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Immunogenicity of baculovirus expressed recombinant proteins of Japanese encephalitis virus in mice

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Genes encoding for the premembrane and envelope (prME), envelope (E) and nonstructural protein (NS1) of Japanese encephalitis virus (JEV) were cloned. Each protein was expressed in baculovirus expression system. Of the three proteins expressed in baculovirus system, only prME had hemagglutination activity. The prME (72 and 54 kDa), E (54 kDa) and NS1 (46 kDa) proteins could be detected by Western blotting in the recombinant virus infected cells. Immunogenicity of the recombinant proteins obtained from infected *Spodoptera frugiperda* (*Sf*-9) cells was examined in mice. The 3 week-old ICR mice immunized intraperitoneally with three recombinant proteins three times were challenged with a lethal JEV. A survival rate was increased from about 7.7% in unimmunized mice to 92.3% in E + prME and only E groups. The complete protection was shown in prME and live vaccine inoculated groups, respectively. We also measured neutralizing antibody and three immunoglobulin subtypes of IgG1, IgG2a and IgG2b in the sera of mice before and after challenge. Titers of IgG1 antibodies were approximately two to three times higher than that of IgG2b antibodies in all the immunized groups as compared to the control group. However, IgG2a antibody level somewhat increased after challenge, indicating T-helper type 1 (Th1) cell response. The results of this study can provide useful information for developing efficacious subunit vaccine against JEV.

Key words: baculovirus, Japanese encephalitis virus, JEV, protective immunity

Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne viral zoonosis of public health importance. Although the incidence of JE has been reported primarily in the far-east and South Asia, JEV is one of the emerging viruses, which are spreading into new area such as Australia [16]. In human, JEV infection can cause a severe central nervous system disease including febrile headache, aseptic meningitis and encephalitis [2]. Viral transmission occurs in an enzootic cycle, involving primarily *Culex* mosquitoes and swine as amplifiers, respectively [29]. Although most JEV infections of domestic animals are asymptomatic, JEV is a causative agent of fetal encephalitis, abortion and stillbirth in pregnant sows and hypospermia in boars [9,27].

The JEV genome is typical of other *Flaviviruses* in that a single open reading frame (ORF) encodes all the viral proteins, which are mainly derived via co-translational proteolytic processing [2]. Two proteins, envelope (E) and non-structural 1 protein (NS1) have been shown to elicit a protective immune response like other *Flaviviruses*. The E protein (54 kDa) is the major envelope glycoprotein of virion and a determinant of viral neurovirulence and neuroinvasiveness [21]. While the NS1 protein (46 kDa) is not incorporated into the assembled virion, it exists in cell-associated, cell-surface, or extracellular nonvirion forms. NS1 antibodies solely are capable of protecting animals against yellow fever and dengue virus mortality [25]. Presumably, protection stimulated by NS1 protein results from the destruction of infected cells before progeny virus is released [26].

In Korea, Anyang 300 strain of JEV, an attenuated live vaccine virus, was developed by continuous passage in chicken fibroblast cells for the protection of pig from reproductive disorders [13]. Since 1980, this live vaccine has been applied to pigs, and reduced the incidence of JEV infection in amplifying host animal. Park [23] reported that the vaccine strain, Anyang 300, belonged to the genotype III. Since 1990s, genotype I of Korean isolate have been identified in Korea. Nam *et al*. [22] reported that the recently isolated JEV strain from Korea was genetically distinct, compared with other JEV strains including the current vaccine strain for human used in Korea. In order to

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determine the efficacy of the current vaccine strain in Korea, antigenic characteristics of the wild JEV isolate needed to be investigated. In addition, the World Health Organization has promoted the development of improved or new vaccine for JEV [3,29].

Previously, we isolated JEV, KV1899 strain (1999 Korean isolate) from the plasma of growing pigs. The complete nucleotide sequence of the isolate was determined (GenBank accession number AY316157), showing its phylogenetic lineage to Ishikawa stain and classified into genotype I. In this study, we developed three recombinant baculoviruses encoding prME, E and NS1 proteins. The expressed proteins mixed with IMS1313 adjuvant were evaluated for immunogenicity in mice. In addition, we also investigated the ability of baculovirus-expressed proteins to protect mice against lethal JEV challenge.

Materials and methods

Cells and viruses

TF104 cells were regularly maintained in α-MEM (GibcoBRL, USA) supplemented with 5% fetal bovine serum (FBS), penicillin (100 unit/ml), streptomycin (100 unit/ml) and amphotericin B (0.25 ug/ml). *Sf-9* and High Five (Hi-5) insect cells were maintained in TC-100 medium (Sigma, USA) with 10% FBS and 1% lactoalbumin hydrolysate. The JEV isolate, (designated KV1899), Anyang 300 (attenuated strain) and Nakayama strain were used in this study. The KV1899 strain was propagated in TF104 cells that were cultured at 37°C in 5% CO_2 incubator.

Preparation of monoclonal antibodies

Five 6-week-old female mice (BALB/c strain) were intraperitoneally inoculated with 0.5 ml of JEV (512 HA unit) mixed with an equal volume of complete Freund's adjuvant (CFA) and boosted 4 weeks later with JEV in incomplete Freund's adjuvant. Blood samples were collected from immunized mice after 2 weeks post-booster inoculation to check humoral immune response. Spleenocytes were fused with SP2/0 myeloma cells using 50% of polyethylene glycol 1500 (Beohringer Mannheim, Germany). After hypoxanthine, aminopterin and thymidine (HAT) selection, culture supernatants from hybridoma cells were analyzed for antibody reactivity against JEV by using indirect fluorescence assay. Single cell clone was generated by limiting dilution and isotypes were determined by using monoclonal antibody isotyping kit (Pierce, USA). The hybridoma cells secreting JEV E and NS1 protein-specific monoclonal antibodies were grown in D-MEM with 10% FBS. The hybridomas were intraperitoneally injected into pristane-primed BALB/c mice for ascitic fluid production.

Construction of plasmids carrying prME, E and NS1 genes of JEV

Genomic RNAs of JEV were extracted from the JEV KV1899 infected culture fluid of TF104 cells. Each JEV forward primer (Table 1) contained a *Bam* HI restriction enzyme site and a start codon. Each reverse primer contained *Eco* RI restriction enzyme site. All the primers were designed based on the genomic sequence of recent JEV isolate, KV1899 (GenBank accession No. AY316157). JEV genes encoding prME, E and NS1 glycoproteins were amplified by reverse transcription and polymerase chain reaction (RT-PCR) using primers for JEV KV1899 strain, separated on 1.5% agarose gels, excised, and ligated into the cloning site of the pGEM-T vector system (Promega, USA). Three gene segments for prME, E and NS1 genes were obtained by *Bam* HI/*Eco* RI double digestion from a pGEM/prME, pGEM/E and pGEM/NS1 plasmid and ligated respectively into the *Bam* HI/*Eco* RI site of baculovirus transfer vector, pBlueBac 4.5/V5-His (Invitrogen, USA) which contains a C-terminal peptide encoding a six-histidine tag for detection and purification (Fig. 1). Each plasmid was transformed into JM 109 cells. The pBlueprME, pBlueE and pBlueNS1 plasmids were extracted and purified by plasmid purification kit (Qiagen, USA).

Transfection and purification of recombinant baculoviruses

For transfection of recombinant plasmids, Bac-N-Blue DNA (Invitrogen, USA) and 10 μg of highly purified each

Table 1. Oligonucleotide primers for cloning and expression of JEV glycoproteins

Primer designation	Oligonucleotide sequence (5'-3')	Orientaion	Genomic region
JENSF(2481-2502)*	CCGGATCCGACACTGG**ATG**TGCCATTGAC	Sense	NS1
JENSR(3728-3748)	CCGAATTCAGCGTCA ACCTGTGATCTA AC	Antisense	
JEF(798-815)	GGCGGATCCGGGA**ATG**GGGAACCGGGATTCC	Sense	E
JER(2457-2477)	CCGAATTCGGCATGCACATTGGTCGCCTA	Antisense	
JEMEF(477-503)	CCGGATCCAGCC**ATG**GAGCTATCATCAAACTTCCAAGG	Sense	prME
JEMER(2457-2477)	CCGAATTCGGCATGCACATTGGTCGCCTA	Antisense	
Bac F	TTTACTGTT TTCGTA ACA GTTTTG	Sense	Baculovirus MCS
Bac R	CAACAACGCACAGAATCTAGC	Antisense	

*Numbers in parentheses indicate the nucleotide sequence position of KV1899 strain (GenBank accession No. AY316157). The underlined sequences show restriction enzyme sites (*Bam* HI or *Eco* RI) and start codons.

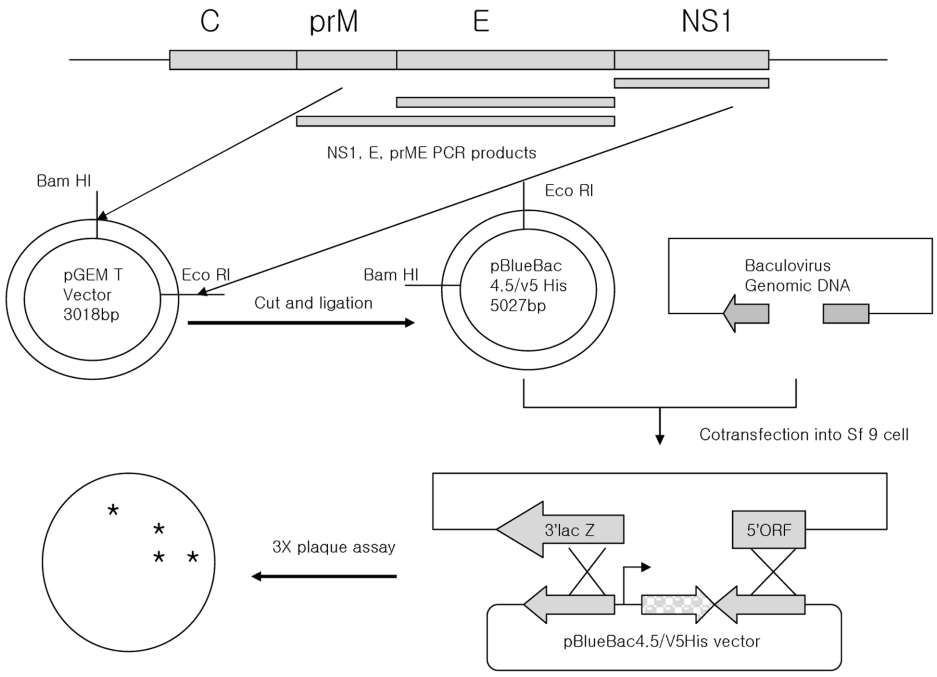


Fig. 1. Cloning and expression strategy of the JEV prME, E and NS1 proteins by recombinant baculoviruses. The recombinant baculoviruses were generated by cotransfection of the transfer vector and a linearized baculovirus genomic DNA, and selected after three times plaque purification.

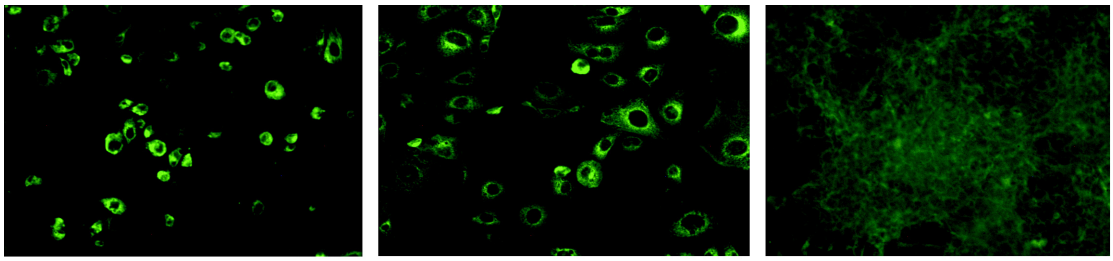
plasmid DNA containing prME, E and NS1 genes were mixed with Cellfectin reagent (Invitrogen, USA) in Grace's insect medium without supplements or FBS, respectively. After incubation for 15 min at room temperature, each transfection mixture was added into the 60 mm dish in which *Sf*-9 cells had been cultivated. After 3 days, cultured recombinant virus was harvested and the cells were incubated continuously by adding fresh medium. To purify recombinant baculovirus, plaque assay was performed in 1% agarose medium containing 150 μg/ml of X-gal. After plaque assay, PCR assay of recombinant virus was used to confirm the isolation of a pure, recombinant plaque using baculovirus specific primers (Table 1). Recombinant BacprME, BacE and BacNS1 viruses were passed three times by infecting *Sf*-9 cells with a multiplicity of infection (MOI) of 0.1 respectively. The third passage level of the recombinant virus was used as virus stock for further experiments and the fourth or fifth passage level of the stock virus was used for protein production in Hi-5 cells.

Immunofluorescence test and preparation of recombinant vaccines

For identification of recombinant protein, *Sf*-9 cells were infected with recombinant baculoviruses in 96-well microplate and incubated for 4 days. Infected cells were fixed with cold acetone at −20°C for 10 min, washed with phosphate buffered saline (PBS, pH 7.2) and then incubated with monoclonal antibodies (MAb) against E, NS1 protein of JEV and six histidines for 1 hr at 37°C. After washing, FITC conjugated anti-mouse IgGs were added and incubated at 37°C for 1 hr. After rinsing with PBS (pH 7.2) and the cells were examined under fluorescence microscope (Olympus IX70, Japan). For production of prME, E and NS1 proteins, Hi-5 cells were cultured in spin culture flask at the stirring rate of 50 rpm. Hi-5 cells were grown at a density of 2×10^5 cell/ml in a total volume of 800 ml and infected with recombinant viral stocks (expressing prME, E and NS1 respectively) at 10 MOI. After incubation for 5 days, the infected Hi-5 cells were harvested and collected after centrifugation at 3,000 g for 15 min. For the immunization of expressed protein of JEV, IMS1313 adjuvant (Seppic, France) and the lysate of recombinant virus infected insect cells (2×10^7 cell/ml) were blended with equal ratio under agitation. In order to purify and quantify three recombinant proteins, Ni-NTA agarose beads (Invitrogen, USA) were used and their proteins were eluted under the native condition. The eluted recombinant proteins were dialyzed against PBS (pH 7.2) at 4°C overnight. Protein concentrations were determined at an absorbance of 280 nm by spectrophotometry (Beckman, USA).

Western blot assay

For the identification of expressed protein, the lysed and sonicated recombinant proteins were dissolved in SDS-PAGE sample buffer with or without 2-mercaptoethanol and boiled for 5 min. Proteins were separated on 12.5% polyacrylamide-SDS gels and transferred electrophoretically to nitrocellulose paper (NP). The paper was blocked with a



(A) (B) (C)

Fig. 2. Immunofloresence of TF104 cells infected with JEV using two anti JEV MAbs. A; JEV-infected TF104 cells reacted with 8G3 for E, B; JEV-infected TF104 cells reacted with 4C11 for NS1, C; Non-infected TF104 cells reacted with two MAbs (8G3, 4C11) mixture.

5% skim milk in TBS (10 mM Tris pH 8.0, 150 mM NaCl) solution for 1 hr and reacted with the culture supernatant from hybridoma cells that secreting monoclonal antibody at room temperature for 1 hr. After washing with TBST buffer containing 0.05% Tween 20 three times, NP was incubated with a 1/2,000 dilution of alkaline phosphatase conjugated rabbit anti-mouse IgG (Promega, UAS) at room temperature for 1 hr. The blots were developed with BCIP/NBT (Invitrogen, USA) substrate.

Antibody assay

Serum samples were collected from the immunized mice at day 28 after first immunization and at day 43, the fifteenth day after challenge. Sera collected from mice were measured for the presence of neutralizing antibody against JEV. The immunoglobulin subtypes of immunized sera were measured by using indirect enzyme linked immunosorbent assay (ELISA). Microplates were coated with acetone extracted whole JEV from mouse brain emulsion corresponding 2 HA unit. After incubation with 200 µl of 3% bovine albumin in PBS, 100 µl of 1 : 50 dilution of the test serum was added to each well. After washing with PBST, bounded proteins were detected with HRP conjugated goat anti-mouse IgG1, IgG2a and IgG2b (Beohringer Mannheim, Germany). Color was detected by adding ABTS (KPL, USA). The serum neutralization (SN) test was carried out by the 50% plaque reduction method. Antibody titer was calculated as the reciprocal of the highest serum dilution resulting in 50% plaque reduction [4]. HA and HI test were carried out using the standard method [6].

Mouse protection assay

Three week-old female ICR mice were divided into 7 groups of 13 mice. The protective immunity of prME, E and NS1 proteins was evaluated according to virulent challenge test. Immunization of each group was carried out by intraperitoneal injection of recombinant protein blended with IMS1313 adjuvant. An additional group of mice was immunized with inactivated or attenuated live vaccine strain (Anyang 300). For the production of inactivated vaccine, the virus containing $10^{7.0}$ $TCID_{50}$/ml was inactivated by 1 mM of binary ethyleneimine (BEI) at 37°C for 18 hr. After neutralization of BEI with 2 mM of sodium thiosulfate, equal volume of Montanide IMS1313 adjuvant was added into inactivated virus with agitation. Immunization was done 3 times at days 0, 14 and 21, respectively. The mice were challenged intraperitoneally with virulent JEV at day 28. The challenge virus was prepared from a 1/10 dilution of a 20% suspension of JEV-inoculated (Nakayama strain) suckling mouse brain. The challenge preparation contained 100 LD_{50} /0.5 ml of JEV. The challenged mice were observed daily for 15 days. Survival rate was recorded for the same period.

Statistical analysis

Data were entered into a database for the statistical analysis program (GraphPAD Prism version 3.02). Difference between the means of experimental groups was analyzed using an independent t-test for statistical analysis. Survival data were expressed as the percentage surviving.

Results

Characterization of JEV monoclonal antibodies

Following fusion of spleen cells from immunized mice, ten hybridoma clones were reactive to JEV antigens by indirect fluorescent assay. After cloning by limit dilution, antibodies from 10 hybridoma clones were characterized by Western blotting, hemagglutination inhibition activity and antibody isotyping. The results showed that nine clones were reacted with JEV E and one clone 4C11 with NS1 proteins, respectively. The 6F10 clone produced an antibody of IgM class, and five clones produced antibodies having HI activity (Table 2).

Expression of JEV proteins in recombinant baculovirus-infected insect cells

JEV genes encoding three prME, E, and NS1 glycoproteins from KV1899 strain were amplified and cloned respectively into a baculovirus transfer vector, pBlueBac4.5/V5-His contained six-histidine tag in the C-terminal region (Fig. 1). The nucleotide sizes of prME, E and NS1 genes cleaved by restriction enzyme from transfer vector were about 2,001,

Table 2. Characterization of monoclonal antibodies against JEV

Designation	Protein specificity*	HI activity**	Isotype	Chain
4B8	E	HI activity	IgG2b	κ
4F3	E	HI activity	IgG2a	κ
4C11	NS1	-	IgG1	κ
5H5	E	-	IgG1	κ
6F7	E	HI activity	IgG1	κ
6F10	E	-	IgM	κ
6G8	E	HI activity	IgG2a	κ
7D9	E	-	IgG3	κ
8F10	E	-	IgG2b	κ
8G3	E	HI activity	IgG2b	κ

*Western blotting, **Ascites.

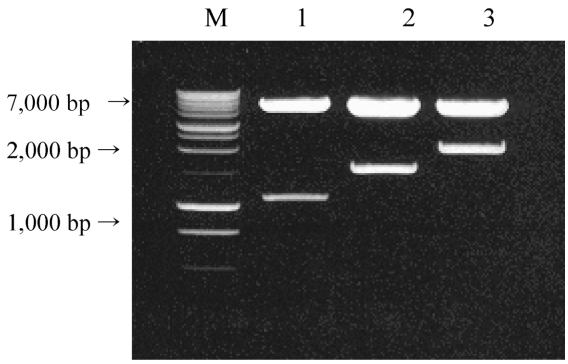


Fig. 3. Cleavage patterns of JEV genes inserted into pBlueBac4.5/V5-His, transfer vector by *Bam* HI/*Eco* RI restriction enzyme treatment. Lane M; 1kb DNA ladder, lane 1; pBlueBac-NS1, lane 2; pBlueBac-E, lane 3; pBlueBac-prME.

1,500 and 1,268 bps each as predicted (Fig. 3). After transfection and plaque assay, recombinant plaques were easily distinguished from non-recombinant, because a transfer vector with *lacZ* gene made recombinant virus blue plaques. Plaque purified prME, E and NS1 recombinant baculoviruses were also identified by IFA (Fig. 4) and confirmed by PCR using baculovirus primers (data not shown). Three JEV recombinant proteins were expressed successfully in *Sf-9* cell respectively. The recombinant baculoviruses were propagated in Hi-5 cells. The three expressed proteins were examined by HA test to determine the quantity of expression. HA titer of the prME protein showed 1:4 only in the infected cell lysate (Table 3). The lysed and sonicated proteins were run on polyacrylamide-SDS gels and followed by Western blotting assay with specific monoclonal antibodies against E and NS1 protein of JEV, respectively. All the three recombinant proteins were also reacted with six-histidine monoclonal antibody. Western blot analysis showed that 72 and 54 kDa protein bands were present in the prME recombinant infected cell lysate, but little present in culture supernatant. In addition, 54 and 46 kDa proteins also were revealed in the E and NS1 recombinant infected cell lysates, respectively (Fig. 5). The prME, E and NS1 proteins were expressed mainly with

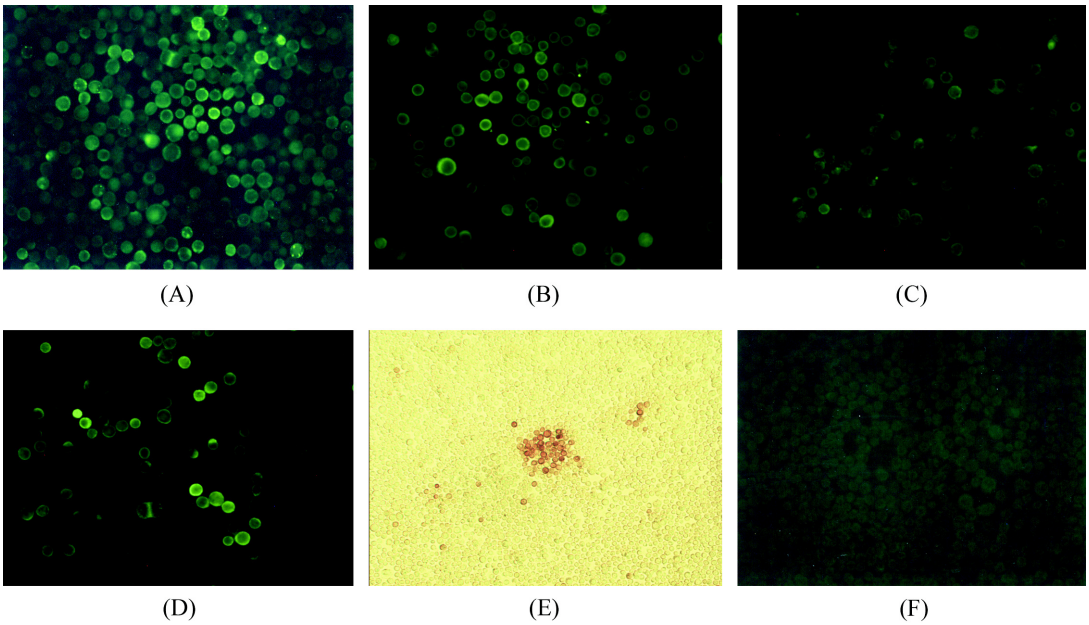


Fig. 4. Detection of recombinant proteins in pBlueprME, pBlueE and pBlueNS1 transfected *Sf*-9 cells by immunofluorescent or immunohistochemical assay with anti-E (4B8, 8G3), NS1 (4C11) or anti-six histidine (Qiagen, Maryland, USA) monoclonal antibodies. A; pBlueprME transfected Sf 9 cells. B; pBlueE transfected Sf 9 cells. C; pBlueNS1 transfected *Sf*-9 cells. D; pBlueE transfected *Sf*-9 cells stained with anti-six histidine Mab. E; pBlueprME transfected *Sf*-9 cells stained with peroxidase linked assay. F; Non-transfected *Sf*-9 cell as a control.

Table 3. Hemagglutination activities of three recombinant proteins from supernatants and cell lysates

Proteins expressed	Supernatant	Cell lysate*
prME	<1:2	1:4
E	<1:2	<1:2
NS1	<1:2	<1:2
Hi-5 cell	<1:2	<1:2

*Hi-5 cells were cultured in 1 liter spin flask, and suspended in 15 ml of PBS.

intracellular form within insect cells. The purified prME, E and NS1 proteins were measured about 4-11 mg from 2 X 10^8 Hi-5 cells (800 ml culture).

Immune response of mice given recombinant prME, E and NS1 proteins

To evaluate the immunogenicity of recombinant proteins, groups of 13 mice were immunized with prME, E, NS1, prME+E or inactivated vaccine emulsified with IMS1313 adjuvant, respectively. After the second booster dose the mice were bled for measuring of neutralizing antibodies. Low-level neutralizing antibodies were demonstrated in sera from mice given baculovirus-recombinant prME or E protein and inactivated vaccine (Fig. 6). Although SN titers before the virulent virus challenge were low at 1 : 2 to 1 : 4 in the prME, E or prME+E immunized mice, SN titers from all the immunization groups were increased from 1 : 4 to 1 : 32 after the lethal JEV challenge. No detectable neutralizing antibody response was observed in the control group.

Immunoglobulin subtypes elicited by immunization with the recombinant proteins

To evaluate whether the immunization of recombinant proteins may affect isotypes of immunoglobulins, three

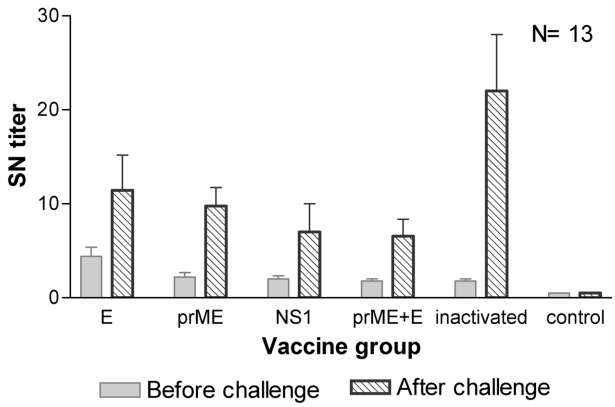


Fig. 6. Comparison of SN titer of immunized mice before and after lethal challenge with JEV. Following the third immunization with each antigen, mice were challenged with 100 LD_{50}/0.5 ml of virulent JEV. Serum neutralization antibodies were tested at day 0 and day 15 after challenge exposure.

immunoglobulin subtypes of IgG1, IgG2a and IgG2b were measured from sera collected before and after the challenge of virus. Following the second booster dose, the IgG1 antibody levels were increased in the E and prME immunized groups (Table 4). The titers of IgG1 antibodies were approximately two and three times higher than those of IgG2b antibody as compared to the control group. Mice immunized with the inactivated JEV vaccine induced relatively low titers of IgG2a and IgG2b antibodies. However, the IgG2a antibody level increased significantly in most of the immunized groups at 2 weeks after the challenge.

Protection of the immunized mice against lethal JEV

In order to investigate whether baculovirus expressed recombinant proteins were biologically functional, groups

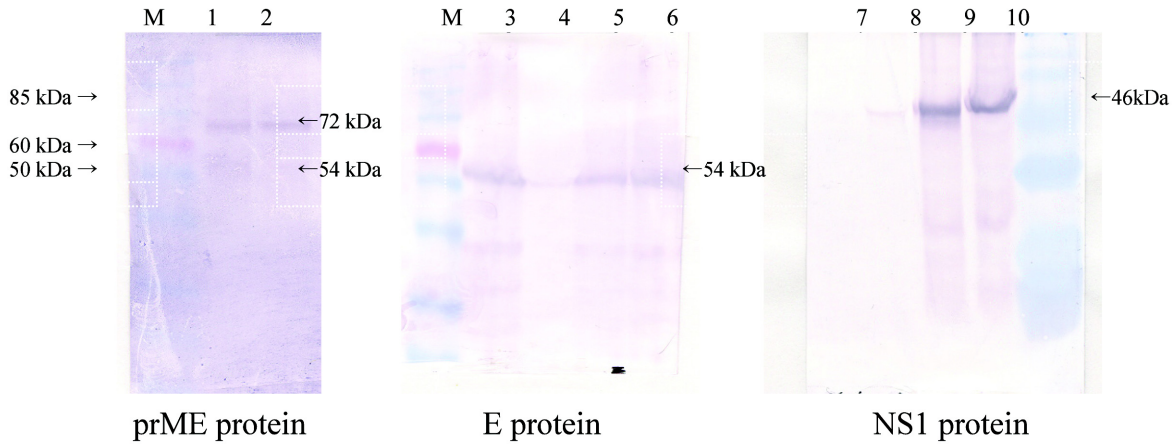


Fig. 5. Western blot analysis of prME, E and NS1 proteins expressed in Hi-5 cells. Western blot was performed under native condition of Hi-5 cell infected with recombinant baculovirus containing prME, E and NS1 genes, respectively. The sizes of prME protein reacted with Mab (8G3) was 72 and 54 kDa. The E and NS1 proteins showed 54 and 46 kDa, respectively. Lane 1 and 2; prME protein, lane 3, 4, 5 and 6; E protein, lane 7; NS1 supernatant, lane 8, 9 and 10; NS1 protein.

Table 4. JEV specific serum IgG subclass levels were determined after immunization and JEV lethal challenge

Immunogen*	IgG1		IgG2a		IgG2b		Ratio of IgG1/IgG2a
	Before**	After**	Before	After	Before	After	
E	0.81±0.11	0.52±0.16	0.26±0.06	0.28±0.10	0.36±0.13	0.33±0.06	3.1:1
prME	0.55±0.32	0.45±0.12	0.15±0.01	0.30±0.05	0.31±0.14	0.33±0.09	3.7:1
NS1	0.20±0.03	0.45±0.04	0.14±0.01	0.25±0.02	0.15±0.01	0.27±0.02	1.4:1
prME+E	0.52±0.16	0.57±0.24	0.16±0.01	0.26±0.07	0.31±0.15	0.34±0.13	3.3:1
Inactivated	0.34±0.03	0.53±0.12	0.11±0.01	0.26±0.06	0.18±0.06	0.37±0.04	3.1:1
Live	NT	0.34±0.12	NT	0.24±0.05	NT	0.23±0.05	-
Control	0.10±0.03	0.12±0.12	0.11±0.01	0.14±0.01	0.12±0.01	0.10±0.02	-

*cell lysate from Hi-5 cells.
**JEV specific serum IgG subclasses were determined at 2 weeks after the third immunization (Before) and 2 weeks after challenge (After), respectively. Antigen-specific ELISA reported as the optical density at 405nm at a serum dilution of 1:50.

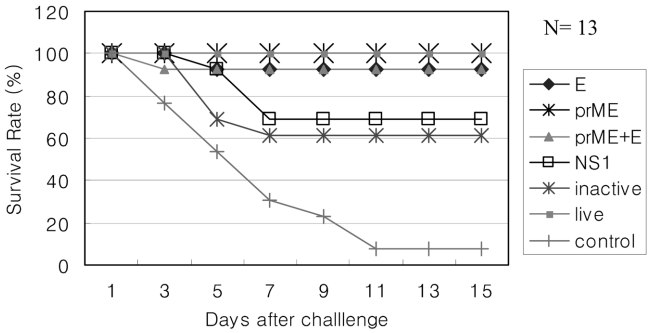


Fig. 7. Survival rate of the immunized mice against JEV challenge. After immunization with the recombinant proteins or JEV, mice were challenged with 100 LD_{50}/0.5 ml of virulent JEV. Percentages of surviving mice in each immunization group are shown at each day post-challenge.

of mice were immunized with either recombinant proteins, live or killed JEV. One week after the second immunizing boost, the mice were challenged with a virulent JEV strain, Nakayama strain (100 LD_{50}/0.5 ml).The results showed that the survival rates of mice immunized with prME or E recombinant glycoproteins were significantly increased over that of control mice as shown in Fig. 7. Over 90 percent of the mice, which received the prME + E or only E glycoproteins were survived against the lethal challenge of JEV. While mice immunized with the NS1 recombinant protein and BEI-inactivated JEV showed 69.2 and 61.5% protection, respectively. Mice immunized with the prME protein and live vaccine showed a complete (100%) protection. In contrast, only 1 (7.7%) out of 13 was survived for 15 days in control group (Fig. 7).

Discussion

In JEV, three viral proteins (prME, E and NS1) have been reported to be capable of inducing protective immunity. The prM protein is part of the immature virion and its proteolytic cleavage generates mature virion [19]. The E protein, a major structural protein of *Flavivirus* virion, appears to play a dominant role in the receptor binding, generation of neutralizing antibodies and induction of a protective immunity [11,20]. In addition to the structural proteins, NS1 protein is also able to elicit a protective immune response during the course of JEV infection in mice [2,7]. However, the role of NS1 in protection against the disease has been controversial [5,13]. Recently, the results from DNA-based vaccination showed that all prM, E and prME proteins were found to play a dominant role in disease protection [5,23].

A number of expression systems have been reported for the JE viral recombinant proteins. Expression of the recombinant E protein has been achieved in several hosts such as *Escherichia coli* [18], insect cells [17,20], yeast [28], and mammalian cells [10]. Although JE viral proteins have been expressed successfully in *Escherichia coli*, the proteins did not elicit neutralizing antibody and protective immunity [18]. This might be due to lack of properly folded and correctly assembled recombinant protein. Recombinant vaccinia virus expressing prM and E proteins alone was highly effective at eliciting protective immunity against JEV challenge in both mice [17] and pigs [12]. Among the viral expression systems, the baculovirus-insect cell expression system provides the advantages of high level expression of the recombinant proteins with proper folding and co- or post translational processing [24].

In this study, the specific genes of KV1899 strain, which belongs to the genotype I were cloned for expression of recombinant proteins. We expressed three recombinant JEV proteins (prME, E and NS1) in insect cells by using the pBlueBac 4.5/V5-His transfer vector. Immunoreactive bands were observed in Western blotting with JEV monoclonal antibodies against E and NS1. Two protein bands (72 and 54 kDa) were shown in prME protein produced by the recombinant virus, suggesting that prME may be undergone to post-translational proteolytic cleavage as reported previously [20]. Expression of the prME, E and NS1 proteins in insect cells was found to remain intracellular. It is possible to expect that anchor region of the E protein acts to retain the

cell association, since the anchor-deleted E proteins of dengue virus were reported to become secretory in insect cells [17].

In order to examine the immunogenicity of recombinant proteins, we immunized the mice with the expressed proteins. Recombinant E protein group acted as better immunogenic than only prME and prME + E groups by SN test (Fig. 6). Compared with inactivated vaccine, the baculovirus expressed proteins were equally antigenic, but elicited only low levels of serum neutralizing antibodies. From these results, although JEV proteins expressed by the recombinant baculovirus showed the weak immune responses, they induced sufficient priming activities for protection. In survival test, recombinant prME protein and live vaccine group showed the complete protection. The prME and E protein groups proved to be a better immunogen in protection against lethal JEV challenge than either inactivated vaccine or NS1 protein. The protection rate mediated by the recombinant E protein was clearly superior to that of E protein expressed in *E. coli* [4]. The absence of glycosylation in *E. coli* might be a possible reason for this difference. The prM gene was included in the prME recombinant plasmid construct, because co-expression of the prM protein is related to maintenance of proper conformation of the E protein and protection of the E protein from irreversible conformational changes in the acidic compartment of the secretory pathway [7,15]. However, in this study the E and prME + E groups were not protected completely after challenge. It was assumed that less glycosylation or low level of E protein was produced in Hi-5 cells. In this regard, Wengler *et al*. [31] presented that the virion associated E glycoprotein of another *flavivirus* such as West Nile encephalitis virus, exist as a trimer, clearly these questions require further study.

The IgG antibody isotype produced as a consequence of immunization reflects the type of T-helper cell involved in immune responses. IgG2a was produced as a consequence of Th1-cell activation which associated with the development of cell mediated immune response. Th2-cells are considered to be the major helper phenotype supporting of IgG1, IgG2b and IgA in the mouse system [1]. Although IgG2a antibodies were previously reported as the major antibody subtype for neutralizing dengue virus, our results revealed that immunization of prME and E proteins induced a higher titer of IgG1 than IgG2a antibodies, indicating Th2-cell responses. In addition, the IgG2a levels of the mice immunized with recombinant proteins were somewhat increased after challenge. This result is in agreement with a previous report [4].

In summary, our results demonstrated that JEV recombinant proteins expressed in *Sf-9* cells stimulated the immune responses capable of mediating significant protection of mice against JEV. Although the SN antibody titers induced by 3 recombinant proteins and inactivated JEV were nearly same levels, the protection rates after challenge varied significantly. So it was thought that specific proteins play a more important role in JEV immunity. This should allow easier monitoring of expressed antigens during the complex purification process required for a vaccine. In further study, animal trial should be done in swine to evaluate the feasibility of application of recombinant proteins. The results presented here may provide valuable information for further developing subunit vaccines to JEV. The expressed recombinant proteins also could be used for the development of diagnostic kits such as ELISA for JE diagnosis.

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