Acta virol. **34** : 228 – 238, 1990

IMMUNOGENICITY OF PEPTIDES CLEAVED BY CYANOGEN BROMIDE FROM JAPANESE ENCEPHALITIS VIRUS ENVELOPE GLYCOPROTEIN E

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Received February 27, 1989

Summary. — Envelope glycoprotein (E) prepared from purified Japanese encephalitis (JE) virus was cleaved with cyanogen bromide (CNBr) followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Mice were immunized with 36 kD, 27 kD, and 8 kD bands from CNBr-cleaved and 54 kD band from control specimens. Neutralization test was positive in one out of two anti-54 kD, in none of the anti-36 kD, and all of anti-27 kD and anti-8 kD sera at 1 : 10 dilution. Geometrical mean ELISA titre was the highest for anti-54 kD followed by anti-36 kD, anti-27 kD, and anti-8 kD sera. Reactivity of these sera to CNBr-cleaved fragments in Western blotting indicated that the 8 kD fragment was a part of the 27 kD but was not included in the 36 kD fragment, while the 27 kD and 36 kD fragments shared an overlapping part. These fragments were located on the E protein by N-terminal amino acid sequencing of each fragment purified by reversed-phase high-performance liquid chromatography and by comparison with the nucleotide sequence of the E protein gene. The 36 kD fragment was located between the third and the ninth methionine and covered most of the N-terminal side of the E protein. In contrast, the 27 kD fragment was located between the fourth and the tenth methionine and included the 8 kD fragment which was situated between the ninth and the tenth methionine near to the C-terminus of the E protein. Denaturation-resistant neutralizing epitope(s) appeared to be present on the 8 kD fragment, but not on the 36 kD fragment.

Key words: CNBr fragments; immunogenicity; Japanese encephalitis virus glycoprotein E; N-terminal amino acid sequence

Introduction

Japanese encephalitis (JE) has been prevalent in several Asian countries; its control is a major objective in the public health (Miles, 1960; Umenai *et al.*, 1985). In Japan, formalin-inactivated and purified JE vaccines were

developed for human use (Hammons *et al.*, 1971) and a similar vaccine was also produced in the Republic of Korea to control the JE. China has developed its own inactivated vaccine from infected hamster kidney cell cultures (Huang, 1982). The control of JE in presently epidemic areas would be greatly facilitated by the development of inexpensive second generation JE vaccines which can be supplied in a large amount.

JE virus belongs to the family *Flaviviridae* with a single-stranded RNA genome of 42 S (Westaway *et al.*, 1985) and contains three structural proteins: M, C, and E (Kitano *et al.*, 1974; Shapiro *et al.*, 1979; Takegami *et al.*, 1982). Kimura-Kuroda and Yasui (1983, 1986) and Kobayashi *et al.* (1984) reported the epitope analysis of JE virus envelope glycoprotein (E) using monoclonal antibodies, and Heinz (1986) reviewed the epitope mapping of tick-borne encephalitis (TBE) and other flavivirus E proteins. Although detailed investigations have been carried out on cyanogen bromide (CNBr)-cleaved fragments from glycoproteins of influenza virus (Ward and Dopheide, 1979), adenovirus (Jornwall and Bahr-Lindstrom, 1981), rabies virus (Dietzschold *et al.*, 1982), and avian myeloblastosis virus (Johnson *et al.*, 1984), similar studies have not been published on flaviviruses except of TBE virus (Heinz *et al.*, 1984) and JE virus (Srivastava *et al.*, 1987).

As a step in developing the second generation of JE vaccine, we examined immunogenicities of three CNBr-cleaved fragments from JE virus E protein and determined their positions by N-terminal amino acid sequencing and comparison with the nucleotide sequence of the E protein gene (Sumiyoshi *et al.*, 1986).

Materials and Methods

Cells and virus purification. *Aedes albopictus* clone C6/36 cells (Igarashi, 1978) and BHK21 cells were grown at 28 °C and at 37 °C, respetively. Both cells were grown in Eagle's medium supplemented with 10% foetal calf serum (FCS) and 0.2 mmol/l each of nonessential amino acids (Eagle, 1959). The origin of JE virus JaOArS982 strain has been described (Hori *et al.*, 1986). The virus was grown in C6/36 cells, precipitated by polyethylene glycol and purified by sucrose density gradient centrifugation as described previously (Srivastava *et al.*, 1987).

Preparation of the E protein. The procedure described by Heinz *et al.* (1984) was followed. Purified virus (800 μg/ml) was disrupted by Triton X-100 at a detergent to protein ratio of 10 : 1 at room temperature for 10 min, and centrifuged into detergent-free 15—50 % sucrose gradient in an SW 50.1 rotor at 40,000 rev/min for 20 hr at 20 °C. Each fraction was examined ELISA, SDS-PAGE and Western blotting, and fractions containing only E protein at high concentration were pooled. E protein was precipitated by 50% trichloroacetic acid, washed twice by acetone and dissolved in 70% formic acid (Merck, F.R.G.).

CNBr-cleavage of E protein. Purified E protein (400—500 μg/ml) was cleaved with CNBr in 70% formic acid at CNBr to protein ratio of 2 : 1 at room temperature for 24 hr in the dark. Control specimen was run similarly without CNBr. Formic acid and residual CNBr were removed from the specimen by dilution with water and lyophilization.

SDS-PAGE. Slab gel method (Studier, 1973) with a discontinuous buffer system (Laemmli, 1970) was used with 15% gel (acrylamide to bisacrylamide ratio of 30 : 0.8) of 1.5 mm thickness. Before electrophoresis specimens containing 10—20 μg protein were solubilized under nonreducing conditions using 1% SDS in 0.1 mol/l iodoacetamide and 0.125 mol/l Tris-HCl, pH 6.8, at 100 °C for 1 min (Durbin and Stoller, 1984). The protein bands were visualized with 0.1% Coomassie Brilliant Blue R 250 in 10% acetic acid and 30% methanol followed by destaining with 10% acetic acid and 30% methanol. M_r of stained bands was estimated by a low M_r electrophoresis calibration kit (Pharmacia, Sweden). The bands corresponding to 36 kD, 27 kD, and 8 kD from

CNBr-treated as well as 54 kD from control E protein were cut out from the gel and used for immunization.

Immunization of mice. Each gel piece was emulsified with Freund's complete adjuvant for the first injection or with incomplete adjuvant for subsequent injections, and inoculated intraperitoneally to BALB/c mice for a total of 6 times at 1 week interval using 0.2 ml/dose. For each injection, the immunogen was newly prepared by SDS-PAGE from CNBr-cleaved or control E protein using 20 μg each. Seven mice were initially immunized for each band, but the number of surviving mice after the last injection was two for 54 kD, four for 36 kD, seven for 27 kD, and four for 8 kD band, respectively. Three days after the last injection, the mice were individually bled and serum was separated for further testing.

Neutralization (N) test. The procedure of Hashimoto *et al.* (1971) was modified using a diluent of 5% FCS in Eagle's medium. JE virus (infected C6/36 cell culture fluid) at an appropriate dilution was mixed with an equal volume of immune mouse sera at dilutions of 1 : 4, 1 : 10, 1 : 40, 1 : 100, 1 : 400, and 1 : 1000. The mixtures were incubated at 37 °C for 1 hr and inoculated to BHK21 cells grown on 24 well plates (0.1 ml/well) after removing cell growth medium. Adsorption was carried out at 37 °C for 1 hr and the cells were covered by 1 ml/well of 1.5 % methylcellulose and 1% FCS in Eagle's medium. After 5 days incubation at 37 °C in a humidified 5% CO_2 atmosphere, the overlay medium was removed by washing with PBS (phosphate buffered saline). The cells were fixed with cold methanol at −20 °C for 30 min and stained with 0.1% Trypan blue to reveal plaques. The N test was scored as positive when more than 50% of plaque-reduction was observed compared with the negative control of virus-diluent mixture. The reciprocal of the highest dilution of test sera which was N-positive was taken as the N-titre.

ELISA. Indirect micro ELISA (Voller *et al.*, 1976) was used to assay anti-viral antibody titres. The 96-well microplate was coated with purified virions (2—3 μg/ml, 100 μl/well) in a coating buffer (0.06 mol/l carbonate-bicarbonate buffer, pH 9.6) at 4 °C overnight. The optimal concentrations of virions to coat the plates were predetermined by checkerboad titration using standard positive and negative sera. The wells were emptied and washed with PBS-T (PBS containing 0.05% Tween 20 and 0.01% NaN_3) 3 times 3 min. each. The wells were reacted at 37 °C for 1 hr with 100 μl of serum (diluted 1 : 100 and 1 : 1000), along with serially diluted standard anti-JE polyclonal mouse serum. The wells were emptied and washed as above, and reacted at 37 °C for 1 hr with 100 μl/well of horseradish peroxidase (HRPO)-conjugated anti-mouse immunoglobulin goat IgG (Cappel, U.S.A.) at 1 : 1000 dilution in PBS-T. The wells were emptied and washed as above, and HRPO reaction was performed at room temperature for 1 hr in the dark with a substrate solution of 0.5 mg/ml of o-phenylene diamine and 0.02% of H_2O_2 in 0.05 mol/l citrate-phosphate buffer, pH 5.0. The reaction was stopped by adding 75 μl/well of 4 N H_2SO_4, and the optical density (OD) at 490 nm was recorded by a micro ELISA autoreader with reference wavelength at 630 nm. Titres of test specimens were calculated by comparing their OD values with those of serially diluted standard positive srum (Igarashi *et al.*, 1981; Morita *et al.*, 1982). Standard anti-positive mouse sera were prepared by repeated intraperitoneal inoculation of purified virions grown in suckling mouse brains.

Western blotting. CNBr-cleaved as well as control E proteins were run on SDS-PAGE and separated peptides were electrophoretically transferred to nitrocellulose membrane (Burnette, 1981; Naser and Miltenburger, 1983). The membrane was blocked with 3% casein and 0.01% NaN_3 in PBS at room temperature for 45 min, washed with PBS and cut into small strips. Each strip was reacted at 37 °C for 3 hr with anti-fragment serum diluted to 1 : 100 or anti-JE polyclonal mouse serum diluted to 1 : 1000 in PBS. The membrane strips were washed in PBS and reacted at 37 °C for 2 hr with HRPO-conjugated anti-mouse immunoglobulin goat IgG (Cappel, U.S.A.) diluted to 1 : 1000 in PBS. The membrane strips were washed with PBS, and the reactivity of peptides and antibodies was visualized by HRPO reaction with 0.03% 4-chloro-1-naphtol and 0.03% H_2O_2 in PBS at room temperature until definite colour was developed.

High-performance liquid chromatography (HPLC). The HPLC system (Waters Model 510, U.S.A.) 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. CNBr-cleaved and lyophilized JE virus E protein was dissolved in 6 mol/l guanidine hydrochloride (enzyme grade, Bethesda Research Laboratories, U.S.A.) in water, and then 1 ml of the specimen was injected to a reversed-phase C-18 column (3.9 × 300 mm). The specimen was eluted with a linear gradient of acetonitril in 0.05% trifluoroacetic acid (TFA). Fractions of 0.5 ml volume were collected, lyophilized, dissolved in 0.1% SDS, and examined for the presence of pep-

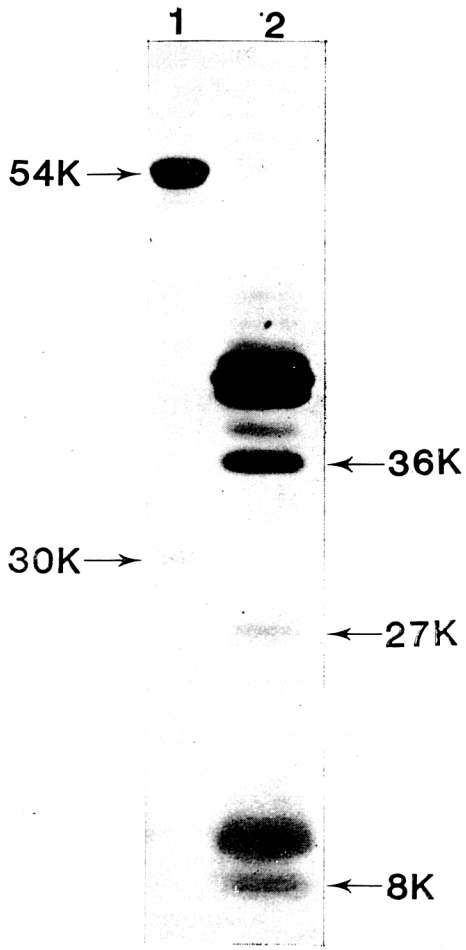

Fig. 1
JE virus E protein was prepared from purified viron and cleaved with CNBr followed by SDS-PAGE and Coomassie blue staining. *1* control, *2* CNBr-cleaved.

tide fragments by SDS-PAGE. Acetonitril (HPLC grade), trifluoroacetic acid (research grade) and SDS (electrophoresis grade) were purchased from Wako Pure Chemicals (Japan).

Amino acid sequencing. Fractions from HPLC containing 70—100 pmol of the 36 kD, 27 kD, or 8 kD fragments were applied to an automatic protein/peptide sequencer (Applied Biosystems Model 477A, U.S.A.) and their N-terminal amino acid residues were determined. Amino acid residues are shown by a single letter code.

Chemicals. Iodoacetamide, acrylamide, bis-acrylamide, and cyanogen bromide were the products of Wako Pure Chemicals Co. Japan.

Results

Immunogenicities of CNBr-cleaved fragments of JE virus E protein

Fig. 1 shows SDS-PAGE pattern of control JE virus E protein (lane 1) and its CNBr-cleaved fragments (lane 2). A major band of uncleaved 54 kD and a minor band of 30 kD were observed in the control specimen. This minor band

Table 1. N test and ELISA against JE virus on sera from mice immunized with CNBr fragments from JE virus E protein

Serum number	Immunogen	N test at 1 : 10* & (endpoint)		ELISA titre & (GMT)	
1	54 kD	+	(100)	17,260	(9,817)
2	54 kD	−	(<10)	5,584	
3	36 kD	−	(<10)	5,335	
4	36 kD	−	(<10)	6,146	
5	36 kD	−	(<10)	4,602	(5,518)
6	36 kD	−	(<10)	6,144	
7	27 kD	+	(100)	nt**	
8	27 kD	+	(40)	3,653	
9	27 kD	+	(40)	847	
10	27 kD	+	(10)	167	(881)
11	27 kD	+	(10)	618	
12	27 kD	+	(100)	681	
13	27 kD	+	(100)	2,154	
14	8 kD	+	(100)	171	
15	8 kD	+	(10)	43	(103)
16	8 kD	+	(10)	14	
17	8 kD	+	(10)	1,085	

* Positive (+) indicate more than 50% plaque reduction at 1 : 10 serum dilution, otherwise negative (−).

** Not tested because of limited amount and not included to calculate GMT.

could be due to acid treatment. On the other hand, multiple bands with M_r of uncleaved 54 kD to cleaved 8 kD were seen in CNBr-treated specimen. Since the bands of 36 kD, 27 kD, and 8 kD were clear and distinct from other bands and reproducible, they were cut out and used for immunization, along with 54 kD band from the control.

The N test and ELISA were carried out in individual sera from mice immunized with each of these bands, and the results using JE virus are summarized in Table 1. One of the two anti-54 kD sera was N-positive at a 1 : 100 dilution, while the other anti-54 kD and all four anti-36 kD sera were N-negative at 1 : 10 dilution. On the other hand, all seven anti-27 kD sera (three at 1 : 100, two at 1 : 40, and two at 1 : 10 dilutions), and all four anti-8 kD sera (one at 1 : 100 and three at 1 : 10 dilutions) were N-positive. Although N titres of individual sera showed considerable fluctuation even with the same immunogen, the results as a whole indicate that some neutralizing epitope(s), which is resistant to acid and SDS treatments, is present on the 27 kD and 8 kD fragments, but not on the 36 kD fragment. ELISA titres of anti-27 kD and anti-8 kD sera showed more individual fluctuations than the N titres (167 to 3,653, and 14 to 1,085, respectively); they did not correlate with N titres, but the ELISA titre of anti-36 kD sera were similar

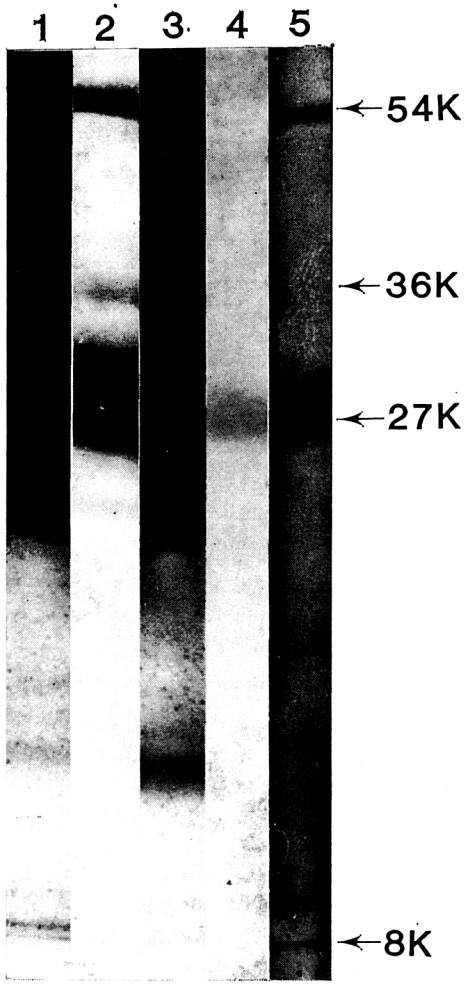

Fig 2
JE virus E protein was cleaved by CNBr and the resulting fragments were separated by SDS-PAGE and reacted by Western blotting
1 anti-54 kD (serum number 1), *2* anti-36 kD (serum number 4), *3* anti-27 kD (serum number 13), *4* anti-8 kD (serum number 14), and *5* anti-JE polyclonal serum. Serum numbers shown in Table 1.

among four mice (4,602 to 6,146), and the difference in ELISA titres of two anti-54 kD sera was about 3-fold and less than the difference in their N-titres. However, geometrical mean titre (GMT) of ELISA was highest for anti-54 kD (9,817), and gradually decreased for anti-36 kD (5,518), anti-27 kD (881), and anti-8 kD (103).

Interrelationship of CNBr-cleaved fragments from JE virus E protein

Fig. 2 shows the reactivity of anti-fragment sera and anti-JE sera with CNBr fragments by Western blotting. Anti-54 kD serum reacted with multiple bands including 54 kD, 36 kD, 27 kD, and 8 kD fragments (lane 1), and anti-36 kD serum reacted with several bands including 54 kD, 36 kD, and

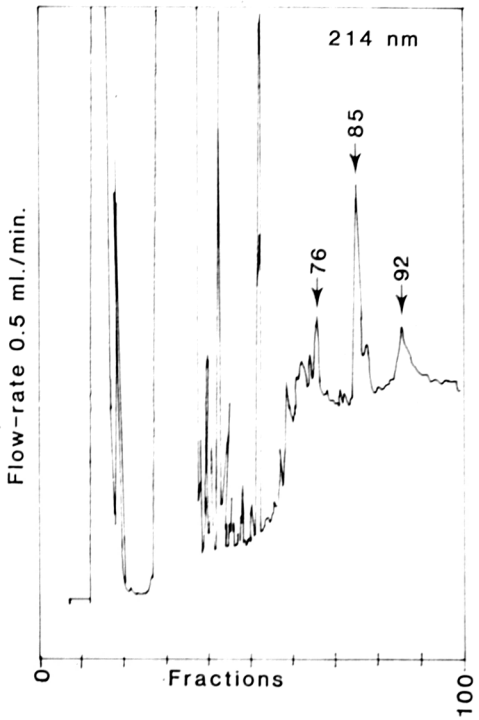

Fig. 3
JE virus E protein was cleaved by CNBr and fragments were separated by Reverse -phase HPLC: The sample (100 μg) of lyophilyzed E protein was dissolved in 6 mol/l guanidine hydrochloride and 1 ml volume was injected into the column C-18 (3.9 × 300 mm). The specimen was eluted with a linear gradient of acetonitrile in 0.05% TFA at a flow rate of 0.5 ml/min.

27 kD but not 8 kD fragments (lane 2). While the anti-27 kD serum reacted with multiple bands including 54 kD, 36 kD, 27 kD and also 8 kD fragments (lane 3), and anti-8 kD serum reacted only with the 27 kD and 8 kD fragments (lane 4). Polyclonal anti-JE serum reacted with several bands from uncleaved 54 kD to cleaved 8 kD including the 36 kD and 27 kD bands (lane 5). This result indicated that the 8 kD fragment is a part of 27 kD but not included in the 36 kD fragment, despite that the 36 kD and 27 kD fragments share a common part.

Physical map of CNBr-cleaved fragments on JE virus E protein

In order to determine the positions of the CNBr-cleaved 36 kD, 27 kD, and 8 kD fragments from JE virus E protein, these fragments were purified by HPLC (Fig. 3). Fraction numbers 76, 85, and 92 contained the 36 kD, 27 kD, and 8 kD fragments, respectively. These fractions were analysed for their N-terminal amino acid residues; the results were compared with the sequence deduced from the nucleotide sequence of JE virus E protein gene reported by Sumiyoshi *et al.* (1986). Eleven residues (INIEASQLAEV) of the 36 kD fragment were identical to the deduced sequence beginning after the third methionine (amino acid number 45 of E protein). While, five out of nine residues (TVG—LV) of the 27 kD fragment agreed with the deduced sequence following the fourth methionine (amino acid number 204), although

four residues did not give clear-cut resolution in sequencing. Eleven residues (EPPFGDSYIVV) of the 8 kD fragment showed complete agreement with the deduced sequence following the ninth methionine (amino acid number 374) of the E protein. From these results and from the interrelationship of these fragments with the M_r, their positions of E protein were located as

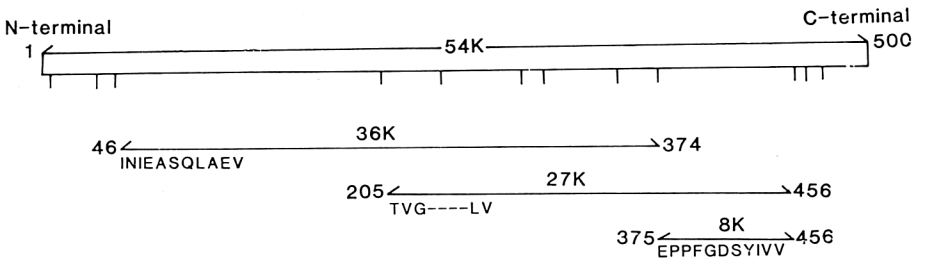

Fig. 4

Physical map showing positions of three CNBr-cleaved fragments on JE virus E protein Vertical bars indicate methionine residues deduced from the nucleotide sequence of the E protein gene. The fragments are shown by horizontal bars with the amino acid number on the E protein at both ends and N-terminal amino acid residues by single letters under the bars.

shown on a physical map (Fig. 4). The 36 kD fragment, which occupies two-third of E protein, begins at amino acid number 46 and ends at 374, and its M_r calculated from the deduced sequence was 35,326. While the 27 kD fragment begins at amino acid number 205 and ends at 456, and its M_r calculated from the deduced sequence was 26,913. The 8 kD fragment is located at the C-terminal end of 27 kD fragment and near C-terminal of E protein (amino acid number 375 to 456), and its M_r calculated from the deduced sequence was 8,648.

Discussion

Studies on several viruses such as influenza virus (Breschkin *et al.*, 1981), reovirus (Burtsin *et al.*, 1982), Venezuelan equine encephalomyelitis virus (Mathews and Roehrig, 1982), Tick borne encephalitis virus (TBE) (Heinz, 1986), and JE virus (Kimura-Kuroda and Yasui, 1983; 1986; Mason *et al.*, 1987) have shown that antibodies directed against critical sites on viral proteins are capable of inactivating certain biological activities of viruses like haemagglutination or infectivity. Heinz *et al.* (1984) reported that digestion of CEE virus E protein with trypsin or α-chymotrypsin released a 9 kD fragment, while chemical cleavage by CNBr released a 19 kD fragment. Both the 9 kD and 19 kD fragments retained reactivity with neutralizing monoclonal antibodies defining a denaturation-resistant antigenic domain. However, the location of these peptide fragments on E protein has not been determined, although (Heinz, 1986) reviewed peptide mapping of CEE and other flavivirus E proteins by monoclonal antibodies, and Mandl *et*

al. (1988) reported the sequence for TBE virus E protein gene. Therefore, it is difficult to relate CNBr-cleaved fragments of JE virus E protein to the fragments of TBE virus. Suppose the 8 kD or 27 kD fragment of JE virus carries similar epitope(s) as the 9 kD and 19 kD fragments of TBE virus, the difference of their M_r could be due to the difference in the amino acid sequences of E protein between these two viruses.

Compared with the model of West Nile virus E protein by Nowak and Wengler (1987), the 8 kD fragment of JE virus covers about 94% of their R3 region and a portion of the hydrophobic anchor region, and contains a hydrophilic beta turn of the R3 region which could be a strong antigenic epitope. The 8 kD fragment also contains Arg-Gly-Asp (RGD) tripeptide, which was reported as crucial for the interaction of a protein containing this sequence with its cell surface receptor (Rouslathi and Pirschbacher, 1986). The possibility that the RGD sequence plays a similar role in the process of JE virus interaction with its host cell receptor should be further investigated. On the other hand, the 27 kD fragment of JE virus covers 87% of the R2 region and a portion of the anchor region in adition to the whole L2 and R3 regions. Since both anti-8 kD and anti-27 kD sera neutralized JE virus and the 8 kD is a part of the 27 kD, the result suggested that denaturation-resistant N epitopes are present on the 8 kD fragment or R3 region. However, the N titres of anti-8 kD or anti-27 kD sera obtained in this study were much lower than those of monoclonal antibodies (Kimura-Kuroda and Yasui, 1983; 1986; Mason *et al.*, 1987). The difference may be due to the insufficient amount or small size of the immunogens in this study. Titre fluctuation of individual mouse immunized with 27 kD or 8 kD fragment could not simply be explained by uneven distribution of immunogens, because N titre was not always parallel to ELISA titre. The result may indicate that N and ELISA epitopes on these immunogens were not evenly recognized by the individual mouse. The 36 kD fragment covers 63% of R1 region and 11% of R3 region besides the whole L1, L2, and R2 regions, and denaturation-resistant N epitopes were not demonstrated on this fragment, although such ELISA epitopes were present. This is compatible with sequence data showing that the R1 region is rich in disulphide bonds and forms a highly conformational structure which can be denatured by acid and/or SDS treatment. Only one out of two mice immunized with 54 kD band produced neutralizing antibodies and similar result was obtained in a separate experiment (data not shown). This result may be explained assuming that mice could not well recognize denaturation-resistant neutralizing epitopes on E protein when its whole molecule was inoculated.

The physical map in Fig. 4 shows that only the 8 kD fragment was the product of complete cleavage, while the 27 kD and 36 kD fragments were the products of partial cleavage by CNBr of JE virus E protein. Biochemical studies have shown that CNBr-cleavage was not complete, when methionine residues had been oxidized (Odani *et al.*, 1971) or when the residue is followed by a serine or threonine residue (Titani *et al.*, 1972). Thelatter possibility was observed for the fourth and the eighth methionine of JE virus E protein.

Acknowledgements. This work was supported by Grant in Aid for Developmental Research No. 61870025 in the year of 1986—1987. Animal experiments of this study were performed at Animal Research Center, Institute of Tropical Medicine, Nagasaki University in collaboration with Dr. S. Matsuo. We are grateful to Prof. G. Matsuda and his staffs in the Department of Biochemistry, Nagasaki University School of Medicine for their technical advice in amino acid sequencing.

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