[原著] Expression of Japanese Encephalitis Virus Envelope Protein by Baculovirus Expression System for the Analysis of Immunopathogenesis

Expression of Japanese Encephalitis Virus Envelope Protein by Baculovirus Expression System for the Analysis of Immunopathogenesis

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ABSTRACT

We have constructed C-terminally truncated Japanese encephalitis virus envelope (E) proteins that ranged in length from 260 amino acids (a. a.) to 380 a. a. of the N-terminal sequence, using the baculovirus expression system, to analyze antigenic sites and examine basic conditions to keep a proper configuration of E protein. Truncated E proteins longer than 304 a. a. residues reacted with patients sera with sequential flavivirus infection, but failed to react with sera with primary flavivirus, even JE virus, infection. The E proteins of 279 a. a. or shorter residues did not react with sera from both patients with primary and sequential flavivirus infection. These results suggest that the main epitopes recognized during the secondary flavivirus infection are different from those recognized during the primary flavivirus infection, and also that existence of the fifth disulphide bond (a. a. #190 and a. a. #287) is involved in the reactivity of recombinant E proteins with patient sera in sequential flavivirus infection. Analysis of the length of expressed membrane (M) protein combined with E protein revealed that M proteins shorter than 37 a. a. residues failed to react with any of patient sera. This suggests that at least the C-terminal 37 a.a. residues of M protein is needed for the proper cleavage between M and E proteins. *Ryukyu Med. J., 14 (1) 25 ~ 32, 1994*

Key words : Japanese encephalitis virus, expressed E protein, patient sera, sequential flavivirus infection, secondary immune response

INTRODUCTION

Japanese encephalitis (JE) virus belongs to the family Flaviviridae and causes encephalitis with high mortality and severe sequelae, producing a serious public health problem in the epidemic areas[1,2]. Complete sequence of the genome had been reported[3]. Similar to other flaviviruses, JE virus contains only three structural proteins, capsid (C), membrane (M), and envelope (E) proteins[3~5]. C protein (13 kD) binds to the positive-stranded genomic RNA forming the nucleocapsid. M (9 kD) and E (53 kD) proteins constitute a lipid-associated envelope of the virion. E protein is a glycoprotein and a major virion antigen responsible for virus neutralization by specific antibody and for several important antigenic properties such as binding flavivirus-, subgroup specific-, and type-specific antibodies[6,7]. JE virus and other flavivirus E's also exhibit hemagglutinating activity[8]. Results of epitope mapping with a library of monoclonal antibodies indicate that the antigenic structure of JE virus E is similar to that of other flavivirus E's that contains several distinct antigenic sites as defined by competitive binding assay[9~14]. Comparison of amino acid sequences shows that there is significant sequence homology among E glycoproteins of different flaviviruses[15~19]. In the areas where not only JE virus but also other members of flaviviruses, such as dengue (DEN) viruses co-circulate, complicated immune responses were observed in patients with sequential flavivirus infection. The phenomena of immune enhancement and DEN hemorrhagic fever (DHF) are thought to be due to the unusual immune response of the sequential flavivirus infection[20,21].

In this study, in order to analyze the antigenic sites on the flavivirus envelope, we expressed JE E proteins in various length, by baculovirus expression system, and examined the basic conditions for keeping a proper configuration of E protein. We also examined the reactivities of the expressed proteins with the sera from patients with primary and sequential flavivirus infection.

MATERIALS AND METHODS

Cells and viruses

Spodoptera frugiperda Sf-9 cell line (ATCC CRL-1711; American Type Culture Collection, Rockville, MD) was grown in TNM-FH medium containing 10% fetal calf serum (FCS) and antibiotics[22,23]. It was used for the propagation and plaque assay of baculoviruses and JE virus. BHK-21 cells were grown in Eagle's minimum essential medium (MEM) containing 10% FCS. *Autographa californica* nuclear polyhedrosis virus (AcNPV, E2 strain) and baculovirus transfer vectors (pAc409, pAc436) were kindly supplied by Dr. Max Summers (Texas A&M University, College Station, TX). JE virus (Nakayama strain) was supplied by the Research Institute for Microbial Diseases, Osaka University. Stock JE virus was prepared as 10% homogenate of infected suckling mouse brain.

Preparation of plasmid DNA and AcNPV DNA

E. coli infected with plasmid M147 were incubated in a shaking incubator for 40 h at 37℃ in LB medium with ampicillin (50 μg/ml). The plasmid DNA were extracted by alkali method and purified by cesium chloride ultracentrifugation[24]. AcNPV-infected Sf-9 cell culture fluid was harvested at 48 h post infection. The culture fluid was clarified by low speed centrifugation, followed by ultracentrifugation at 100,000g for 30 min. The virus pellet was resuspended in 0.1x TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and overlaid over 25-56% sucrose gradient and centrifuged at 100,000g for 90 min[25,26]. Visual viral band was harvested and pelleted by ultracentrifugation at 100,000g for 30 min. The virus pellet was resuspended in 4.5 ml of extraction buffer (100 mM Tris, 90 mM $Na_2EDTA \cdot 2H_2O$, and 200 mM KCl, pH 7.5) and treated with 200μg of proteinase K for 2 h at 50℃, then 0.5 ml of 10% Sarkosyl was added and continued to incubate for another 2 h at 50℃. After the incubation period, the viral DNA was extract twice with phenol/chloroform/isoamyl alcohol (25 : 24 : 1), and precipitated by ethanol. The viral DNA was resuspend in 0.1x TE and heated for 15 min at 65℃.

Construction of recombinant baculoviruses

Plasmid M147 DNA, which codes for the 5' noncoding region, C, M, E and part of NS1 (nucleotides from #34 to #2538) of JaOH0566 strain of JE virus, was kindly supplied by Dr. Igarashi, Institute of Tropical Medicine, Nagasaki University. In order to obtain a series of C-terminally truncated and full-size E's, plasmid M147 was digested with various restriction endonucleases described in Fig. 1. The resulting fragment was inserted in frame into *Sma*I site of the baculovirus transfer vector with T4 ligase (Fig. 2). Cloned transfer vector DNA (2 μg) was cotransfected with AcNPV genomic DNA (1μg) into Sf-9 cells by the calcium phosphate precipitation method, and the cells were incubated for 4 days at 28℃[27,28]. The recombinant virus resulting from homologous recombination between the plasmid and the virus DNA in the polyhedrin gene region formed occlusion-negative plaques, and was isolated and cloned 3 times in Sf-9 cells. The procedures are outlined in Fig. 2.

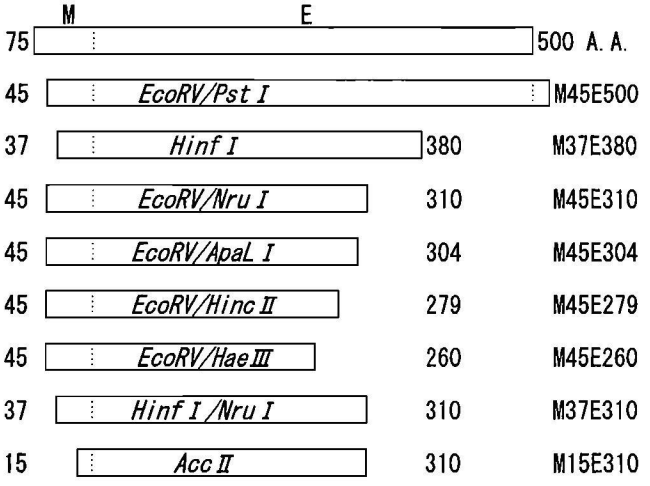

Fig. 1. Map of the JE E regions inserted in the recombinant baculovirus. Numbers on the left show the numbers of C-terminal a. a. residues of M proteins. Numbers on the right show the numbers of N-terminal a. a. residues of E proteins. The restriction endonucleases used for excision were written in the column. The name of the recombinant virus was written on the right side.

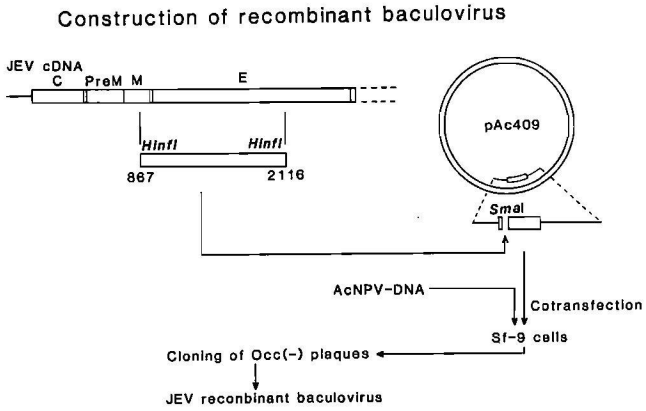

Fig. 2. Construction of recombinant baculovirus containing the envelope gene region of JEV. Occ: occlusion.

PAP Staining

PAP staining method was used as described elsewhere[29]. Sf-9 cells in 96-well flat-bottomed multiplates (Sumitomo Bakelite) were infected with the recombinant viruses or JE virus and cultured for 40 h at 28℃. The cells were then fixed with 3.6% formalin for 10 min and treated with 0.1% Triton X-100 for 5 min at room temperature. After washing twice with phosphate buffered saline (PBS), the cells were blocked with PBS con-

taining 2% FCS (PBS-2FCS) for 1 h at 37℃. The cells were allowed to react sequentially with diluted human serum, rabbit anti-human gamma globulin (1: 1,000, Dakopatts), goat anti-rabbit IgG (1: 1,000, Cappel), rabbit PAP complex (1: 5,000, Jackson Laboratory), and substrate (0.3 mg/ml of 3, 3'-diaminobenzidine plus 0.01% H_2O_2). The cells were reacted with each reagent for 30 min at 37℃ and with substrate for 5 min at room temperature. After each reaction, the cells were washed twice with PBS. Uninfected cells were treated in the same way and used as control. The stained cells were observed under a light microscope. In each experiment, positive and negative control sera were included to standardize the staining conditions.

Western blot analysis of the recombinant protein

Sf-9 cells infected with recombinant virus were incubated for 2-3 days at 28℃. The cells were washed twice with PBS and lysed with Laemmli's lysis buffer[30]. The proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane[31]. The membrane was blocked for 1 h with 10% Block Ace (Dainihon Seiko, Co.) in PBS containing Tween 20 (PBST). The membrane was sequentially reacted with anti-JE hyperimmune rabbit serum, goat anti-rabbit serum, rabbit PAP complex, and substrate as described in PAP staining section.

Enzyme-linked immunosorbent assay (ELISA)

Indirect micro-ELISA was used with modifications[32,33]. Formalin-inactivated, purified JE vaccine concentrate, which was kindly supplied by Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University, was used as coating antigen.

Test sera

The following human sera were used: 1) 13 sera from JE patients and 41 sera from DEN patients were obtained in Chiang Mai, Thailand. 2) 3 sera from JE patients were obtained in Okinawa. The diagnoses of JE and DEN infections were confirmed either by virus isolation from acute-phase sera or by demonstrating an increase in antibody titer in convalescent phase sera by neutralization test. The sera were heated for 30 min at 56℃ and diluted in PBS containing 3% FCS (PBS-3FCS).

Immunofluorescent staining of cell surface

Indirect immunofluorescent antibody method was used[34]. Recombinant virus- and mock-infected Sf-9 cells were cultured for 40 h at 28℃. The unfixed cells were reacted with anti-JE hyperimmune rabbit serum for 30 min, then with FITC-conjugated goat anti-rabbit immunoglobulin for 30 min. The stained cells were observed under a fluorescence microscope.

Immunization

Balb/c mice were given an intraperitoneal injection (i.p.) with $5X10^6$ cells of either M37E380-, M45E310-, or AcNPV-infected cells suspended in PBS. On 7 and 14 days after injection, the same materials were injected i. p. and the animals were sacrificed 8 days later.

Neutralization tests

The sera from mice immunized with recombinant-, or parent strain AcNPV-infected cells, were heat-inactivated for 30 min at 56℃ and diluted in Eagle's MEM containing 5% FCS. Fifty percent focus reduction neutralization test on BHK-21 cells applying PAP staining method was employed[29].

RESULTS

Construction of recombinant baculoviruses containing various length JE virus E genes

A 1249 base pair (bp) DNA fragment containing the coding region of C-terminal 37 amino acid (a. a.) residues of M protein plus N-terminal 379 a. a. residues of E protein of JE virus was excised with *Hinf*I from plasmid M147 DNA. Both ends of the fragment were converted to blunt-ends by Klenow fragment and then cloned into *Sma*I site of transfer vector pAc409 using T4 ligase. The cloned pAc409 DNA was cotransfected with AcNPV DNA into Sf-9 cells. Recombinant virus was isolated from the infected cells by selecting progeny virus with occlusion-negative plaque phenotype. After 3 cycles of successive plaque purification, stock of the recombinant virus (M37E380) was obtained. The DNA fragments with 1074, 1056, 981, 925, and 1727bp were excised with restriction endonucleases *Eco*RV and *Nru*I, *Eco*RV and *Apa*LI, *Eco*RV and *Hinc*II, *Eco*RV and *Hae*III, and *Eco*RV and *Pst*I, respectively. These fragments were cloned into transfer vector pAc436, and the recombinant viruses (M45E310, M45E304, M45E279, M45E260 and M45E500) were obtained as described above. These recombinant viruses directed the synthesis of truncated and full-size JE E proteins in the form of a fusion protein in which C-terminal 1, 5, 12, or 28 a.a. residues of the polyhedrin protein were fused to the C-terminal of the JE virus E proteins (i. e., 1 a.a. to M37E380, 5 a. a. to M45E260, 12 a. a. to M45E500 and 28 a. a. to M45E310, M45E304 and M45E279).

Expression of JE E protein in insect cells

In order to determine whether JE E protein was synthesized in the recombinant virus-infected cells, Sf-9 cells were infected with the recombinant virus and cultured for 40 h at 28℃. The cells were then fixed and stained by PAP method. The recombinant virus directed the synthesis of JE viral antigens which reacted with anti-JE hyperimmune rabbit serum, while mock-infected cells did not (Fig. 3-A, B). Sf-9 cells infected with M45E310, M45E304, M45E279, M45E260 and M45E500 also reacted with anti-JE hyperimmune rabbit serum (data not shown).

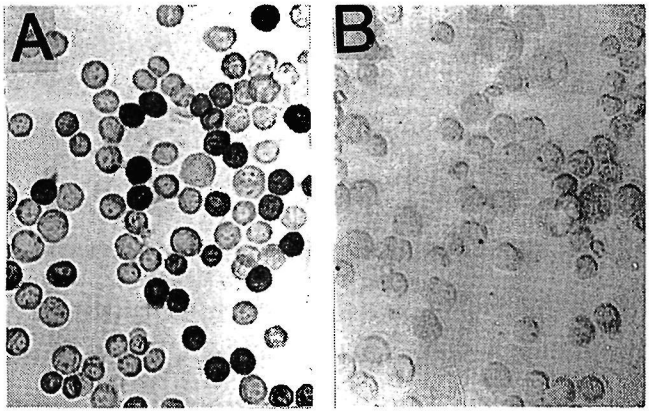

Fig. 3. PAP staining of recombinant virus (M37E380) -infected cells (A), and mock-infected Sf-9 cells (B), using anti-JE hyperimmune rabbit serum. X300.

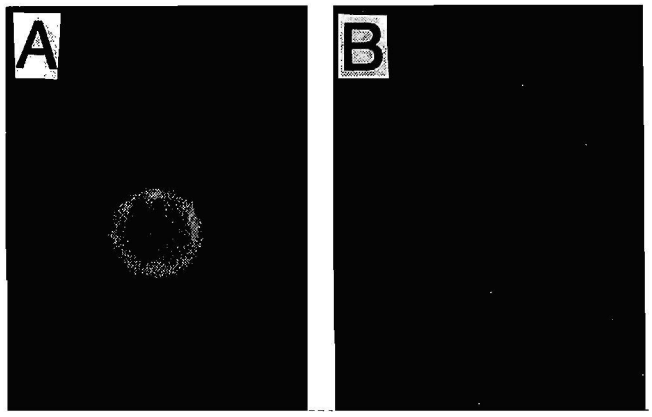

Fig. 4. Surface staining of recombinant virus (M37E380) -infected cells (A), and mock-infected Sf-9 cells (B), using anti-JE hyperimmune rabbit serum. X450.

Characterization of the recombinant protein

Authentic E protein in JE virus-infected cells migrates to the surface of the infected cells. In order to determine whether E protein expressed by the recombinant virus possess the same nature, immunofluorescent staining was undertaken on unfixed cells. As shown in Fig. 4-A, surface staining of Sf-9 cells infected with the recombinant virus (M37E380) was apparent.

Western blot analysis

The M37E380-infected cell lysate was resolved on 10% SDS-PAGE under reduced and non-reduced conditions, and analysed by Western blotting using anti-JE hyperimmune rabbit serum (Fig. 5). Under reduced condition, a protein band with a molecular weight (MW) of approximately 40 kD reacted with the anti-JE rabbit serum, which was not observed in uninfected cell lysate (Fig. 5, lane B and C). The expected MW of the recombinant protein deduced from the sequence data was about

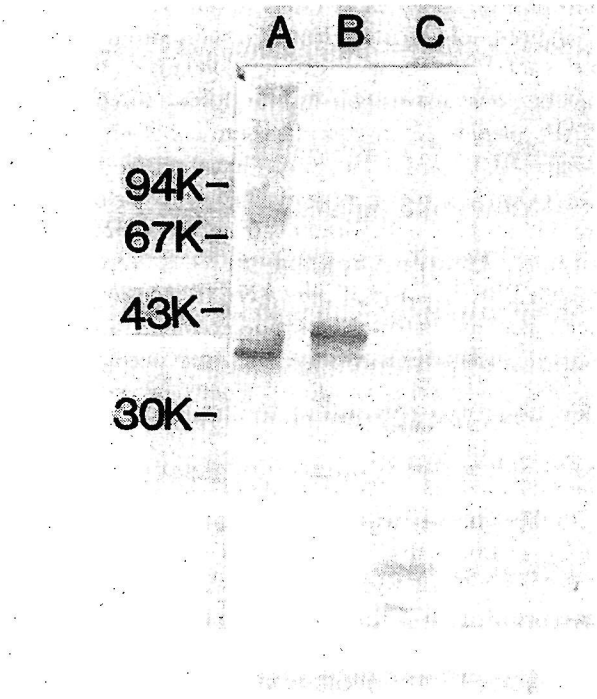

Fig. 5. Western blot analysis of recombinant virus (M37 E380) -infected cells under non-reduced (lane A) and reduced (lane B) conditions, and mock-infected cells (lane C), using anti-JE hyperimmune rabbit serum. Left column shows molecular size.

41 kD. Under non-reduced condition, a band with slightly faster mobility was reacted with the same antiserum (Fig. 5, lane A). With the patient sera in secondary flavivirus infection, much less reaction of the 40K protein was observed under reduced condition than that under non-reduced condition (data not shown).

Reactivities of JE and DEN sera with recombinant protein

JE and DEN sera were diluted 1: 1,000 in PBS-3FCS and examined by the PAP staining method. The recombinant protein expressing full-size E reacted with all of the 4 kinds of sera (Table 1). Anti-JE hyperimmune rabbit serum reacted with all the recombinant proteins. On the other hand, DEN patient serum and anti-DEN 4 rabbit serum reacted with the recombinant proteins expressed by M37E380, M45E310 and M45E304 viruses, but not by M45E279 and M45E260. It was unexpected that JE sera having no DEN antibody failed to react with any of the truncated recombinant proteins, although it reacted with full-size E recombinant protein, as well as, JE virus-infected cells. It is generally accepted that sequential flavivirus infection in human results in the production of high titered and broadly reactive antibodies compared with that of primary infection. In order to compare the reactivity of various sera with the recombinant proteins, the sera were appropriately diluted to obtain equal ELISA titers. At the same time, they were tested by neutra-

Table 1. Results of PAP-staining of Sf-9 cells infected with recombinant baculovirus

Sf-9 cells infected with	JE patient (1:1000)	DHF patient (1:1000)	Anti-JEV rabbit (1:1000)	Anti-D4 rabbit (1:1000)
M45E500	+	+	+	+
M37E380	−	+	+	+
M45E310	−	+	+	+
M45E304	−	+	+	+
M45E279	−	−	+	−
M45E260	−	−	+	−
M37E310	−	+	+	+
M15E310	−	−	+	−

(JEV:Japanese encephalitis virus ; DHF : Dengue hemorrhagic fever ; D4 : Dengue type 4 virus)

Table 2. Results of PAP-staining of Sf-9 cells infected with recombinant baculovirus (M37E380, M45E310, M45E304)

Response	N antibody to JE	DEN	YF	PAP staining	No. of samples
Primary	−	+		−	4
	+	−		−	4
Secondary	−	+		+	3
	+		+	+	3
	+	+		+	9
None	−	−		−	8
			Total		31

Table 3. Immune response to recombinant baculovirus-infected cells

Immunized with	Antibody titer ELISA	NT
M37E380	1135	133
M45E304	430	35
M45E279	‹100	‹10
AcNPV	‹100	‹10

lization test against JE, DEN and yellow fever viruses, and classified into 6 groups based on the immunological responses : (1) Primary JE virus infection, (2) primary DEN virus infection, (3) sequential infection with two or more DEN serotypes (DHF), (4) sequential infection with JE and yellow fever viruses, (5) sequential infection with JE and DEN viruses, and (6) non-flavivirus infection. The reactivities of these sera with the recombinant proteins are shown in Table 2. The sera from the patients with primary JE and DEN infections did not react with any of the truncated recombinant proteins, while those with sequential infections reacted with the recombinant proteins.

The effect of M protein on the reactivity of recombinant E protein

In order to examine the effect of C-terminal M protein on the reactivity of E protein, 3 different recombinant viruses were constructed in which C-terminal JE M protein was expressed in different length with E proteins (i.e., M45E310, M37E310 and M15E310) (Fig. 1). DEN patient serum (with sequential DEN infection), anti-JE and anti-DEN rabbit sera reacted with the recombinant proteins expressed by M45E310 and M37E310 viruses, but not by E15M310, although the latter protein reacted with anti-JEV hyperimmune rabbit serum (Table 1).

Immunogenic properties of recombinant proteins

Sera from the mice immunized with 3 kinds of recombinant virus- and AcNPV-infected cells were harvested and antibody titers were assayed by ELISA and neutralization test. Both ELISA and neutralizing antibodies to JE virus were detected in sera from mice immunized with M37E380- and M45E304-infected cells, while the antibody was not detected in those immunized with M45 E279- and AcNPV-infected cells (Table 3).

DISCUSSION

We constructed a series of recombinant baculoviruses in which polyhedrin genes of baculovirus genomes were replaced with various length of E genes of JE virus. The recombinant baculovirus directed the synthesis of JE E fusion proteins. The sera from patients with primary JE and DEN virus infections reacted with the recombinant full-length E protein, but not with any of the truncated E proteins. On the other hand, the sera from patients with sequential flavivirus infection reacted with the truncated E's longer than 304 a.a. residues, but not with those shorter than 279 a.a. residues. Full-length E of JE virus contains 500 a.a. residues including 12 Cys residues, all of which are conserved in some 20 flaviviruses that have been sequenced. In western blot analysis, under non-reduced condition, a band with slightly faster mobility than the one with reduced condition was observed, indicating that the recombinant proteins contain disulphide bond(s) (Fig. 5). In West Nile virus (WN), another flavivirus, each of the 12 Cys residues appeared to be involved in the formation of disulphide bond[35]. If the disulphide bonds in JE virus are the same as those in WN virus, the truncated recombinant proteins were reactive to the sera from sequential flavivirus infection containing from the first to the fifth disulphide bonds of N-terminal region (i.e., the bonds between the Cys positions at 3-30, 60-121, 74-105, 92-116, and 190-287)[36]. Those proteins which did not react with patient sera, lacked the fifth disulphide bond at 287-Cys position. These results suggest that the existence of the fifth disulphide bond is involved in the reactivity of the recombinant proteins with patient sera. In YF virus, C-terminal region of M protein functions as a signal sequence for the translocation of E across the rough endoplasmic reticulum. The cleavage between M and E occurs after a Ser residue

(a.a. # 286), and could be catalyzed by a cellular protease such as signalase[37]. It has been reported that C-terminal 37 a.a. residues of YF M is a hydrophobic membrane associated protein. In our study, the recombinant protein, M15E310, which is shorter than 37 a.a. failed to react with patient sera, while M37E310 and M45E310 were reactive. These result suggests that at least C-terminal 37 a.a. residues of M may be needed for the proper cleavage between M and E protein. Uncleaved M might interfere with the configuration of the recombinant E protein.

Mice immunized with M37E380- and M45E304-infected cells developed neutralizing antibodies to JE virus. While those immunized with M45E279-infected cells showed no detectable antibody. In order to produce N-antibody, E protein should be longer than 304 a.a. residues. This result again shows the importance of the existence of the fifth disulphide bond in the recombinant protein.

The truncated recombinant E proteins (M37E380, M45E310, and M45E304) reacted with JE virus specific 8 monoclonal antibodies (MAb). Some of them showed neutralizing activity to JE virus (data not shown). This indicates that the recombinant proteins contain JE specific neutralizing epitope. However, from the result of the reactivity with patient sera, this neutralizing epitope might not be the same one as those recognized on primary JE virus infection.

Secondary antibody response after sequential flavivirus infection have been observed in natural or experimental human infections, such as YF and DEN; YF, JE and DEN; YF and JE; and in multiple DEN infections[38-42]. A rapid production of high titered antibody with broad reactivity was observed in the subject who had been preexposed to a member of flaviviruses. The truncated E proteins reacted with the sera showing secondary antibody response, but did not or faintly reacted with the sera showing primary antibody response. This result suggests that the epitopes recognized during the sequential flavivirus infection might be different from those during primary flavivirus infection. The recombinant proteins we obtained was not specific for JE virus but cross-reactive among flaviviruses. These proteins might be useful in the analysis of immune responses of sequential flavivirus infection, especially for the elucidation of dengue hemorrhagic fever, which occurred after sequential DEN virus infection.

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