Immunogenicity of Japanese Encephalitis Virus Envelope Glycoprotein E Prepared by Four Different Methods

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Abstract: Envelope glycoprotein (E) of Japanese encephalitis (JE) virus was prepared from purified virion by Triton X-100 (TX-100) treatment (E-TX), polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) after reduction (E-2ME-SDS) or without reduction (E-IAA-SDS), and high performance liquid chromatography after denaturation with guanidine hydrochloride (E-HPLC). E-2ME-SDS, E-IAA-SDS and particularly E-HPLC were less immunogenic than E-TX, complete virion or formalin-inactivated JE vaccine, which were almost similarly immunogenic to produce anti-JE ELISA and neutralizing (N) antibodies. E-HPLC produced low but definite N antibodies with highest N/ELISA ratio among all immunogens tested. The result indicated that most of the JE virus E protein N and ELISA epitopes were conformational, but some N epitope(s) were resistant to denaturation and could be preserved in E-HPLC better than the ELISA epitopes.

Key words; Japanese encephalitis virus, Envelope glycoprotein (E), Immunogenicity

INTRODUCTION

Japanese encephalitis (JE) has been an acute viral disease of public health impact in Asian countries (Miles, 1960; Umenai *et al.*, 1985). JE virus, a member of mosquito-borne flaviviruses, possesses single-stranded RNA genome of approximately 11Kb (Westaway *et al.*, 1985). Its spherical enveloped virion of 45−50 nm diameter contains 3 structural proteins: core protein (C), membrane protein (M), and envelope glycoprotein (E) (Shapiro *et al.*, 1971; Kitano *et al.*, 1974; Takegami *et al.*, 1982), similar to other flaviviruses (Stollar, 1969). Among multiple E protein epitopes, neutralizing (N) epitopes were considered to be most essential for the protection of JE (Takegami *et al.*, 1982; Kimura-Kuroda and Yasui, 1983, 1986, 1989). We have been studying immunogenicity of JE virus E protein with the objective to develop the second generation vaccine for the control of JE in presently epidemic areas. Our previous report showed that most of the E protein epitopes were

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conformational depending on the disulfide bonds which could be destroyed by reduction (Srivastava *et al.*, 1987).

In this paper we describe immunogenicity of JE virus E protein prepared from purified virion by 4 different methods, in comparison with purified virion and conventional inactivated JE vaccine.

Materials and Methods

Cells: *Aedes albopictus* clone C6/36 cells (Igarashi, 1978) and BHK21 cells were grown at 28℃ and at 37℃, respectively, using the same Eagle's growth medium (GM) in Earle's saline supplemented with 10% heat-inactivated fetal calf serum (FCS) and 0.2 mM each 7 nonessential amino acids (Eagle, 1959).

Virus: Origin of JE virus strains, wild JaOArS982 and classical Nakayama, was described by Hori *et al.* (1986). Purified virion of both strains were prepared from infected C6/36 cell culture fluid by polyethylene glycol precipitation and ultracentrifugation as described before (Srivastava *et al.*, 1987). Formalin-inactivated and purified JE vaccine concentrate (Nakayama strain) was generously supplied by the Research Foundation for Microbial Diseases of Osaka University, Japan.

Preparation of E protein: Four methods were used to prepare E protein from purified JE virion JaOArS982 strain:

(1) TX-100 treatment was performed as described by Heinz *et al.* (1984) and Srivastava *et al.* (1990). Purified virion (500 μg/ml) was disrupted by TX-100 using detergent: protein ratio of 10:1 at room temperature for 10 minutes, and centrifuged into detergent-free 15−50% sucrose gradient in an SW 50.1 rotor of a Beckman Model L8M ultracentrifuge at 40,000 rpm for 20 hours at 20℃. Fractions were examined by ELISA and SDS-PAGE, and those containing only E protein at high concentration were pooled as E-TX.

(2) Specimen of 20−25 μg protein was disrupted in a sample buffer containing 5% 2-mercaptoethanol (2ME), 1% SDS, 10% sucrose and 125 mM Tris-HCl, pH 6.8, at 100℃ for 1 minute and separated by slab gel SDS-PAGE (Studier, 1973) with discontinous buffer system (Laemmli, 1970) and 10% gel of 1 mm thickness (acrylamide to bisacrylamide ratio of 30:0.8). Protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue R250 in 10% acetic acid and 30% methanol followed by destanining with 10% acetic acid and 30% methanol by diffusion. The band corresponding to the molecular weight of E protein (54Kd) was taken as E-2ME-SDS.

(3) Similar specimen was disrupted in a sample buffer as above but containing 0.1 M iodoacetamide instead of 2ME (Durbin and Stollar, 1984), and speparated as above to prepare E-IAA-SDS.

(4) Specimen of 800 μg/ml was dissolved in 6 M guanidine hydrochloride and injected into a reversed-phase C-18 column (0.9×300mm) of the HPLC system (Waters Model 510), which consisted of 2 pressure pumps, automated gradient controller, Model 481 Lambda-Max LC spectrophotometer, Model 741 data module, and Advantec fraction collector

Model SF-139. The specimen was eluted by a linear gradient of acetonitril in 0.05% trifluoroacetic acid into fractions of 0.5ml volume, which were lyophilized and dissolved in 0.1% SDS to analyze by the SDS-PAGE. Fractions containing E protein were pooled as E−HPLCC.

Mouse immunization: Immunogens were emulsified with Freund's complete adjuvant for the first injection or incomplete adjuvant for subsequent injections, and inoculated to BLAB/c mice for a total 4 intraperitoneal injections with 1 week interval and 13−25 μg/0.25 ml/dose/mouse. The mice were individually bled 1 week after the last injection and sera were separated for antibody assay.

ELISA: Indirect micro ELISA of Voller *et al.* (1976) was followed with some modifications. The 96-well microplate was coated with 2−3 μg/ml purified JE virion (either JaOArS982 or Nakayama strain), or formalin-inactivated and purified Nakayama strain JE vaccine at 1:50 dilution in coating buffer (100 μl/well) at 4℃ overnight. The wells were emptied and washed with PBS-T (phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 and 0.01% NaN_3) 3 times 3 minutes each. The wells were reacted at 37℃ for 1 hour with 100 μl of test sera at 1:100 or 1:1,000 dilution along with serially diluted standard anti-JE polyclonal mouse serum. The wells were emptied and washed as above, and reacted at 37℃ for 1 hour with 100 μl/well of hoseradish peroxidase (HRPO)-conjugated anti-mouse IgG goat IgG (Cappel, USA) at 1:1,000 dilution in PBS-T. The wells were emptied and washed as above, and HRPO reaction was carried out at room temperature for 1 hour in the dark with substrate solution of 0.5 mg/ml *o*-phenylenediamine dihydrocholoride (OPD) and 0.02% H_2O_2 in 50 mM citrate-phosphate buffer, pH 5.0. The reaction was stopped by adding 75 μl/well 4 N H_2SO_4 and optical density (OD) at 490 nm was recorded by micro ELISA autoreader with reference wavelength of 630 nm. Titers of test specimens were calculated by comparing their OD with those by serially diluted standard positive serum (Igarashi *et al.*, 1981; Morita *et al.*, 1982). Standard anti-JE mouse serum was prepared by repeated intraperitoneal inoculation of purified JE virus grown in suckling mouse brains.

N test: The procedure of Hashimoto *et al.* (1971) was modified to use diluent of 5% FCS in Eagle's medium and 24-well instead of 96-well plates. JE virus (JaOArS982 or Nakayama strain)-infected C6/36 cell culture fluid was diluted to approximately 200 PFU/0.1 ml and was mixed with an equal volume of test sera serially diluted in 10-fold steps. The mixtures were incubated at 37℃ for 1 hour, and inoculated to BHK21 cells on 24 well plates (0.1 ml/well) after removing GM. adsorption was carried out at 37℃ for 1 hour, and cells were covered by 1ml/well of 1.5% methylcellulose and 1% FCS in Eagle's medium. After 5 days incubation at 37℃ in humidified 5% CO_2-atmosphere, the overlay was removed by rinsing with PBS. The cells were fixed with cold methanol for 30 minutes and stained with 0.1% Trypan blue to reveal plaques. The N test was scored positive when plaque-reduction was more than 50% compared with the negative control of virus-diluent mixture. Reciprocal of the highest dilution of N-positive test sera was recorded as N-titer.

Chemicals: Acetonitrile (HPLC grade), trifluoroacitic acid (research grade), SDS (electrophoresis grade), acrylamide, bisacrylamide, and OPD were purchased from Wako Pure Chemicals Co., Japan. Guanidine hydrochloride (enzyme grade) was the product of Bethesda Research Laboratories, USA.

Statistical analysis; The method described by Snedecor (1952) was followed.

RESULTS

Table 1. showed anti-JE ELISA and N antibody titers of sera from individual mouse immunized with different immunogens. The data were summarized into Table 2 with geometrical mean titer (GMT) both in arithmetic and logarithmic scales and standard deviation (SD) in logarithmic scale. Statistical difference between GMT for the same serum measured by different antigens was shown in Table 3. All 8 mice immunized with purified virion (No. 1−8), 10 mice immunized with E-TX (No. 9−18), and 5 mice immunized wtih JE vaccine (No. 39−43) produced almost similar level of anti-JE ELISA titers. However, the titer was highest to the JaOArS982 virion, followed by the Nakayama virion and lowest by the Nakayama vaccine, although the difference between the latter two was not statistically significant for the JaOArS982 immunogen. This tendency was also observed for the immunogen of heterologous Nakayama vaccine, indicating higher reactogenicity of the JaOArS982 than the Nakayama antigen. Immunogenicty of the E-2ME-SDS, E-IAA-SDS, and E-HPLC appeared to be much less than the above-metioned immunogens (JaOArS982 virion, E-TX, and Nakayama vaccine) both by the ELISA and N test. However, these values could not directly be compared with each other, because the amount of each immunogen was not normalized. Different from the results observed by JaOArS982 virion and E-TX or Nakayama vaccine, the ELISA titers assayed by the JaOArS982 virion and the Nakayama virion were almost the same for E-2ME-SDS, E-IAA-SDS, and E-HPLC immunogens, indicating that the strain-specific ELISA epitope (s) could be destroyed by denaturation procedures.

In order to compare antibody titers obtained by different immunogens, the ratio of N/ELISA titer was calculated for each individual mouse serum as shown in Table 4. The data were summarized into Table 5 with GMT, both in arithmetic and logarithmic scales, and SD in logarthmic scale. The statistical significance of the difference between the GMTs was shown in Table 6 for each combination of immunogens. Except for the titer obtained by the JaOArS982 virion and Nakayama vaccine as assayed by the Nakayama strain, which showed unusually high value, the N/ELISA ratios obtained by E-TX, E-2ME-SDS, and E-IAA-SDS were almost similar to that by the JaOArS982 virion as assayed by the homologous virion. In contrast, the N/ELISA ratio was significantly higher by the E-HPLC than other immunogens (Tables 4 and 5). This result may indicate that nonneutralizing ELISA epitope (s) were selectively destroyed by guanidine hydrochloride denaturation, while denaturation-resistant N epitope (s) were relatively preserved.

Table 1. ELISA and N titer of individual mouse serum obtained by immunization with purified virion or E protein preparations of JaOArS982 strain or formalin-inactivated Nakayama strain vaccine of JE virus

Immunogen	Mouse No.	ELISA titer assayed by			N titer assayed by	
		JaOArS982 virion	Nakayama virion	Nakayama vaccine	JaOArS982	Nakayama
JaOArS982 virion	1	128,000	1,000	1,000	1,000	1,000
	2	125,000	1,000	1,000	1,000	1,000
	3	90,000	500	18,370	1,000	1,000
	4	66,000	500	21,490	100	1,000
	5	81,000	500	4,900	100	1,000
	6	87,000	500	4,560	100	1,000
	7	129,000	1,000	17,480	1,000	1,000
	8	65,000	4,320	500	1,000	1,000
JaOArS982 E-TX	9	67,000	500	2,900	1,000	100
	10	39,000	500	1,640	100	10
	11	72,000	500	10,780	1,000	100
	12	108,000	500	19,710	10	10
	13	100,000	500	6,240	100	10
	14	95,000	500	17,340	100	10
	15	106,000	500	2,540	100	10
	16	95,000	500	4,560	1,000	100
	17	66,000	500	6,870	100	<10
	18	106,000	500	3,200	10	<10
JaOArS982 E-2ME-SDS	19	1,780	2,320	353	<10	10
	20	1,560	500	203	10	10
	21	5,410	2,730	416	10	10
	22	1,370	1,260	152	10	<10
	23	500	500	112	<10	<10
	24	3,000	1,650	489	<10	10
	25	1,160	1,360	250	<10	<10
	26	2,500	1,690	259	<10	<10
JaOArS982 E-IAA-SDS	27	3,160	3,150	408	<10	<10
	28	7,320	5,100	431	<10	<10
	29	3,510	2,090	436	<10	<10
	30	2,030	1,320	112	<10	<10
	31	3,700	1,690	131	<10	<10
	32	5,990	3,730	498	<10	<10
	33	2,630	1,870	142	<10	<10
	34	2,190	2,060	288	<10	<10
JaOArS982 E-HPLC	35	171	218	50	100	100
	36	387	495	121	10	10
	37	749	787	419	10	10
	38	125	220	401	10	10
Nakayama viccine	39	192,300	500	6,870	1,000	100
	40	27,700	500	3,280	100	100
	41	67,300	500	8,080	1,000	1,000
	42	87,600	500	16,430	100	100
	43	45,900	500	6,620	100	100

Table 2. Geometrical mean ELISA and N titers with logarithm and standard deviation of mouse sera obtained by immunization with purified virion or E protein preparations of JaOArS982 strain or formalin-inactivated Nakayama strain vaccine of JE virus

Immunogen	ELISA assayed by			N assayed by	
	JaOArS982 virion	Nakayama virion	Nakayama vaccine	JaOArS982	Nakayama
JaOArS982 virion	31,405 (4,497±0.015)	849 (2.929±0.419)	4,083 (3.611±0.419)	421 (2.625±0.268)	1,000 (3.0±0.0)
JaOArS982 E−TX	81,846 (4.913±0.020)	500 (2.699±0.0)	5,546 (3.744±0.131)	126 (2.1±0.433)	17 (1.240±0.290)
JaOArS982 E−2ME−SDS	1,312 (3.188±0.175)	1,285 (3.109±0.077)	252 (2.401±0.048)	6.5 (0.812±0.024)	7.1 (0.850±0.026)
JaOArS982 E−IAA−SDS	3,532 (3.548±0.034)	2,366 (3.374±0.034)	241 (2.382±0.081)	5 (0.699±0.0)	5 (0.699±0.0)
JaOArS982 E−HPLC	279 (2.445±0.141)	370 (2.568±0.076)	168 (2.225±0.198)	18 (1.25±0.667)	18 (1.25±0.667)
Nakayama vaccine	67,920 (4.832±0.057)	500 (2.699±0.0)	7,228 (3.859±0.062)	251 (2.4±0.3)	104 (2.02±0.002)

Titer below 10 was shown as 5.

Table 3. Significance test on the difference between anti−JE ELISA and N antibody titers of mouse sera measured by purified virion or E protein preparations of JaOArS982 strain or formalin-inactivated Nakayama strain vaccine of JE virus

Immunogen	ELISA assayed by			N assayed by JaOArS982 vs Nakayama
	JaOArS982 virion vs		Nakayama virion vs Nakayama vaccine	
	Nakayama virion	Nakayama vaccine		
JaOArS982 virion	$p<0.001$	$p<0.001$	$0.05<p<0.1$	$0.05<p<0.1$
JaOArS982 E−TX	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.01$
JaOArS982 E−2ME−SDS	$0.8<p<0.9$	$p<0.001$	$p<0.001$	no difference
JaOArS982 E−IAA−SDS	$0.05<p<0.1$	$p<0.001$	$p<0.001$	no difference
JaOArS982 E−HPLC	$0.001<p<0.01$	$0.4<p<0.5$	$0.02<p<0.05$	no difference
Nakayama vaccine	$0.02<p<0.05$	$0.001<p<0.01$	$p<0.001$	$0.1<p<0.2$

Table 4. N/ELISA titer (and log) of individual mouse serum obtained by immunization with purified JE virion or E protein from JaOArS982 strain or formalin-inactivated Nakayama strain vaccine

Immunogen	Mouse No.	N/ELISA titer $\times 10^3$ and (log) assayed by	
		JaOArS982	Nakayama
JaOArS982 virion	1	7.8 (0.892)	1,000 (3.0)
	2	8.0 (0.903)	1,000 (3.0)
	3	11.1 (1.045)	2,000 (3.301)
	4	1.5 (0.176)	2,000 (3.301)
	5	1.2 (0.079)	2,000 (3.301)
	6	1.1 (0.041)	2,000 (3.301)
	7	7.8 (0.892)	1,000 (3.0)
	8	15.4 (1.188)	231 (2.365)
JaOArS982 E−TX	9	14.9 (1.173)	200 (2.301)
	10	2.6 (0.415)	20 (1.301)
	11	13.9 (1.143)	200 (2.301)
	12	0.1 (−1.046)	20 (1.301)
	13	1.0 (0.0)	20 (1.301)
	14	1.1 (0.041)	20 (1.301)
	15	0.9 (−0.027)	20 (1.301)
	16	10.5 (1.021)	200 (2.301)
	17	1.5 (0.176)	10 (1.0)
	18	0.094 (−1.027)	10 (1.0)
JaOArS982 E−2ME−SDS	19	2.8 (0.447)	4.3 (0.633)
	20	6.4 (0.806)	20 (1.301)
	21	1.8 (0.255)	3.7 (0.568)
	22	7.3 (0.863)	4.0 (0.602)
	23	10 (1.0)	10 (1.0)
	24	1.7 (0.230)	6.1 (0.785)
	25	4.3 (0.633)	3.7 (0.568)
	26	2.0 (0.301)	3.0 (0.477)
JaOArS982 E−IAA−SDS	27	1.6 (0.204)	1.6 (0.204)
	28	0.7 (−0.155)	1.0 (0.0)
	29	1.4 (0.146)	3.0 (0.477)
	30	2.5 (0.398)	3.8 (0.580)
	31	1.4 (0.146)	3.0 (0.477)
	32	0.8 (−0.097)	1.3 (0.144)
	33	1.9 (0.279)	2.7 (0.431)
	34	2.3 (0.362)	2.4 (0.380)
JaOArS982 E−HPLC	35	584.8 (2.928)	458.7 (2.662)
	36	25.8 (1.412)	20.2 (1.305)
	37	13.4 (1.127)	12.7 (1.104)
	38	80.0 (1.903)	45.5 (1.658)
Nakayama vaccine	39	5.2 (0.716)	200 (2.301)
	40	3.6 (0.556)	200 (2.301)
	41	14.9 (1.173)	2,000 (2.301)
	42	1.1 (0.041)	200 (2,301)
	43	2.2 (0.342)	200 (2.301)

Table 5. Geometrical mean N/ELISA titer with logarithm and standard deviation of mouse sera obtained by immunization with purified virion or E protein preparations of JaOArS982 or Nakayama strain of JE virus

Immunogen	N/ELISA titer $\times 10^3$ (log SD)	
	JaOArS982	Nakayama
JaOArS982 virion	4.48 (0.652±0.221)	1,178 (3.071±1.860)
JaOArS982 E−TX	1.538 (0.1869±0.632)	37.7 (1.541 ±0.290)
JaOArS982 E−2ME−SDS	3.689 (0.567 ±0.090)	5.518 (0.742 ±0.140)
JaOArS982 E−IAA−SDS	1.447 (0.160 ±0.040)	2.093 (0.321 ±0.039)
JaOArS982 E−HPLC	69.582 (1.842 ±0.626)	42.170 (1.682 ±0.479)
Nakayama vaccine	3.678 (0.566 ±0.106)	2,000 (3.301 ±0.0)

Table 6. Statistical significance of the N/ELISA titer difference by different immunogens of JE virus JaOArS982 virion or E protein preparation or Nakayama strain vaccine for the production of anti-JE N/ELISA antibody titers in mice

Immunogen to be compared	Assayed by	
	JaOArS982	Nakayama
JaOArS982 virion vs E-TX	$0.1<p<0.2$	different population
JaOArS982 virion vs E-2ME-SDS	$0.6<p<0.7$	different population
JaOArS982 virion vs E-IAA-SDS	different population	different population
JaOArS982 virion vs E-HPLC	$0.001<p<0.01$	$0.1<p<0.2$
JaOArS982 virion vs Nakayama vaccine	different population	different population
JaOArS982 E-TX vs E-2ME-SDS	different population	$0.001<p<0.01$
JaOArS982 E-TX vs E-IAA-SDS	different population	different population
JaOArS982 E-TX vs E-HPLC	$0.001<p<0.01$	$0.6<p<0.7$
JaOArS982 E-2ME-SDS vs E-IAA-SDS	$0.001<p<0.01$	$0.01<p<0.02$
JaOArS982 E-2ME-SDS vs E-HPLC	different population	$0.01<p<0.02$
JaOArS982 E-2ME-SDS vs Nakayama vaccine	$0.9<p$	different population
JaOArS982 E-IAA-SDS vs E-HPLC	different population	$0.01<p<0.02$
JaOArS982 E-IAA-SDS vs Nakayama vaccine	$0.01<p<0.02$	different population
JaOArS982 E-HPLC vs Nakayama vaccine	different population	different population

Discussion

Although most of the JE virus E protein epitopes were conformational (Srivastava *et al.*, 1987), one of the cyanogen bromide-cleaved 8Kd fragment of E protein was immunogenic to produce anti-JE N antibodies in mice, indicating the presence of denaturation-resistant N epitope(s) on this fragment which located from amino acid (AA) 375 to 456 near C-terminal of the E protein (Srivastava *et al.*, 1990). As shown in our previous and present studies, the E protein band on SDS-PAGE was not highly immunogenic to elicit anti-JE N antibodies, in contrast to the 8Kd fragment or the E-HPLC. These results may indicate that denaturation proccedures for SDS-PAGE and for HPLC could expose or preserve various epitopes on JE virus E protein in different ways, so that the 8Kd fragment or E-HPLC could elicit relatively high N antibodies compared with ELISA antibodies. The denaturation-resistant and possibly linear epitope(s) on JE-virus E protein could be a hopeful candidate(s) of the seccond generation JE vaccine, because of the stability against various purification procedures when the gene was expressed by the recombinant DNA technology. Mason *et al.* (1987, 1989) reported that the JE virus E protein fragment with minimum size of 95 amino acids (AA) from AA 303 to 396 expressed by recombinant *Escherichia coli* was reactive with anti−JE N monoclonal antibodies (MCA) and the presence of 2 cysteine residues were essential for this reactivity. However, their gene product was not immunogenic to elicit anti-JE N antibodies. The 8Kd fragment of our previous report corresponded to AA 375−496 without any cysteine residues, indicating that the epitope(s) did not depend on disulfide bonds. Similar immunogenicity of both E-2ME-SDS and E-IAA-SDS also supported this possibility. In the case of other flaviviruses, Wengler and Wengler (1989) reported negative data against sequential epitopes on the E protein of West Nile virus. While, Roehrig *et al.* (1989) showed that one of the synthetic peptides No. 17 (AA 356−376) of Murray Valley encephalitis virus E protein was reactive to N-MCA and immunogenic to elicit N antibodies. Winkler *et al.* (1987) reported that a 9Kd tryptic peptide fragment of tick-borne encephalitis virus E protein was immunoreactive with strong tendency of renaturation, but its localization on the E neither its immunogenicity was not shown.

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