321

IMMUNOGENICITY OF SLOWLY SEDIMENTING ANTIGEN OF JAPANESE ENCEPHALITIS VIRUS ENVELOPE GLYCOPROTEIN ISOLATED FROM INFECTED CULTURE FLUIDS

GOLAM MASUD MOHAMMAD SHAMEEM

Abstract: Slowly sedimenting antigen (SE) and the rapidly sedimenting antigen (RE), associated with envelope glycoprotein E were prepared from the concentrated infected culture fluids of Japanese encephalitis (JE) virus. Mice were immunized by each antigen before and after inactivation of virus infectivity by ultraviolet (UV) irradiation. The immunogenicity of the antigens determined by the plaque reduction neutralization test (PRNT), indirect ELISA and hemagglutination-inhibition (HI) test, indicated that SE was almost equally immunogenic as RE, and that the PRNT epitopes on both antigens were more immunogenic before UV-inactivation than after the inactivation.

INTRODUCTION

JE is an acute viral encephalitis and is a serious public health problem in many Asian countries (Umenai *et al.*, 1985). The etiologic agent, JE virus, belongs to the Flaviviridae family (Westaway *et al.*, 1985) comprising about 70 viruses (Calisher *et al.*, 1989), of which many are pathogens for humans and domestic animals (Monath, 1986). Current JE vaccine has been developed as formalin-inactivated and highly purified virion from infected mouse brains and has successfully been used for JE control in Japan, Republic of Korea, and some other countries (Hammon *et al.*, 1971; Huang, 1982; Oya, 1988; Igarashi, 1988). Besides rapidly sedimenting virus antigens associated with complete virion, slowly sedimenting hemagglutinin or soluble complement-fixing antigen (SCF) of flaviviruses have been described in the infected mouse brain homogenates or in culture fluids by sucrose gradient sedimentation (Igarashi *et al.*, 1963; Kitaoka and Nishimura, 1965; Smith *et al.*, 1970; Shameem *et al.*, 1989). However the immunogenicity of SE has not well been documented. In order to clarify the immunogenicity of SE and RE, we examined anti-JE titer of the sera from mice immunized with SE or RE before and after UV-inactivation.

MATERIALS AND METHODS

Cells and virus

    Aedes albopictus, clone C6/36, cells (Igarashi, 1978) were grown at 28°C and BHK21 cells

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Department of Virology, Institute of Tropical Medicine, Nagasaki University,
12-4 Sakamoto-machi, Nagasaki 852, Japan

at 37°C in Roux bottles with 750 cm^3 volume. The cell growth medium was Eagle's minimum essential medium supplemented with 0.2 mmol/l each of 7 nonessential amino acids (Eagle, 1959) and 10% heat-inactivated fetal calf serum (FCS). The origin of a wild strain JE virus, JaOArS982, was described by Hori et al. (1986), and seed virus was prepared in C6/36 cells at 28°C.

Preparation of slowly sedimenting and rapidly sedimenting antigens of JE virus

The SE and RE were isolated by sucrose gradient sedimentation of concentrated infected culture fluids as described in our previous communication (Shameem et al., 1989). In this experiment specimens were harvested 36 hr after infection for both cell lines, but incubated at 28°C for C6/36 and 37°C for BHK21 cells, respectively.

Inactivation of the infective virus by UV-irradiation

Immunogens (2.5 ml) were spread on a petri dish (60 mm) and exposed to 2 UV bulbs (20 W) at a distance of 42 cm for 5 minutes. After the exposure, infectivity of the virus was undetectable (Sachiko Matsuo, personal communication).

Immunization of mice

BALB / c mice were immunized by 4 intraperitoneal injections at 1 week interval (0.1 ml/mouse/dose), using either UV irradiated or unirradiated SE and/or RE fractions, which were mixed with an equal volume of Freund's complete adjuvant for the first injection or incomplete adjuvant for the subsequent injections. One week after the last injection, mice were individually bled and serum was separated for further test.

Plaque reduction neutralization test (PRNT or N)

This procedure was carried out as described by Hashimoto et al. (1971) with some modification. Serial two fold serum dilutions beginning from 1:10 were made using the diluent of 5% FCS in Eagle's medium and mixed with an equal volume of the seed virus diluted to yield 100-200 PFU/0.1 ml. For virus control the working dilution of the virus was mixed with an equal volume of the diluent. The mixtures were incubated for 90 min at 37°C in humidified atmosphere containing 5% CO_2. At the end of the incubation, the mixture was inoculated to the monolayer of BHK21 cells (0.1 ml/well) grown on 24-well polystyrene tissue culture plate. Adsorption was carried out at 37°C for 2 hr and the cells were covered with 1.25% methyl cellulose in the maintenance medium (cell growth medium from which serum concentration was reduced to 2%). Plates were incubated for 5 days in the same condition as above and cells were fixed with cold methanol, stained with 0.1% trypan blue in 0.9% NaCl at room temperature for 1 hr to reveal the plaques. The N was scored as positive when more than 50% plaque reduction was observed compared with the negative control of virus-diluent mixture. Reciprocal of the highest dilution of the test serum with positive plaque reduction was considered as N titer.

Sandwich ELISA

The modified procedure of Voller et al. (1976) was described in our previous communication (Shameem et al., 1989).

Indirect micro ELISA

This was carried out according to Voller *et al.* (1976) using purified JE antigen (100 µg/ml). ELISA titer of test specimens was calculated by comparing the OD with those by serially diluted standard positive serum as described before (Igarashi *et al.*, 1981; Morita *et al.*, 1982). Standard anti-JE mouse serum was prepared by repeated intraperitoneal inoculation of purified JE virus (Srivastava *et al.*, 1987).

Hemagglutination-inhibition (HI) test

The procedure was carried out according to Clarke and Casals (1985) using goose red blood cells in virus adjusting diluent (VAD) at pH 6.6.

Statistical analysis

Student's t test was performed according to the standard procedure.

RESULTS

Mouse immunogenicity of SE and RE before UV-inactivation

In the initial experiment, mice were immunized with SE or RE fractions of both C6/36 and BHK21 cells without UV-inactivation. The antibody titer of individual serum from immunized mice was shown in Table 1. When antibody titers raised by SE and RE were compared, SE immunogen from C6/36 cells produced less N and HI titers than RE from the same cells, and these differences were statistically significant (Table 3). While, both SE and

Table 1   Immunogenicity of SE and RE before UV-irradiation, as shown by titers in log for N, ELISA and HI

Serum No.	Immunogen	N Titer	N Mean	ELISA Titer	ELISA Mean	HI Titer	HI Mean
1 - 1	C6/36 SE	1.30	1.68±0.29 (GMT 48)	3.85	4.28±0.40 (GMT 19,054)	1.90	2.05±0.31 (GMT 112)
1 - 2		1.60		4.72		1.90	
1 - 3		1.90		4.04		1.90	
1 - 4		1.90		4.49		2.51	
2 - 1	C6/36 RE	2.20	2.20±0.25 (GMT 158)	4.16	4.29±0.24 (GMT 19,498)	2.56	2.66±0.30 (GMT 457)
2 - 2		2.51		4.65		2.51	
2 - 3		1.90		4.22		2.51	
2 - 4		2.20		4.14		3.11	
3 - 1	BHK21 SE	1.90	2.05±0.21 (GMT 112)	4.49	4.49±0.01 (GMT 30,902)	2.20	2.36±0.22 (GMT 229)
3 - 2		2.20		4.48		2.51	
4 - 1	BHK21 RE	2.20	2.08±0.16 (GMT 120)	4.06	4.22±0.38 (GMT 16,595)	2.81	2.69±0.27 (GMT 490)
4 - 2		2.20		4.48		2.51	
4 - 3		2.20		4.72		2.51	
4 - 4		1.90		3.89		3.11	
4 - 5		2.20		3.96		2.51	

Note: GMT = Geometric mean titer

Table 2　Immunogenicity of SE and RE before UV-irradiation, as shown by
titer ratio of ELISA/N and ELISA/HI

Serum No.	Immunogen	ELISA/N			ELISA/HI		
		Titer	Mean	GMT	Titer	Mean	GMT
1 - 1	C6/36　SE	2.55	2.60±0.40	398	1.95	2.22±0.41	166
1 - 2		3.12			2.82		
1 - 3		2.14			2.14		
1 - 4		2.59			1.98		
2 - 1	C6/36　RE	1.96	2.09±0.17	123	1.65	1.38±0.35	24
2 - 2		2.14			1.14		
2 - 3		2.32			1.71		
2 - 4		1.94			1.03		
3 - 1	BHK21　SE	2.59	2.44±0.22	272	2.29	2.13±0.23	134
3 - 2		2.28			1.97		
4 - 1	BHK21　RE	1.86	2.07±0.31	119	1.25	1.53±0.57	34
4 - 2		2.26			1.97		
4 - 3		2.52			2.21		
4 - 4		1.99			0.78		
4 - 5		1.76			1.45		

Table 3　Statistical analysis on N, ELISA and HI titer
and titer ratio differences raised by SE and
RE before UV-irradiation

Immunogens	log titer and titer ratio	P value
SE and RE of C6/36	N	$0.02 < P < 0.05$
	ELISA	>0.10
	HI	$0.02 < P < 0.05$
	ELISA/N	>0.10
	ELISA/HI	$0.02 < P < 0.05$
SE and RE of BHK21	N	>0.10
	ELISA	$0.02 < P < 0.05$
	HI	>0.10
	ELISA/N	>0.10
	ELISA/HI	>0.10

RE from C6/36 cells produced similar ELISA titers. In the case of immunogen from BHK21 cells, both SE and RE produced similar N and HI titers, while ELISA titer produced by RE was less than the titer by SE and the difference was statistically significant (Table 3). The antibody titers produced by immunogens from C6/36 and BHK21 cells were almost the same except that N titer by SE antigen from C6/36 cells was lower than the titer by other antigens (Table 1). Since immunogens of SE and RE from C6/36 cells and SE from BHK21 cells

possessed comparable ELISA titers as shown in Table 4, the results may indicate low immunogenicity of SE from C6/36 cells to induce N antibodies. Lower ELISA titer by RE from BHK21 cells may be explained by its slightly less ELISA titer (Table 4). The antibody titers were compared by their ratios (ELISA/N and ELISA/HI), and the results were shown in Table 2. The SE from C6/36 cells showed higher ELISA/HI ratio than RE from the same cells with statistically significant difference, while other ratios did not show significant difference between SE and RE. In this series of experiment a high mortality rate of mice around 40% was observed probably due to encephalitis caused by residual infective virus in the immunogens (data not shown). Therefore, it was conceivable that the antibody titers and their ratios in Tables 1 and 2 might reflect the effect of viral antigens produced in mice after virus replication, besides immunogenicity of inoculated specimens. Therefore, subsequent experiments were performed by immunogens which had been inactivated by UV-irradiation.

Mouse immunogenicity of SE and RE after UV-irradiation

Each immunogens of SE and RE from C6/36 and BHK21 cells were irradiated by UV as described in the Materials and Methods, and ELISA titers of these immunogens were shown in Table 4. The ELISA titer of SE from both C6/36 and BHK21 cells was reduced around 78%, while the titer reduction of RE was less (around 42-43%).

The antibody titers of individual mouse serum raised by UV-inactivated immunogens were shown in Table 5. Only the HI titer produced by RE from BHK21 cells was higher than the titer by SE from the same cell, and the difference was statistically significant (Table 7).

The titer ratio (ELISA/N and ELISA/HI) of the individual mouse serum was shown in Table 6. In this case, only the ELISA/HI produced by SE from C6/36 cells was higher than the titer by RE from the same cells, and the difference was statistically significant.

Comparison of the immunogenicity before and after UV-irradiation

The effect of UV-irradiation on the immunogen was evaluated by comparing the antibody titers (N, ELISA, HI) as well as their ratios (ELISA/N, ELISA/HI) raised by each immunogen before and after UV-irradiation. The statistically significant difference was observed only for the reduction in HI titer produced by RE from C6/36 cells and the increase in ELISA/N ratio produced by RE from BHK21 cells (Table 8). Other immunogenicities were apparently not affected by UV-irradiation.

Table 4　ELISA titer of immunogens before and after UV-irradiation

Immunogen		ELISA titer of immunogen		% Reduction in ELISA titer
		before UV	after UV	
C6/36	SE	232	50	78.45
C6/36	RE	206	116	43.69
BHK21	SE	229	50	78.15
BHK21	RE	186	107	42.48

Table 5   Immunogenicity of SE and RE after UV irradiation, as shown by
titers in log for N, ELISA and HI

Serum No.	Immunogen	N		ELISA		HI	
		Titer	Mean	Titer	Mean	Titer	Mean
1 - 1	C6/36 SE	2.20	1.54±0.54 (GMT 35)	4.58	4.42±0.20 (GMT 26,302)	1.90	1.96±0.13 (GMT 91)
1 - 2		1.00		4.58		1.90	
1 - 3		1.00		4.11		1.90	
1 - 4		1.90		4.48		2.20	
1 - 5		1.60		4.35		1.90	
2 - 1	C6/36 RE	2.20	1.98±0.29 (GMT 95)	4.29	4.40±0.17 (GMT 25,118)	2.51	2.28±0.29 (GMT 191)
2 - 2		2.20		4.23		1.90	
2 - 3		1.90		4.51		2.20	
2 - 4		1.60		4.58		2.51	
3 - 1	BHK21 SE	2.20	1.84±0.39 (GMT 69)	4.64	4.43±0.23 (GMT 26,915)	2.51	2.20±0.22 (GMT 158)
3 - 2		1.60		4.51		1.90	
3 - 3		1.30		4.61		2.20	
3 - 4		2.20		4.35		2.20	
3 - 5		1.90		4.08		2.20	
4 - 1	BHK21 RE	1.90	1.90±0.24 (GMT 79)	4.35	4.50±0.12 (GMT 31,622)	2.51	2.66±0.39 (GMT 457)
4 - 2		1.90		4.58		3.11	
4 - 3		2.20		4.44		2.20	
4 - 4		1.60		4.61		2.81	

DISCUSSION

From the results of our immunological studies it appears that the SE of JE virus is immunogenic in mice to induce N, ELISA and HI antibodies, and the immunogenicity of both antigens, SE and RE, were almost comparable. Although UV-irradiation totally inactivated infectivity and partially destroyed ELISA antigenicities in both SE and RE, they still retained their immunogenicities. The different UV-susceptibility of ELISA antigenicity between SE and RE from both C6/36 and BHK21 cells may be explained by different target size of the particle. The RE is the complete virion and possesses larger size and greater number of repetitive epitope units than SE, which is smaller size with less number of repetitive units. It is reasonable to imagine that RE with larger number of repetitive ELISA antigenic epitopes is more resistant to UV-irradiation than SE. Kimura-Kuroda and Yasui (1983) reported that at least five topographically distinct antigenic determinants including N, HI, and ELISA epitopes, were present on E protein and the epitopes of HI and N were separated from each other by monoclonal antibodies.

Recently, Mason et al. (1991) reported that the JE vaccinia virus recombinants possessing JE virus cDNA inserts produced particulate form of antigen containing M (membrane) and E proteins of JE virus. The antigen migrated in sucrose gradients slower than the complete virion, similar to the SE, and induce high level of anti-JE N antibodies and conferred protection against lethal JE virus infection.

Table 6 Immunogenicity of SE and RE after UV-irradiation, as shown by titer ratio of ELISA/N and ELISA/HI

Serum No.	Immunogen	ELISA/N			ELISA/HI		
		Titer	Mean	GMT	Titer	Mean	GMT
1 - 1	C6/36 SE	2.38	2.88±0.47	758	2.68	2.46±0.21	288
1 - 2		3.58			2.68		
1 - 3		3.11			2.21		
1 - 4		2.58			2.28		
1 - 5		2.75			2.45		
2 - 1	C6/36 RE	2.09	2.45±0.46	281	1.78	2.12±0.25	131
2 - 2		2.03			2.33		
2 - 3		2.61			2.31		
2 - 4		2.98			2.07		
3 - 1	BHK21 SE	2.44	2.60±0.50	398	2.13	2.23±0.28	170
3 - 2		2.91			2.61		
3 - 3		3.31			2.41		
3 - 4		2.15			2.15		
3 - 5		2.18			1.88		
4 - 1	BHK21 RE	2.45	2.59±0.32	389	1.84	1.84±0.31	69
4 - 2		2.68			1.47		
4 - 3		2.24			2.24		
4 - 4		3.01			1.80		

Table 7 Statistical analysis on N, ELISA and HI titer and titer ratio differences raised by SE and RE after UV-irradiation

Immunogens	log titer and titer ratio	P value
SE and RE of C6/36	N	>0.10
	ELISA	>0.10
	HI	>0.10
	ELISA/N	>0.10
	ELISA/HI	0.02< P <0.05
SE and RE of BHK21	N	>0.10
	ELISA	>0.10
	HI	0.02< P <0.05
	ELISA/N	>0.10
	ELISA/HI	>0.05

328

Table 8 Statistical analysis on the difference in antibody titers and titer ratios obtained by immunogens before and after UV-irradiation

Immunogens	P value				
	N	ELISA	HI	ELISA/N	ELISA/HI
SE of C6/36	>0.10	>0.10	>0.10	>0.10	>0.10
RE of C6/36	>0.10	>0.10	<0.001	>0.10	>0.10
SE of BHK21	>0.10	>0.10	>0.10	>0.10	>0.10
RE of BHK21	>0.10	>0.10	>0.10	$0.02 < P < 0.05$	>0.10

At present, inactivated JE vaccine has been manufactured from infected mouse brain homogenates, by purifying formaline-inactivated complete virion using primarily ultracentrifugation (Takaku *et al.*, 1968). Till now, little or no attention has been paid for the recovery of SE during JE vaccine preparation, and most of the SE would have been lost in the supernatant of ultracentrifugation. It would be worthwhile to consider the immunogenicity of SE in the crude vaccine preparation before ultracentrifugation in order to recover more immunogens.

Some flaviviruses have been shown to produce slowly sedimenting antigen along with rapidly sedimenting antigen or complete virion in the mouse brain homogenates or in tissue culture system (Igarashi *et al.*, 1963; Smith *et al.*, 1970; Shameem *et al.*, 1989). This information has permitted us to predict that some form of 'incomplete virion' is synthesized in the early period of replication of JE virus. In the case of dengue type 2 virus, another important member of flavivirus, Cardiff *et al.* (1971) argued that most of the incomplete virus or top component was associated with several unrelated phenomena, viz. defective interfering particles, adsorbing interfering particles, pleomorphic aggregates of coat structures, macromolecular capsid precursors and some naturally occurring particles which morphologically resemble infectious form except that they did not contain no core structure. Alternatively, SE may represent a protein synthesized during the course of viral replication which might be released into the medium, besides complete virion, sharing several biological properties possessed by envelope glycoprotein E.

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