encephalitis in China. Advances in Virus Research 27:71–101.

Kanamitsu M, Ashimoto N, Urasawa S, Katsurada M, Kimura H (1970): A field trial with an improved Japanese encephalitis vaccine in a nonendemic area of the disease. Biken Journal 13:313–328.

Kimoto T, Yamada T, Ueba N, Kunita N, Kawai A, Yamagami S, Nakajima K, Akao M, Sugiyama S (1968): Laboratory diagnosis of Japanese encephalitis comparison of the fluorescent antibody technique with virus isolation and serologic tests. Biken Journal 11: 157–168.

Kitano T, Yabe S, Kobayashi M, Oya A, Ogata T (1986): Immunogenicity of JE Nakayama and Beijing-1 vaccines. JE & HFRS Bulletin 1:37–41.

Kobayashi Y, Hasegawa H, Oyama T, Tamai T, Kusaba T (1984): Antigenic analysis of Japanese encephalitis virus by using monoclonal antibodies. Infection and Immunity 44(1):117–123.

Konishi E, Pincus S, Paoletti E, Shope RE, Burrage T, Mason PW (1992): Mice immunized with a subviral particle containing the Japanese encephalitis virus PrM/M and E proteins are protected from lethal JEV infection. Virology 188:714–720.

Mackay CR (1991): T cell memory: the connection between function, phenotype and migration pathways. Immunology Today 12(6): 189–192.

Monath TP (1988): Japanese encephalitis A—plague of the orient. New England Journal of Medicine 319(10):641–643.

Morens DM, Halstead SB, Pepik PM, Putvatana R, Raybourne N (1985): Simplified plaque reduction neutralization assay for dengue viruses by semimicro methods in BHK-21 cells: comparison of the BHK suspension test with standard plaque reduction neutralization. Journal of Clinical Microbiology 22(2):250–254.

National Quarantine Service (1992): Statistics on Japanese encephalitis control in Taiwan Areas pp 4.

Ogata M, Nagao Y, Jitsunari F, Kikui R, Kitamura N (1970): Vaccination with complete adjuvant-added inactivated virus vaccine of Japanese encephalitis to swine for preventing viremia (with specific reference to the effects of vaccination on viremia; epidemiological study on Japanese encephalitis 35). Acta Medica Okayama 24:579–587.

Okumura K, Metzler CM, Tsu TT, Herzenberg LA, Herzenberg LA (1976): Two stages of B-cell memory development with different T-cell requirements. Journal of Experimental Medicine 144:345–357.

Okuno T, Okada T, Suzuki M, Kobayashi M, Oya A (1968): Immunotyping of different strain on Japanese encephalitis virus by anti-

body absorption, hemagglutination-inhibition and complement-fixation tests. Bulletin of the World Health Organization 38:547–563.

Okuno T, Tseng PT, Hsu ST, Huang CT, Kuo CC, Lin CC (1975): Japanese encephalitis surveillance in China (province of Taiwan) during 1968–1971. I. Geographical and seasonal features of case outbreaks. Japan Journal of Medical Science and Biology 28:235–253.

Okuno Y, Okamoto Y, Yamada A, Baba K, Yabuuchi H (1987): Effect of current Japanese encephalitis vaccine on different strains of Japanese encephalitis virus. Vaccine 5:128–132.

Oya A (1988): Japanese encephalitis vaccine. Acta Pediatrica Japonica 30(2):175–184.

Poland JD, Cropp CB, Craven RB, Monath TP (1990): Evaluation of the potency and safety of inactivated Japanese encephalitis vaccine in US inhabitants. Journal of Infectious Disease 161:878–882.

Russell PK, Nisalak A (1967): Dengue virus identification by the plaque reduction neutralization test. Journal of Immunology 99(2): 291–296.

Takaku K, Yamashita T, Yoshida I, Osanai T, Goda H, Akashi M, Sugahara Y, Kagawa H, Amano T, Kunita N, Inoue K, Toyoshima K (1971): Vaccine potency testing methods and their significance. In Hammon WMcD, Kitaoka M, Downn WG (eds): "Immunization for Japanese Encephalitis." Baltimore: Williams & Wilkins, pp 258–265.

Umenai T, Krzysko R, Bektimirov TA, Assaad F (1985): Japanese encephalitis: current world status. Bulletin World Health Organization 63(4):625–631.

Vaughn DW, Hoke CH (1992): The epidemiology of Japanese encephalitis: prospects for prevention. Epidemiologic Review 14:197–221.

Wilson IA, Cox NJ (1990): Structural basis of immune recognition of Influenza virus hemagglutinin. Annual Review of Immunology 8:737–771.

Yasuda A, Kimura-Kuroda J, Ogimoto M, Miyamoto M, Sato T, Takamura C, Kurata T, Kojima A, Yasui K (1990): Induction of protective immunity in animals vaccinated with recombinant vaccinia viruses that express encephalitis virus. Journal of Virology 64: 2788–2795.

Zanetti M, Sercarz E, Salk J (1987): The immunology of new generation vaccines. Immunology Today 8(1):18–25.