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Short communication

Cloning, expression and purification of envelope proteins E1 and E2 of western equine encephalitis virus and potential use of them as antigens in immunoassays

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Abstract

The genes encoding envelope proteins E1 and E2 of western equine encephalitis virus (WEEV) were respectively cloned into a prokaryotic T7 RNA polymerase-regulated expression vector. The recombinant C-terminal 6×His-tagged WEEV E1 and E2 were expressed in bacteria as inclusion bodies that were subsequently solubilized with 8 M urea, purified by immobilized metal ion affinity chromatography and finally refolded using an arginine system. The purified 6×His-tagged proteins showed 50 kDa bands as revealed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, consistent with the expected sizes of WEEV E1 and E2. The potential of the recombinant WEEV E1 and E2 as antigens for serologic tests to detect anti-WEEV antibodies for diagnosis of WEEV infection was assessed by an enzyme-linked immunosorbent assay with anti-WEEV polyclonal antibodies obtained from the mice infected with WEEV. The anti-WEEV antibodies bound the recombinant WEEV E1 and E2 in a dose dependent manner. On the contrary, antibodies against Venezuelan equine encephalitis virus with a genetic background and a disease spectrum very similar to WEEV, did not bind to the recombinant WEEV E1 and E2. Our results suggest that the recombinant WEEV E1 and E2 possess predominant antigenicity of WEEV and have the potential to be used as antigens in immunoassays to detect anti-WEEV antibodies for serological diagnosis of WEEV infection so as to eliminate the need for preparation of cell culture-derived viral antigens, which is time-consuming, expensive, laborious, tedious, and hazardous.

Keywords: Western equine encephalitis virus; Recombinant E1/E2; Prokaryotic expression; Antigens; Enzyme-linked immunosorbent assay

1. Introduction

Western equine encephalitis (WEE), a mosquito-borne disease of domestic animals and humans in

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North, Central, and South America, is caused by WEE virus (WEEV). WEEV has been responsible for large, periodic, and extensive epizootics and epidemics of encephalitis in equines and humans (Johnston and Peters, 1996; Reisen and Monath, 1988). WEEV poses threats to livestock industry and human health and is therefore classified as one of potential biological warfare and terrorism agents.

WEEV is a member of alphaviruses, which are a group of enveloped viruses with a positive sense, single-stranded RNA genome. A subgenomic positive-stranded RNA (the 26S RNA) is identical to the 3′ one-third of the genome and serves as the translational template for the structural proteins, capsid (C), E3, E2, 6 K and E1 (Strauss and Strauss, 1994). E1 and E2 project from the virus envelope as spikes and are directly involved in contact with the host immune system and elicitation of the host immune responses (Das et al., 2004). They are highly conserved among different WEEV strains (Nagata et al., 2006). Serological diagnosis of WEEV infection is mainly based on detection of anti-envelope proteins antibodies (Calisher et al., 1983). Typically, WEEV antigens for immunoassays are produced by infection of susceptible cultured cells with the virus followed by purification and inactivation of the virus, or extraction of viral antigens (Boctor et al., 1989; Reisen et al., 1996). Since WEEV is virulent, the process for production of the antigens may expose the personnel to the possible danger of WEEV infection and therefore requires biosafety level 3 (BSL-3) containment. This process is time-consuming, expensive, tedious, and laborious due to large volumes of mammalian cells and reagents conducted in BSL-3 containment. Furthermore, the antigens can vary in quantity from preparation to preparation and such antigen preparations may still be infectious and must be handled accordingly. An alternative way is to produce recombinant viral envelope proteins in a protein expression system without replication of the homologous virus (Kobayashi et al., 2000; Yamakawa and Furuuchi, 2001). Since recombinant proteins are non-infectious, they can be manipulated in non-specialized laboratories. Bacterial expression of recombinant proteins is one of the protein expression systems. Recombinant proteins can be expressed in large amounts in bacteria in a short time since bacteria grow fast. In addition, production of recombinant proteins in bacteria is inexpensive. Therefore, the bacterial expression system is widely used to produce recombinant proteins.

In this study, we respectively cloned the genes that code for E1 and E2 of WEEV strain Fleming into a bacterial expression vector and determined if the recombinant WEEV E1 and E2 would be suitable as antigens in an enzyme-linked immunosorbent assay (ELISA) for detection of anti-WEEV antibodies.

2. Materials and methods

2.1. Virus culture and purification

Seed stock of WEEV strain Fleming (American Type Culture Collections, ATCC) was made by inoculation of Vero cells (ATCC) with virus suspension at a multiplicity of infection of less than 0.1. All experiments with live virus were carried out in the Defence Research and Development Canada-Suffield (DRDC-Suffield) BSL-3 Containment facilities, in compliance with Health Canada and Canadian Food Inspection Agency Guidelines.

2.2. Extraction of viral RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

The total viral RNA of WEEV strain Fleming was prepared by the lysis of virus using the Qiagen Rneasy Mini kit. RT-PCR was performed using an One-Step RT-PCR kit (Qiagen, Mississauga, ON) with 0.1 μg of the viral RNA and a pair of primers (forward primer, 5′-AAGCTTCCGCCAAAATGTTTCCATACCCT-CAG-3′ and reverse primer, 5′-TCTAGAGTGTATAT-TAGAGACCCATAGTGAGTC-3′) flanking the open reading frame of the genes encoding the whole structural proteins. The reverse transcription reaction was performed for 30 min at 45 °C and then PCR was started by an increase in temperature to 95 °C for 15 min followed by 40 cycles of amplification (94 °C for 10 s, 68 °C × 30 s, and 68 °C × 4 min) and final extension (72 °C × 10 min). The purified PCR product was cloned into a pcDNA 3.1 TOPO vector (Invitrogen). The resulting plasmid was designated as pcDNA-WEEV-26S.

2.3. Construction of recombinant WEEV E1 and E2 expression plasmids

The genes encoding WEEV E1 and E2 were respectively amplified by PCR using E1 primers (forward, 5′-ATGTTCGAACATGCGACCACTGTG-3′ and reverse, 5′-TCTACGTGTGTTTATAAGCATA-GAGC-3′) and E2 primers (forward, 5′-ATGAGCAT-TACCGATGACTTCACA-3′ and reverse, 5′-TGCGT-TGGTTGGCCGAATACAGC-3′) from pcDNA-WE-EV-26S plasmid and then inserted at the upstream of 6×His tag sequence of a prokaryotic T7 RNA poly-merase-regulated expression vector, pCRT7/CT TO-PO (Invitrogen). Resultant plasmids, pCRT7WEEV E1 and E2 were verified by restriction analysis. E1 and E2 DNA sequences were further confirmed by DNA sequencing.

2.4. Expression, purification and refolding of recombinant WEEV E1 and E2

pCRT7WEEV E1 and E2 were respectively transformed into chemically competent *Escherichia coli* BL 21 (DE3) pLys cells. The recombinant WEEV E1 and E2 expressed in *E. coli* as inclusion bodies, were solubilized, purified, and refolded, as previously described, with minor modification (Hu et al., 2003, 2002). Briefly, after induction for 3 h by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C, *E. coli* transformants were harvested by centrifuga-tion. The cell pellet was resuspended and sonicated in urea buffer. The sonicate was centrifuged and the pellet was solubilized in 8 M urea. Purification of the recombinant WEEV E1 or E2 was performed on Immobilized Metal Ion Affinity Chromatography (IMAC) (Qiagen). The purified recombinant WEEV E1 or E2 was refolded in 1 M arginine buffer.

2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining and 6×His protein tag staining

In order to check the purity of the recombinant WEEV E1 and E2, the purified protein samples (1 μg each) were electrophoresed in 10% SDS-PAGE gels under reducing (5% 2-mercaptoethanol) condition. The bands were visualized by Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Mississauga, ON) staining.

In order to confirm 6×His-tagged proteins, the bands on another gel were stained using a Gelcode 6×His protein tag staining kit (Pierce Biotechnology, Rock-ford, IL), and then visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

2.6. ELISA

The antigenicities of the recombinant WEEV E1 and E2 were examined by an ELISA. Nunc maxisorp™ flat bottomed 96 well plates (Canadian Life Technologies, Burlington, ON) were coated overnight at 4 °C with the recombinant WEEV E1 or E2 at 10 μg/ml in carbonate bicarbonate buffer, pH 9.6. After blocking, the plates were incubated for 1.5 h with various dilutions (1:4860–1:20) of the mouse ascitic fluid containing anti-WEEV poly-clonal antibodies obtained from mice infected with WEEV strain Fleming or a mouse ascetic fluid without the WEEV infection (negative control). In addition, the plates were also incubated with various dilutions of the mouse ascetic fluid containing anti-WEEV strain B11 to check the strain specificity of the recombinant WEEV E1 and E2, and 50 μg/ml of anti-Venezuelan equine encephalitis virus (VEEV) monoclonal antibodies (1A4A1 and 5B4D6) to check the species specificity of the recombinant WEEV E1 and E2. After washing, the plates were incubated for 1 h with a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin antibody (Caltag Laboratories, Burlingame, CA). Finally, the plates were washed and developed for 30 min by a tetramethylbenzidine microwell peroxidase substrate system (Kirkegaard and Perry Laboratories, Gath-ersburg, MD). The reactions were read at an absorbance of 615 nm by a microplate autoreader (Molecular Devices, Sunnyvale, CA).

3. Results

3.1. Cloning, expression and purification of recombinant WEEV E1 and E2

The total viral RNA of WEEV strain Fleming was reverse-transcripted and then amplified by PCR. The

amplified PCR product was inserted to a pcDNA 3.1 vector. Afterwards, the gene encoding for WEEV E1 or E2 was amplified by PCR and inserted at the upstream of the 6×His-tag sequence of a pCRT7 bacterial expression vector. The DNA sequence of 6×His-tagged WEEV E1 or E2 was 1410 or 1362 bp in length, encoding 470 or 454 residues each with a molecular weight of ~50 kDa. The recombinant WEEV E1 or E2 was expressed in *E. coli*, purified via IMAC, and folded using an arginine system. SDS-PAGE with Coommasie blue staining and 6×His protein tag staining demonstrated that the molecular weights of the purified 6×His-tagged recombinant proteins were ~50 kDa, corresponding to the predicted sizes (kDa) of the recombinant WEEV E1 and E2 (Fig. 1). The yields of the purified recombinant WEEV E1 and E2 were respectively about 20 mg per liter of culture medium with the purity of about 90% and recovery rate of around 75%.

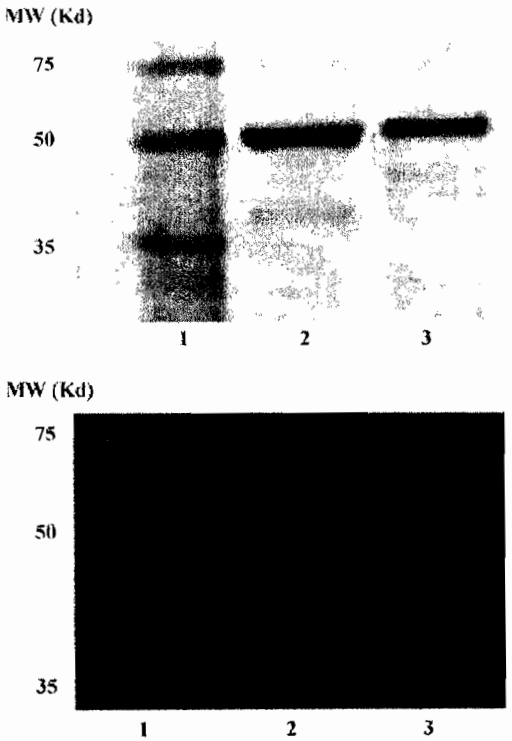

Fig. 1. SDS-PAGE with Coomassie blue staining (upper part) and 6×His protein tag staining (lower part) for analysis of recombinant WEEV E1 and E2. Lane 1, molecular weight; lane 2, recombinant WEEV E1; lane 3, recombinant WEEV E2.

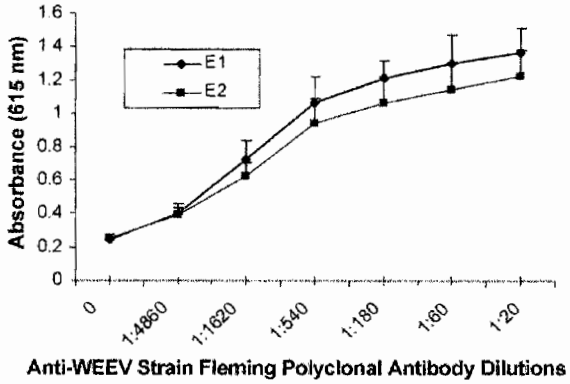

Fig. 2. Antigenicity analysis of recombinant WEEV E1 and E2 using anti-WEEV strain Fleming polyclonal antibodies by ELISA.

3.2. Antigenicities of recombinant WEEV E1 and E2

The antigenicities of the recombinant WEEV E1 and E2 were examined by ELISA with anti-WEEV polyclonal antibodies. The anti-WEEV strain Fleming polyclonal antibodies bound to the E1 or E2 in a dose dependent manner (Fig. 2). Both the LODs for the WEEV E1 and E2 to be used to detect anti-WEEV antibodies were about 1:5000. Meanwhile, anti-WEEV strain B11 polyclonal antibodies also bound to the recombinant WEEV E1 or E2 in a similar manner as anti-WEEV strain Fleming polyclonal antibodies did (data not shown). On the contrary, negative control and two anti-VEEV monoclonal antibodies did not bound to the recombinant WEEV E1 nor E2 (data not shown).

4. Discussion

The detection of specific antibodies by serological methods is very useful in the diagnosis of infection. The immune responses to E1 and E2 have been reported in models of experimental WEEV infections (Roehrig, 1993). During alphavirus infection, the envelope proteins E1 and E2 are the main targets of the host immune system (Mendoza et al., 1988). The E1 and E2 have the greatest potential as diagnostic reagents of the detection of anti-WEEV antibodies. Immunoassays employing the inactivated whole virus or extracted viral

proteins as antigens have been used widely to detect anti-WEEV antibodies for serological diagnosis of WEEV infection. Using the recombinant WEEV E1 and E2 as antigens for detection of anti-WEEV antibodies will eliminate the use of the inactivated whole virus or extracted viral proteins, which is difficult, slow, expensive, and hazardous to produce. In our study, the recombinant WEEV E1 and E2 from strain Fleming were expressed in a bacterial expression system due to low cost, high productivity, and rapid process of the bacterial expression system. The antigenicities of the recombinant WEEV E1 and E2 were examined by the ELISA using the anti-WEEV strain Fleming polyclonal antibodies. The recombinant WEEV E1 and E2 exhibited strong binding activity towards the anti-WEEV strain Fleming polyclonal antibodies, indicating that the recombinant WEEV E1 and E2 have kept the predominant antigenicity of WEEV strain Fleming. Different WEEV strains display very high nucleotide and amino acid identity. There is more than 96% nucleotide and amino acid identity in the structural protein genes among 8 different WEEV strains although these 8 strains can be divided into two groups by a cluster diagram (Nagata et al., 2006). Therefore, the recombinant WEEV E1 and E2 from strain Fleming were expected to be cross-bound by the antibodies against other WEEV strains. As a matter of fact, the polyclonal antibodies against B11, a strain from group B, was confirmed to bind to the recombinant WEEV E1 and E2 from strain Fleming in a similar manner as the polyclonal antibodies against Fleming, a strain from group A did. Obviously, these recombinant WEEV E1 and E2 from strain Fleming are not strain specific and might be used as antigens to detect anti-WEEV antibodies without strain specificity.

In addition, the species specificities of the recombinant WEEV E1 and E2 were confirmed by showing no cross-reactivity with the two monoclonal antibodies against VEEV, a virus with a genetic background and a disease spectrum very similar to WEEV.

Although glycosylation in the bacterial expression is not available and there are two glycosylation sites on WEEV E1 and E2, respectively (Pletnev et al., 2001), the recombinant WEEV E1 and E2 without glycosylation still remained high levels of the antigenicity of WEEV. Therefore, the recombinant WEEV E1 and E2 have the potential to be used as antigens in immunoassays for serological diagnosis of WEEV infection and for characterization of anti-WEEV antibodies. Further analysis of the recombinant WEEV E1 and E2 in experimental animal models may shed new light on their roles in the pathogenesis of WEEV infection.

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