Development of a Japanese encephalitis virus-like particle vaccine in silkworms using codon-optimised prM and envelope genes

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Abstract

We have successfully prepared a Japanese encephalitis virus (JEV) – Nakayama virus like particle (NVLP) vaccine using synthetic codon-optimized prM and E genes. The expression of the recombinant JEV Nakayama-BmNPV (JEV-NNPV) virus was determined in infected silkworm Bm-N cells by fluorescence and Western blot analysis. The recombinant was inoculated into silkworm pupae and the yield of Nakayama VLP (NVLP) reached a peak in the homogenates after 3 days. Additionally, in the peptide analysis of infected pupae homogenate, it appeared approximately 300–500 µg E protein/pupa were produced. When purified the above eluates on the discontinuous sucrose density gradient centrifugation,
NVLP showed a strong hemagglutination (HA) activity by using chicken red blood cell in phosphate-buffered saline (PBS) free from Mg$$^{++}$$ and Ca$$^{++}$$ ions. The immune antisera against NVLP strain could efficiently neutralize the plaque formation of Nakayama, Beijing-1 and Muar strains, showing tendency of much higher reaction with heterologous Muar strain than homologous Nakayama strain. Our findings suggest that the JEV-NVLP may be useful for JEV epidemic control in many endemic areas of Asian countries as a widely effective and less expensive JE vaccine.

Keywords: Immunology, Vaccines, Infectious disease

1. Introduction

The first confirmed epidemic of Japanese encephalitis disease was reported in Japan and Korea in 1924, damaging that 6000 or more patients infected and 60% of them died at the outbreak [1]. In 1935 and 1949, the causative viruses were isolated from patients in Tokyo and Beijing, and they were serologically, virologically characterised and designated the Nakayama and Beijing-1 strains, respectively. The ecological background of JEV and its transmission through mosquitoes has been investigated in detail [2, 3]; however, JEV epidemics caused the death of approximately another 5,000 individuals before the mid-1960s [4]. Owing to ecological maintenance of the swine industry [2], the use of mouse brain-derived JE vaccine was enforced by the Japanese government in 1954 [1], the incidence of Japanese encephalitis has decreased dramatically. However, Japanese encephalitis is still a major health threat, with numbers of patients showing symptoms in recent years [5, 6, 7], and JE vaccines have been distributed throughout many parts of the world to combat this disease [8, 9]. Notably, despite the use of effective vaccines, JEV has actively and silently circulated in the mosquito-swine ecological system. Moreover, the incidence of Japanese encephalitis has increased in many parts of Asian and other Pacific countries [10, 11].

In recent years, the quality of the JE vaccine has been greatly improved by introducing the different biotechnologies including Vero cell derived vaccine production, transfected cell lines, etc. [8, 12, 13, 14, 15, 16, 17]. JE vaccines produced in Vero cells have been used as safe and efficacious vaccines [15]. Similarly, adjuvant Vero cell-derived vaccines have been reported to elicit potent antibody responses in mice and horses [16]. Additionally, potent antibody responses and enhanced expression of JEV-E protein have been reported in a Drosophila cell line [17]. Another type of JEV-VLP vaccine has also been produced in cloned BHK cells following genetic engineering, and the resulting cells showed increased JEV-E protein production [18]. Moreover, several JEV-VLP vaccines have been produced using the baculovirus system [19, 20, 21].
However, despite the development of the above-mentioned vaccines, novel vaccines that are inexpensive, safe, and efficacious are still needed in developing countries. On the other hand, the molecular mechanisms of viral morphogenesis and the role of protective JEV E antigen have been well characterized [22]. In the 11-kb, single-stranded, positive-sense RNA JEV genome, a single, long open reading frame (ORF) synthesises a single polyprotein, which is then cleaved into 11 structural (C, pr M, E) and nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins.

In this study, we describe the biological, morphological, and immunological characteristics of a VLP vaccine containing the E protein of JEV Nakayama strain based on its important role as a protective antigen [22].

2. Materials and methods

2.1. Cells and viruses

Silkworm Bm-N cells were maintained in TC-100 medium and minimal essential medium containing 10% foetal calf bovine serum. For construction and expression of target vaccine DNA, the P6E strain of the baculovirus *Bombyx mori* nuclear polyhedrosis virus-BmNPV [21] was used to generate the recombinant baculovirus-JEV Nakayama virus. Baculovirus was multiplied in Bm-N cells. Vero cells (cat. no. JCRB 9013, lot no. 11292002; Japanese Collection of Research Bioresources [JCRB] cell bank) were also used for propagation of the Nakayama, Beijing-1, and Muar JEV strains, which were obtained from the National Institute of Infectious Diseases (Tokyo, Japan). The strains were propagated in Vero cells in MEM containing 10% calf foetal serum.

2.2. Construction and generation of recombinant JEV-NPV

The methods for construction and preparation of JEV-Nakayama-BmNPV (JEV-NNPV) were described previously [23]. To enhance expression in silkworms, prM/E genes were codon-optimized for silkworms as described previously. Synthesis of the designed prM/E DNA was carried out by GENEWIZ (Saitama, Japan). The resulting DNA was inserted into the pBM-8 plasmid as described previously [24] using the In-Fusion technique (Clontech, Mountain View, CA, USA) to generate pBM-8-JEV-NNPV. Subsequently, pBM-8-JEV-NNPV and linearized BmNPV-NNPV DNA were cotransfected into serum-free cultured Bm-N cells. After 5 h of incubation, the medium was changed to foetal calf serum-enriched TC-100 medium, and the cells were incubated for an additional 5–7 days. The resulting medium was obtained for subsequent virological examination. The harvested medium was centrifuged at 3,000 rpm for 5 min to separate the sedimented cell debris. The resultant supernatant was used as recombinant virus (JEV-NNPV).
2.3. Fluorescence imaging and western blot analysis

Bm-N cells were infected with recombinant JEV-NNPV baculovirus harbouring the codon-optimised JEV-E gene and maintained in MEM containing 10% calf serum at 25 °C in a 5% CO₂ incubator for 1–3 days. After confirmation of the infection based on cytopathic effects, Bm-N cells on the cover slip glass were washed in phosphate-buffered saline (PBS), fixed, and permeabilised. The cells were then treated with mouse or rabbit antiserum against JEV, followed by treatment with fluorescein isothiocyanate (FITC)-conjugated anti-mouse or rabbit IgG antibodies (West Grove, PA, USA). For western blot analysis, goat polyclonal anti-JEV rabbit serum was obtained from NIID (Tokyo, Japan), and FITC-conjugated rabbit anti-goat IgG-AP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. The final signal was detected using ECL plus reagents (GE Healthcare, Port Washington, NY, USA).

2.4. 50% Plaque Reduction Neutralization Test (PRNT₅₀)

Neutralizing antibodies to JEV were measured by PRNT₅₀ in Vero cells. Each JEV strain was mixed by equal volume with four-fold serial dilutions (1:40 to 1:10,240) of sera from rabbit or mice immunized with VLP antigens, and then incubated at 35 °C for 90 min. Vero cell monolayers were inoculated with these mixtures in 6-well plates and incubated at 35 °C for 90 min. Subsequently, overlay medium containing 1% methylcellulose was added and the cells were incubated at 35 °C for 4–6 days. The cells were fixed using a 3.7% formalin/PBS solution and stained using methylene blue. The PRNT₅₀ titre, expressed as the logarithm (log₁₀) of the reciprocal serum dilution, was calculated based on plaque numbers compared with those in the control wells, containing only a virus-diluent mixture (non-serum control).

2.5. Extraction of JEV-NVLP from silkworm pupa homogenates

The recombinant baculovirus (JEV-NNPV) consisting of codon-optimized DNA of JEV Nakayama strain was inoculated into silkworm pupa. After three days, 0.01% formalin and phenylthiourea were added, and the inactivated pupae were then homogenised with three cycles of sonication. The homogenates were centrifuged at 16,000 rpm for 30 min. Estimation of E protein was carried out by slab gel electrophoresis (e-PAGEL; Atto). The resulting supernatant was further centrifuged for 4 h [24] and subjected to the electrophoresis.

2.6. Purification of VLP antigens

The silkworm pupae homogenates were first centrifuged at 16,000 rpm for 30 min using SW28 rotor with a Beckman centrifuge, and partially purified by centrifugation at 32,000 rpm for 16 h on a 10–50% (w/w) discontinuous sucrose
gradient in SW 50.1 rotor. The resultant sucrose gradient specimen which produced 20 fractions was subjected to the sucrose concentration, protein concentration and HA activity analyses.

2.7. Hemagglutination test

In this study, we found a phenomenon that silkworm derived NVLP could apparently agglutinate chicken RBC in PBS free from Mg\(^{++}\) and Ca\(^{++}\) ions (PBS (−)) and we carried out the HA test by using a chicken RBC which prepared by common PBS (−) (pH 7.4). NVLP samples were diluted 2-fold serially with 50 μL PBS (−) in 96 microtitre plates, and 50 μL of 0.5% (v/v) chicken RBC in PBS (−) was added and mixed well. After 30 min at room’s temperature, the HA plates were read as previously described [24].

2.8. Immunization with the JEV-NVLP vaccine

Mouse and rabbit experiments were approved and carried out in accordance with the Fundamental Rules for Animal Experiments and the Guidelines for Animal Experiments Performed at The Institute of Biological Resources published by the Animal Welfare and Animal Care Committee including Animal Ethics Committee of the Institute of Biological Resources, Okinawa, Japan. The partially purified JEV-NVLP was used for immunisation in rabbits and mice. JEV-E protein contents were adjusted to 20 μg per dose into a mouse (inbred ddY, female, 4 weeks old; Japan SLC) and 40 μg per dose in a rabbit (Std: NZW, female, 10 weeks old, Japan SLC), as adjuvant or incomplete adjuvant vaccines. In the first immunization (first dose), NVLPs were mixed with complete adjuvant. In the second immunisation (second dose), incomplete adjuvant vaccine was used after a 2-week interval. A third immunisation (third dose) without adjuvants was given after 4 weeks, and then, blood samples were collected after 2 weeks.

3. Results

3.1. Construction and expression of the target DNA coding structural gene

The target DNA for vaccine production was designed to include the complete pr M, and target E genes. When we designed the chimeric DNA, assumed the pr M protein may play an important role in morphogenesis and that introduction of silkworm-derived codons may enhance the expression of the E protein. Based on these assumptions, 2001 silkworm codon-optimised E genes were designed. Of these, 1557 (77.8%) sequences were derived from the authentic E gene of human JEV-Nakayama strain and the remaining 444 (22.2%) sequences were designed to include original silkworm codons which were frequently used in silkworm (Fig. 1).
As shown in Fig. 2A, recombinant JEV-NNPV expressed E protein, demonstrating E protein-specific fluorescence of JEV monoclonal antibody (a) and JEV rabbit antiserum (c). Negative control did not show any fluorescence (b, d). Apparently, the expression of JEV Nakayama E protein in the cell lysates was determined by western blot analysis and it appeared to increase with time (Fig. 2B(a)). The reason that E protein was undetected in the culture fluids was more likely due to lack of a signal peptide in the front of prM protein (Fig. 2B(b)).

3.2. Production and purification of VLPs in silkworm pupae

The recombinant JEV-NNPV was injected into silkworm pupae, and the expression of E protein antigen was examined based on ELISA, western blot and slab gel electrophoresis analyses. As shown Fig. 3A(a), ELISA titres peaked on day 3 after infection. Also, marked expression of the E protein was observed on day 3 by western blot analysis of purified VLPs (Fig. 3A(b)). The content and position of E protein included in a large number (30) of pupae homogenates were confirmed by slab gel electrophoresis shown in Fig. 3B(a) and the migration profiles of the peptides from infected pupae homogenates (Fig. 3B(b)). The result was also reflected in the proportion (%) data of Exp. 2 (Fig. 3C) and its molecular weight was estimated 53 kDa similar to that of JEV control (Fig. 3A(b) and 3B(a)). To enhance E protein yields, the JEV-NVLP recombinant was further inoculated into 30 silkworm pupae. After three cycles of pupae homogenates-sonication, 9,480 μg of E protein was recovered from the homogenates of 30 pupae (Fig. 3C). From tentative calculations, each silkworm pupa appeared to produce more than 300 μg.
E protein. Furthermore, in the second experiment, 15,840 μg E protein was recovered from 30 pupae, resulting in production of 528 μg E protein/pupa.

To establish the first level purification method, the pupae homogenate obtained from the centrifugation at 16,000 rpm for 30 min was partially purified using small scale sucrose gradient. For example, the harvested homogenates, the resulting eluates were further centrifuged on a 10–50% (w/w) linear sucrose gradient in SW50.1 roter, producing 20 fractions. The fractions were characterised by the sucrose concentration, protein concentration and HA activity tests as shown in Fig. 4A. The centrifuged pupae-homogenates showed two protein peaks in fractions 5 (first) and 12 (second) (Fig. 4A). The first peak located in the 40% sucrose fraction supported the possible presence of the heavy VLP based on HA activity (Fig. 4A) and the migration profile in the electrophoresis (Fig. 4B). The second peak may have been derived from the host components because definite E protein was not detected in the second peak (Fig. 4B).

Fig. 2. Confirmation of the expression of JEV-NVLP E protein by fluorescent antibody analysis. A, (a), anti-JEV monoclonal antibody treated and recombinant-infected (JEV-NNPV) BmN cells. (b), noninfected BmN cells (negative control). (c), recombinant infected and anti-JEV rabbit antiserum treated cells. (d), normal rabbit serum treated cells (negative control). B, Determination of the expression of JEV-Nakayama E protein by western blot analysis. (a), pupae cell lysate and (b), supernatant specimens. JEV-C and MM represent JEV-control and molecular weight marker, respectively.
3.3. Immunogenicity and immune responses of JEV-NVLP

We investigated the immunogenicity of the JEV-NVLP vaccine produced in silkworm pupae by measuring antibodies produced in rabbits. Plaques produced in Nakayama strain-infected Vero cells were extremely small, as compared with Beijing-1 and Muar strains (Fig. 5A).

Immune responses of JEV-NVLP was examined in rabbits immunized with three doses of NVLP vaccine. Antiserum against JEV-NVLP showed significant PRNT$_{50}$ mean titres ($\log_{10}$), 3.9, 3.1 and 4.7 against three challenge viruses, homologous Nakayama, heterologous Beijing-1 and Muar strains, respectively. Notably, the antiserum to Nakayama VLPs had a much higher titre against
Fig. 4. Partial purification of NVLP specimens on 10–50% (w/w) linear sucrose gradient and fractionated into 20 fractions. A, Fractions were examined by the sucrose concentration, protein concentration and HA activity tests. B, Protein migration profile analyzed in the electrophoresis.

Fig. 5. Plaque neutralization tests with antibodies produced in rabbits immunized with NVLP antigens. A, Plaque phenotypes of the JEV Nakayama, Beijing-1 and Muar strains in Vero cells. B, PRNT_{50} titres of NVLP-rabbit antibody against three challenge viruses. C, Antibodies production elicited by immunization of one and three doses of JEV-NVLP antigen in mice.
heterologous Muar strain (4.7) than that of homologous Nakayama strain (3.9) (Fig. 5B).

Next, we further investigated the immune responses of JEV-NVLP in mice immunised with one and three doses of NVLP vaccine. As shown in Fig. 5C, higher neutralising titre was observed for the JEV-Muar strain with three dose, like as observed in rabbits.

4. Discussion

In this study, we prepared large amounts of JEV-NVLP vaccine in silkworm pupae through successful construction of codon-optimised chimeric DNA and generation of recombinant JEV-NNPV. The NVLP product appeared to contain VLPs of approximately 30–200 nm in diameter covered with densely arranged short projections (data not shown).

The recombinant baculovirus JEV-NNPV produced large amounts of NVLP antigen in silkworm pupae; based on this yield, a suitable amount of E protein was also confirmed. For example, the yield of E protein extracted from pupae homogenates reached approximately 300–500 μg/pupa, with the protein identified as a 53-kDa peptide band. Notably, the production of JEV-E protein has recently been reported using a Drosophila cell line [15], baculovirus-insect cells, and human cells [16], with suitable yields. For example, the production of JEV-E antigen in recombinant insect cells reached about 20 μg/mL on day 6 after infection [17]. Additionally, Bu et al. [15] reported a production level of 15–20 μg/mL JEV-glycoprotein as a JEV-VLP vaccine in a human cell line. When compared with the above production, the yield (approximately 300–500 μg/pupa) of JEV-E protein in the present silkworm pupae appeared to be higher. Moreover, high antibody titres against JEV-NVLP were observed in rabbit and mouse.

For example, antibodies against JEV-NVLP produced in rabbits showed more than 3.9 and 3.1 PRNT_{50} titres (log_{10}) for homologous Nakayama and heterologous Beijing-1 strains, both GIII viruses. In contrast to this strain, the titre for the heterologous Muar (GV) strain was estimated to be 4.7.

JEV is classified into five genotypes (GI-GV) based on its genomic sequence [25], of them the GIII strain was most widely distributed and most frequently isolated in JE endemic areas until the 1990s. Therefore, all licenced inactivated and live-attenuated JE vaccines are derived from GIII JE viruses. However, the major genotype of JEV isolated in Japan changed from GIII to GI in the early 1990s [26] and in recent years, a similar genotype shift has been observed in south Korea, northern Vietnam, China, Taiwan, Thailand [27, 28, 29], and India since 2009 [30].
One GV JEV strain was first isolated from a patient with encephalitis in Malaysia in 1952, and it remained the only one instance of this genotype for over 50 years [27]. Recently, the GV JEV genome has been identified in Culxex mosquitoes in China in 2009 and in South Korea in 2010 and 2012 [31, 32]. A group in China succeeded in isolating a new GV strain from C. tritaeniorhynchus [32]. These findings lead to the possibility that other species of Culxex mosquitoes may be involved in the natural transmission cycle of the GV strain of JEV. Thus, GV JEV may be emerging in other endemic areas, and that is why it is necessary to be more focused on investigating the dynamics of circulating JEV strains and development of its vaccine used in these areas.

In summary, based on the above virological background, we have attempted to prepare potent JE vaccines for a variety of epidemic viruses including five genotypes (GI-GV). Thus, the silkworm system may be useful for large-scale preparation of effective and inexpensive JE vaccines for future emerging viruses.

**Declarations**

**Author contribution statement**

Sayaka Matsuda, Reiko Nerome, Kenichi Maegawa, Akira Kotaki, Shigeo Sugita, Kazunori Kawasaki, Kazumichi Kuroda, Ryoji Yamaguchi, Tomohiko Takasaki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kuniaki Nerome: Wrote the paper.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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