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Evaluation of a Western Equine Encephalitis recombinant E1 protein for protective immunity and diagnostics

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Abstract

The E1 and E2 glycoproteins of Western Equine Encephalitis (WEE) are candidate antigens for WEE subunit vaccine development. We have cloned the E1 gene of WEE virus and expressed it in *Escherichia coli* as inclusion bodies. The inclusion bodies were successfully solubilised, refolded and the immunogenicity of this unglycosylated protein was assessed in mice. Immunization of mice with recombinant E1 protein generated both humoral and cell-mediated immune responses, indicating the recombinant E1 protein is immunogenic. Challenge of E1-immunized mice with live WEE virus demonstrated little or no protection from this *E. coli*-derived non-glycosylated subunit.

Keywords: WEE; E1 glycoprotein; Refolding; Humoral response; Cell-mediated immune response; Subunit vaccine; Diagnostics

1. Introduction

The Western Equine Encephalitis (WEE) virus is one of about 20 viruses that have been assigned to the alphavirus genus of the Togaviridae family. The new world alphavirus include WEE and this virus causes encephalitis in humans and severe disease in horses. WEE is endemic in Western North America although varieties have been isolated from different parts of the world. The alphavirus virion is approximately 71 nm in diameter and consists of four major components: the glycoprotein shell, the plasma membrane, the nucleocapsid core and genomic RNA (Tellinghuisen et al., 1999). All alphaviruses share a number of structural, sequence and functional similarities including a genome with two polyprotein gene clusters (Strauss and Strauss, 1994; Schlesinger and Schlesinger, 1996). The non-structural proteins are translated directly from the genomic RNA and are encoded by genes located along the first two-thirds of the 5′ end of the genome. A subgenomic positive strand RNA (26S RNA) transcribed from the negative strand RNA, is identical to the 3′ one-third of the genomic RNA and serves as the mRNA for a single structural polyprotein that is post-translationally cleaved into five individual proteins: capsid, E3, E2, 6k and E1 (Cancedda et al., 1975; Garoff et al., 1980; Schlesinger, 1980; Rice and Strauss, 1981). The envelope surrounding the nucleocapsid is a lipid bilayer derived from the plasma membrane of the host cell and carries the two virally encoded glycoproteins E1 and E2, both approximately 55 kDa. The E1 and E2 glycoproteins each contain two glycosylation sites (Pletnev et al., 2001), which undergo N-linked glycosylation with mannose-rich oligosaccharides (Sefton, 1977) in the lumen of the rough endoplasmic reticulum (Garoff et al., 1978; Bonatti et al., 1979). The envelope glycoproteins are key target proteins for the development of vaccine because they include epitopes that elicit neutralizing antibodies. One mechanism by which the immune system protects an animal from viral disease is the production of neutralizing antibodies, which prevent spread of the virus by interfering with viral binding or entry into uninfected cells.

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In this study, we report the successful cloning of the E1 gene of WEE, its expression in and purification from *Escherichia coli* inclusion bodies and the evaluation of the immunogenicity of recombinant E1 protein in mice. The E1 recombinant protein and E1-specific MAb could be used for the development of effective diagnostic and therapeutic measures against WEE.

2. Materials and methods

2.1. Bacterial strain, vector and chemicals

The vector pET22b+, *E. coli* BL21 (DE3) strain, Bugbuster™ and Benzonase™ were purchased from Novagen Inc (Madison, USA). The *E. coli* Top 10F strain was purchased from Invitrogen (Burlington, Canada). Oligonucleotides were synthesized by the Department of Biochemistry, University of Alberta, Edmonton, AB, Canada. The Taq DNA polymerase, T4 DNA ligase, Isopropyl *β*-D-1-thiogalactopyranoside (IPTG), DMEM, penicillin, streptomycin and L-glutamine (PSG) and FBS were purchased from Gibco BRL (Burlington, Canada). Restriction enzymes were purchased from New England Biolabs Ltd. (Mississauga, Canada). Acrylamide: bisacrylamide, pre-stained low range protein molecular weight markers and Biorad protein assay reagent were purchased from Bio-Rad Laboratories Ltd. (Mississauga, Canada). Glutathione (GSH and GSSG) was purchased from Roche Diagnostics (Laval, Quebec, Canada). Plasmid DNA isolation (QIAprep) and gel extraction kits (QIAquick) were obtained from Qiagen Inc (Mississauga, Canada). dNTPs, Hybond ECL nitrocellulose membrane and the ECL Western blotting kit were purchased from Amersham BioSciences (BaiedUrfe, Quebec, Canada). The Polymyxin B resins, TiterMax Gold adjuvant, MTT [(3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide)], mitomycin C, L-arginine and goat anti-mouse-horseradish peroxidase (GAM-HRPO) were purchased from Sigma (Oakville, Canada). [Diammonium 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate)] (ABTS) and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) peroxidase substrate were purchased from Kirkegaard & Perry Laboratory Inc (Gaithersburg, USA). The 11D2 anti-E1 WEE hybridoma was obtained from Defence Research and Development Canada–Suffield (DRDC Suffield), Alberta, Canada (Long et al., 2000a).

2.2. Construction of the recombinant E1 expression plasmid

All standard cloning methods were carried out as described by Sambrook et al. (1989). The E1 gene was PCR amplified from the original 26S clone in pcDWXH-7 vector (Netolitzky et al., 2000) using gene-specific primers (5′ Primer: 5′ CTC GCG GCC CAG CCG GCC ATG AGC TCC TTC GAA CAT GCG ACC ACT GTG 3′ and 3′ Primer: 5′ GAA TTG GCC TCG GGG GCC AAG CTT TCT ACG TGT GTT TAT AAG CAT 3′) designed from the published sequence (Hahn et al., 1988). The PCR mixtures contained 200 μM of each dNTPs, 0.4 pmole of each primer, 1 Unit Taq DNA polymerase, 5 μl 10× PCR buffer in 50 μl volume and PCR cycle was set at 94/55/72 °C, 1 min/45 s/1 min, respectively, for 30 cycles. The gel purified PCR product was digested with *Sac*I and *Hin*dIII and ligated with *Sac*I and *Hin*dIII digested *E. coli* expression vector pET22b+. The ligation mixtures were used to transform the *E. coli* Top 10F cells by electroporation (Gene pulser, Biorad) followed by 1 h incubation at 37 °C. The transformants were spread onto Luria Bertani (LB) agar (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, 1.5% Agar agar, pH 7.5) plates containing 100 μg/ml carbenicillin. Recombinant colonies were screened by plasmid DNA isolation (Qiagen plasmid DNA isolation kit) and digested with *Sac*I and *Hin*dIII. The digested products were analysed in 1% agarose gel electrophoresis for the correct size clones.

2.3. Functional clone analysis

The plasmid containing the correctly oriented E1 gene (pDS03) was used to transform *E. coli* BL21 (DE3) by heat shock method (Sambrook et al., 1989) for recombinant protein expression. *E. coli* BL21 (DE3) transformants were propagated in 10 ml LB medium containing 100 μg/ml of carbenicillin and incubated at 37 °C with shaking at 250 rpm until an OD_{600} of ∼0.4 was reached. The bacterial culture was induced with 1 mM IPTG and grown for another 3 h at 30 °C. The bacterial culture was harvested and total cell lysate was prepared by addition of sample buffer (50 mM Tris, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) to the pellet and heated at 95 °C for 5 min. Total cell proteins were analysed by SDS-PAGE using 10% polyacrylamide gel performed according to Laemmli (1970) with a Biorad Mini Protean II apparatus. The protein gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 30% methanol.

2.4. Western blot analysis

Total cell proteins were electrophoresed on SDS-PAGE and electroblotted onto the Hybond ECL nitrocellulose membrane according to Towbin et al. (1979) using Trans blot apparatus (BioRad) following manufacturer's instructions. The membrane was blocked with 5% skim milk in PBST (0.1% Tween 20 in PBS, pH 7.3) for 2 h. The membrane was washed four times with PBST and incubated for 1 h with 11D2 MAb (Long et al., 2000a) that specifically binds to E1 glycoproteins. After washing four times with PBST, the membrane was incubated with GAM-HRPO (1:10,000 dilution in 1% dialysed BSA) for 1 h. Finally, the membrane was washed again and enhanced chemiluminiscent (ECL)-based detection was performed according to manufacturer's instructions.

2.5. E1 protein expression and purification

Bacterial cultures were propagated and induced as previously described. To isolate periplasmic soluble protein, the cell pellet was suspended in 5% of original culture volume in periplasmic extraction buffer (50 mM Tris, 20% (w/v) sucrose, 1 mM EDTA pH 8.0) and incubated on ice for 45 min. Spheroplasts were centrifuged at 11,000 × *g* for 15 min at 4 °C and the supernatant containing periplasmic soluble proteins was collected. The remaining insoluble fraction was used to isolate inclusion bodies as described below. The periplasmic soluble and insoluble proteins were analysed by Western blot to determine the localization of expressed protein.

The insoluble fraction was suspended in Bugbuster™ reagent (5 ml/g of cell pellet), mixed and incubated in ice for 5 min. Benzonase™ (5 U/ml of Bugbuster™ reagent) was added and incubated at room temperature for 20 min with gentle shaking. The insoluble fraction was separated by centrifugation at 11,000 × *g* for 15 min at 4 °C. The pellet was washed twice with diluted Bugbuster™ reagent (1:10 in sterile water). Finally, inclusion bodies were solubilised in 50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 6 M urea buffer (5 ml/g of cell pellet) and incubated in ice for 1 h. The insoluble material and cell debris were separated from soluble denatured protein by centrifugation at 11,000 × *g* for 30 min at 4 °C and the supernatant was collected. Protein was quantified by Bradford's method (Bradford, 1976). Solubilised denatured protein was adjusted to 100 μg/ml with TA buffer (50 mM Tris, pH 8.0, 0.4 M L-arginine) and refolding was done in the presence of 1.0 mM GSH (glutathione, reduced), 0.1 mM GSSG (glutathione, oxidized) for 3 days at 4 °C. Final dialysis was done in PBS at 4 °C. Refolded proteins were separated by SDS-PAGE and analysed by Western blot using the E1-specific 11D2 MAb.

2.6. Enzyme Linked Immunosorbant Assay (ELISA)

The direct ELISA was done by coating of the antigen in Nunc 96-well ELISA microplates. Wells were coated with recombinant E1 antigen in PBS or in carbonate buffer, pH 9.6 containing 0.02% (w/v) sodium azide (10 μg/ml in 100 μl volume) overnight at 4 °C. The plates were washed three times with PBST and blocked with 1% dialysed BSA in PBS for 2 h at room temperature or for 1 h at 37 °C. After incubation, the plates were washed with PBST, serum samples serially diluted in PBS were added and plates were incubated for 2 h at room temperature. Plates were washed and incubated with GAM-HRPO (1:10,000 dilution in 1% dialysed BSA) for 1 h at room temperature. After the final wash with PBST, TMB peroxidase or ABTS substrate was added at 100 μl/well and OD_{650} or OD_{405}, respectively, was taken after 20–30 min using an ELISA V_{max} kinetic microplate reader (Molecular Devices Corp, California, USA).

2.7. Removal of bacterial endotoxin from E1 protein

Polymyxin B immobilized on agarose was used to remove bacterial endotoxin from protein solutions (Issekutz, 1983) according to manufacturer's instructions (Sigma). Briefly, Polymyxin B resin was washed with three to five times with 0.1 M ammonium bicarbonate buffer, pH 8.0 and packed in a 5 ml disposable syringe. The column was equilibrated and washed with 100 ml of the same buffer and the protein sample (pre-dialysed in the same buffer) was loaded onto the column. The protein samples were eluted with 0.1 M ammonium bicarbonate buffer, pH 7.8 and 1 ml fractions were collected. Finally, the eluted sample was dialysed against PBS, pH 7.3 and stored at 4 °C. But no confirmatory test was done to ensure LPS reduction or removal.

2.8. Virus culture and purification

The WEE strain 71V-1658 was kindly provided by Dr. Nick Karabatsos, Centers for Disease Control, Fort Collins, CO; WEE Fleming strain was purchased from ATCC (Mannanas, VA). Cell culture was maintained in accordance with established methods. Minimal essential media containing 5% fetal calf serum (5% MEM) was used to grow Vero (CCL-81) cells (ATCC). Seed stocks of WEE strains were made by inoculation of Vero cells with virus suspensions at a multiplicity of infection (MOI) of less than 0.1. The supernatants were clarified by centrifugation, aliquoted and stored at −70 °C. The WEE virus was inactivated according to the published method (Long et al., 2000b). All experiments with live virus were carried out in the Defence Research and Development Canada–Suffield Biological Level-3 Containment facilities following recommended guidelines from Health Canada and the Canadian Food Inspection Agency.

2.9. Immunizations of mice with recombinant E1 protein or inactivated WEE virus

BALB/c mice (6–8 weeks) were obtained from Health Sciences Laboratory Animals Services (HSLAS) of the University of Alberta, Edmonton, Canada. Animal treatment and care were carried out according to the Canadian Council of Animal Care guidelines or the Animal Care Committee at DRDC Suffield. Five mice were immunized intraperitoneally with 50 μg/mouse of endotoxin-free recombinant E1 antigen emulsified with an equal volume of TiterMax Gold adjuvant. Two weeks following primary immunization, mice were boosted with the same amount of antigen emulsified in TiterMax Gold adjuvant. After the 3rd and 4th week, mice were immunized with the same amount of antigen diluted in PBS. Control mice were immunized with only Titer Max Gold adjuvant.

For experiments in which mice were challenged with live virus, purified recombinant E1 protein or inactivated WEE virus vaccine (SALK WEE-inactivated vaccine) in PBS, were administered to 17–25 g BALB/c female mice by an

intramascular route of injection. A dose of 30 μg/mouse of E1 protein in a volume of 50 μl/mouse or 50 μl/mouse of SALK WEE-inactivated vaccine was diluted to a final volume of 100 μl/mouse. For the initial dose, the formulations were diluted 1:1 in TiterMax Gold and subsequent boosts were diluted in Hanks Balanced Saline Solution (HBSS). The immunization schedule was 0, 14 and 28 days, followed by virus challenge at day 49.

2.10. Evaluation of immune responses

Blood samples were collected from each mouse after the 5^{th} week by tail vein bleeding and serum was separated from cells by standard methods (Coligan et al., 1995). Antibody titers were determined by direct ELISA, as described earlier.

Spleens were removed aseptically 2 weeks following the final injection. Single cell suspensions were prepared by macerating the spleens in DMEM medium containing 1% (v/v) penicillin, streptomycin and L-glutamine. Cell suspensions were pelleted and the cell pellet was gently suspended in 5 ml ACK lysis buffer (0.15 M NaCl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). Cell suspensions were incubated at room temperature for 5 min, washed three times with DMEM medium containing 10% (v/v) FBS, 1% (v/v) PSG and suspended in complete DMEM medium containing 10% (v/v) FBS, 1% (v/v) PSG, 0.05 mM 2-mercaptoethanol and 0.2 ng/ml IL-2. For stimulator cells, cells from naïve mice were counted by haemocytometer and 2.5×10^7 cells were incubated with or without 10 μg/ml endotoxin-free E1 antigens for 1 h at 37 °C in a CO_2 atmosphere. After incubation, 50 μg/ml mitomycin C was added and cells were incubated for 20 min at 37 °C in a CO_2 atmosphere. At the end of the incubation, cells were washed with DMEM medium containing 10% (v/v) FBS, 1% (v/v) PSG three times, suspended in complete DMEM medium and diluted to 10^6 cell/ml. Responder cells from immunized mice were aliquoted to 96-well tissue cultures plate in triplicate at 10^6 cells/well. Amount of 100 μl/well of stimulator cells +/− antigen were added to each respective well and incubated for 3–4 days at 37 °C in a CO_2 atmosphere. Cell survival and cell proliferation assays were done using published methods (Mosmann, 1983). Briefly, 10 μl/well of a sterile, 5 mg/ml MTT solution was added to each well at day 4 of culture and plates were incubated for 4 h at 37 °C in a CO_2 atmosphere. Formazan crystals were solubilised by the addition of HCl–isopropanol solution and vigorous mixing. The plates were read at OD_{570} with OD_{650} reference to negate the effect of cell debris and precipitated proteins.

2.11. Challenge of WEE-immunized mice with live virus

Live virus was administered to the mice intranasally using a volume of 50 μl/mouse, containing 1.5×10^3 Plaque Forming Units (PFUs) diluted in HBSS. Mice were anaesthetized with sodium pentobarbital (50 mg/kg body weight), given intraperitoneally. When the animals were unconscious, they were carefully supported by hand with their nose up and the virus suspension in HBSS gently applied with a micropipette into the nostrils. The applied volume was naturally inhaled into the lungs. Infected animals were observed daily, for up to 14 days post-infection. The survival rates of the treatment and non-treatment control groups were compared using the two-tailed *t*-test and one-way analysis of variance, using Graph-Pad Prism ver. 2.0 (GraphPad Software, San Diego, CA). Values were considered statistically significant at P-values < 0.05.

3. Results

3.1. Plasmid construction and functional clone analysis

The entire structural gene (26S) of WEE has been previously cloned and sequenced (Netolitzky et al., 2000). The E1 glycoprotein gene was PCR amplified from the original 26S clone and cloned into pET22b+ vector in the *Sac*I and *Hin*dIII sites (Fig. 1). The E1 gene was cloned in the correct reading frame with the *pel*B leader sequence and under the control of the T7 promoter and *lac* operator. All the resultant recombinant clones were analysed by PCR and then by restriction enzyme digestion fragment mapping. Correct sized clones were selected for protein expression analysis.

The plasmid pET22b+ containing E1 gene was transformed into the *E. coli* expression host BL21 (DE3) and stable transformants were screened for the expression of E1. Six positive clones were tested to select for the best productive clone. To verify that the transformed cells expressed recombinant E1 protein, cells were induced with or without IPTG, and total cell lysates were prepared. It was observed from SDS-PAGE that the clones were expressing E1 protein at different levels but all with the desired band at ∼55 kDa (data not shown). The E1/5 clone was chosen for further

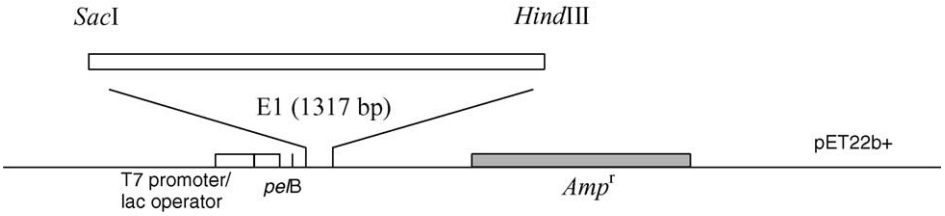

Fig. 1. Cloning of E1 gene in pET22b+. E1 gene was PCR amplified and cloned into the *Sac*I and *Hin*dIII sites of pET22b+ as described under Section 2.

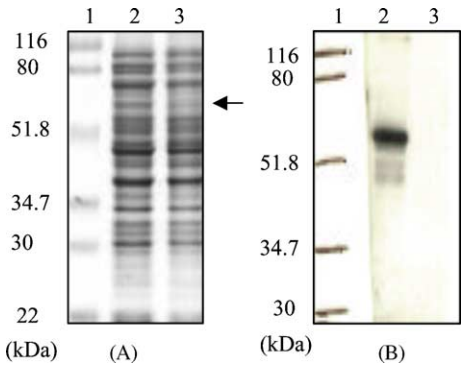

Fig. 2. Total cell proteins were separated on SDS-PAGE, stained with Coomassie Brilliant Blue (A) and blotted on nitrocellulose membrane and probed with 11D2 MAb followed by HRPO-conjugated goat anti-mouse IgG (B). Lane 1: pre-stained low range molecular weight markers, lane 2: induced culture and lane 3: uninduced culture.

analysis (Fig. 2A). Expression of the recombinant E1 protein was confirmed by Western blot analysis using the 11D2 MAb (Fig. 2B). This clone was selected for large scale of production and purification of recombinant E1 protein.

3.2. Purification of recombinant protein

To determine the subcellular distribution of the recombinant E1 protein, transformed cells were induced with 1 mM IPTG and both soluble and insoluble fractions were isolated, followed by analysis by SDS-PAGE and Western blotting. The expression of the recombinant E1 protein was almost exclusively in inclusion bodies. The periplasmic soluble extract of the induced culture and the total protein of the uninduced culture did not have detectable ~55 kDa bands in Western blots (Fig. 3A) indicating the majority of the recombinant E1 was contained in inclusion bodies. In addition to the E1 protein, several other bands were present, so the recombinant E1 protein was further purified using Bugbuster™ protein extraction reagent and Benzonase™, which is a nuclease. Finally, 6 M urea was used to solubilise the inclusion bodies and the denatured soluble protein was clarified by centrifugation. Renaturation of the protein was done by refolding in TA buffer in the presence of the GSH/GSSG (redox pair) over a period of 66 h at 4 °C. Finally, arginine was removed by dialysis on PBS at 4 °C. The refolded E1 protein was analysed by SDS-PAGE (Fig. 3B) and the 11D2 MAb showed strong binding to the E1 protein in Western blot (Fig. 3C). The smear below the 55 kDa band in Western blots may be degraded products and needs to be confirmed.

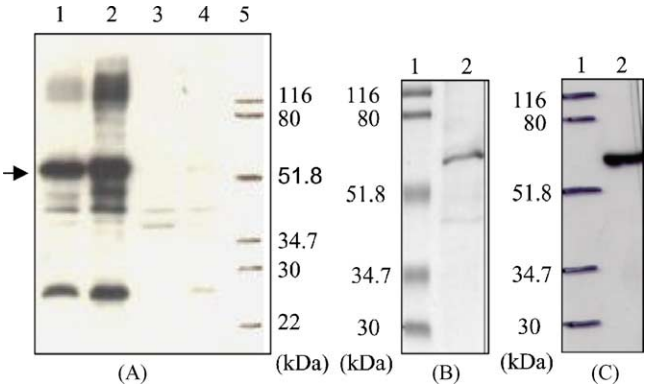

Fig. 3. Analysis of subcellular distribution of recombinant E1 protein by Western blot (A). Samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with 11D2 MAb followed by HRPO-conjugated goat anti-mouse IgG. Lane 1: total cell protein, lane 2: inclusion bodies, lane 3: periplasmic soluble protein, lane 4: total cell protein of uninduced culture and lane 5: pre-stained low range molecular weight markers. SDS PAGE (B) and Western blot analysis (C) of refolded E1 protein. Lane 1: pre-stained low range molecular weight markers and lane 2: refolded E1 protein.

3.3. Evaluation of serum from mice immunized with inactivated WEE virus with recombinant E1 protein

Mice immunized with inactivated virus (formalin-inactivated WEE vaccine) were completely protected against intranasal challenge with either WEE 71V-1658 or WEE Fleming strain. The recombinant E1 protein was tested for use as a solid phase diagnostic antigen to detect anti-WEE serum antibodies. Sera taken from these mice 2 days before challenge and titrated against fixed concentrations of E1 antigen exhibited very high antibody titers to the recombinant viral antigen (Fig. 4). It appears that the refolded recombinant E1 protein has significant relevant native epitopes recognized by inactivated WEE virus-immunized mouse serum. This ELISA data also demonstrated that the recombinant E1 antigen could be useful as a reagent for monitoring antibody

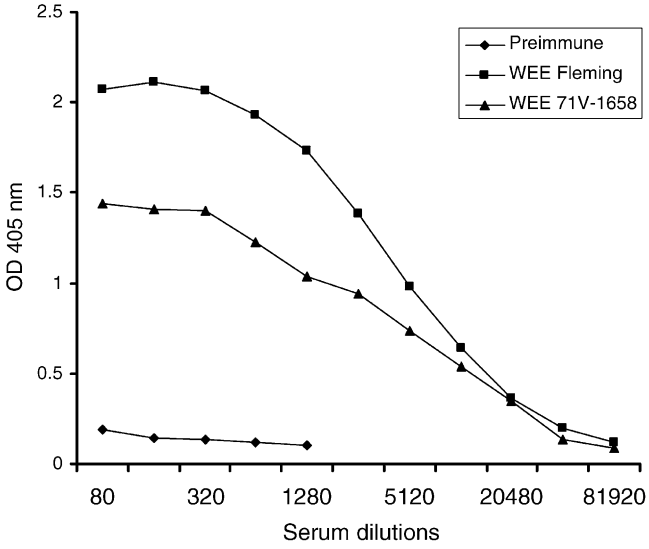

Fig. 4. Evaluation of serum from mice immunized with inactivated WEE virus with recombinant E1 protein. ELISA plates were coated with 10 μg/ml of recombinant E1 antigen and incubated with serially diluted mouse serum from animals prior to immunization (pre-immune) or after immunization with whole inactivated virus. Binding was detected with goat anti-mouse-HRPO and followed by ABTS solution and OD was measured at 405 nm.

titers in sera of individuals infected or vaccinated (as a confirmation of titers) with inactivated WEE virus. However, it is important to confirm this observation at very early time points following infection.

3.4. Immunogenicity of E1 recombinant protein in naïve mice

After removal of endotoxin from purified and refolded E1, the recombinant E1 antigen was injected into mice and both humoral and CMI responses were tested. Serially diluted serum from each mouse was titrated for anti-E1 antibodies using an ELISA with purified, recombinant E1 protein adsorbed to the solid phase. All E1-immunized mice developed strong antibody titers against E1 protein in comparison with mice immunized with only adjuvant (Fig. 5). These results showed that recombinant E1 protein induced very good humoral responses in mice.

Recombinant E1 protein also elicited a CMI response as demonstrated by the in vitro, antigen-specific cell proliferation of spleen cells. The splenocytes of each mouse in the group were specifically stimulated by the in vitro presentation of E1 antigen, as indicated by cell proliferation (Fig. 6) and responses were significantly higher than the control sample ($P < 0.01$). Thus, both humoral and CMI responses were strongly elicited against purified recombinant E1 antigen in BALB/c mice.

3.5. WEE challenge study

BALB/c mice were immunized intramuscularly with recombinant E1 protein or inactivated WEE virus vaccine then

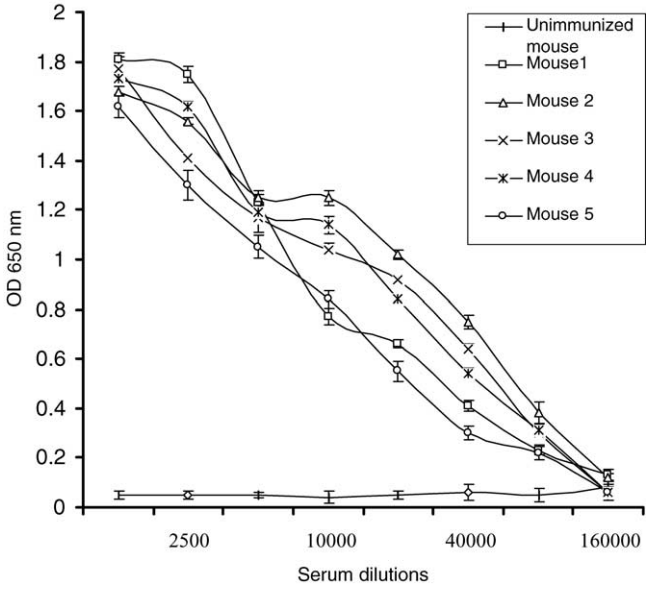

Fig. 5. Immunogenicity of recombinant E1 protein in naïve mice. ELISA plates were coated with 10 μg/ml of recombinant E1 antigen and serially diluted mouse serum was added. Binding was detected with goat anti-mouse-HRPO followed by TMB-peroxidase substrate. Each point represents the mean of three values.

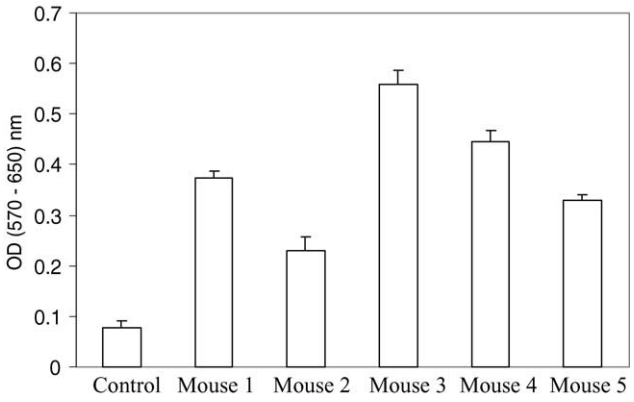

Fig. 6. CMI response to recombinant E1 protein. Splenocytes were isolated from immunized and naïve mice. Responder cells from immunized mice were aliquoted to 96-well tissue cultures plate in triplicate at 10^6 cells/well. 100 μl of stimulator cells +/− antigen were added to each respective well and incubated for 3–4 days at 37 °C in a CO_2 atmosphere. Amount of 10 μl/well of a sterile, 5 mg/ml MTT solution was added to each well at day 4 of culture and plates were incubated for 4 h at 37 °C in a CO_2 atmosphere. Formazan crystals were solubilsed by the addition of HCl-isopropanol solution and vigorous mixing. Optical density was taken at OD_{570} with an OD_{650} reference.

intranasally challenged with lethal doses of one of two different WEE virus strains. Control animals were immunized with only saline (mock vaccine). None of the control mice challenged with WEE virus survived at the end of 14 days. The recombinant E1 vaccinated mice resulted in only 25% survival (statistically not significant) when challenged with WEE 71V-1658 (Table 1). However, none of the mice survived when challenged with WEE Fleming strain. The survival rate for E1-immunized groups was the same at day 21. The E1 gene was cloned from the structural gene 26S of strain WEE 71V-1658 (Netolitzky et al., 2000) suggesting that some of the E1 epitopes that elicited protective immunity could be strain specific. Although the purified E1 protein did not elicit protective immunity as effectively as did inactivated WEE virus, our data indicated the purified protein could elicit protective immunity in some mice.

4. Discussion

The alphavirus envelope consists of a lipid bilayer in which multiple copies of two virus-encoded glycoproteins

Table 1
Live virus challenge study of BALB/c mice immunized with recombinant E1 protein or parental inactivated WEE virus

Antigen	Challenge virus	Survival (%)
Recombinant E1 antigen	WEE 71V-1658	2/8 (25)
	WEE Fleming	0/8 (0)
Inactivated WEE virus	WEE 71V-1658	8/8 (100)
	WEE Fleming	5/6 (83)
Saline only	WEE 71V-1658	0/8 (0)
	WEE Fleming	0/7 (0)

are embedded (Harrison et al., 1992). The viral glycoproteins are produced as a polyprotein precursor that is processed by intra-cellular enzymes during its insertion into the endoplasmic reticulum and transport to the cell membrane. The E2 glycoprotein has a cell recognition function, whereas E1 facilitates fusion of the viral and cellular membrane in an acidic environment (Garoff et al., 1980; Dubuisson and Rice, 1993; Kielian, 1995). Since both E1 and E2 play important roles for entry of the virus particle into the host, they are potential candidates for subunit vaccine formulations. Recombinant protein expression and purification is useful and advantageous in comparison to whole virus, which requires more precautions in handling. Recombinant subunit vaccines are desirable due to their safety compared with whole attenuated or inactivated virus, which can present a risk of infection in some subjects.

In the present study, we have cloned the E1 gene, expressed and purified the recombinant protein and evaluated its antigenicity in mice. The bacterial expression system is not suitable for expression of glycosylated eukaryotic proteins because bacteria cannot perform glycosylation. However, their expression level is usually very high in comparison to eukaryotic systems. Some foreign genes can be expressed as soluble protein, as well as inclusion bodies and the latter presents an advantage with respect to the purification of expressed protein. Most recombinant proteins are expressed in *E. coli* as inclusion bodies and different strategies have been reported to extract and renature proteins from inclusion bodies (Clark, 1998; Liu et al., 2001; Guo et al., 2003; Kurucz et al., 1995; Lu et al., 2001; Sanchez et al., 1999; Wei et al., 1999; Hevehan and Clark, 1997; ShengFeng et al., 2003; Das et al., 2004).

The E1 protein was expressed and purified in *E. coli* as inclusion bodies. The final yield of refolded E1 protein was 10–15 mg/L of initial bacterial culture. The purity of refolded protein was judged by SDS-PAGE and Western blot data indicated that 11D2 MAb reacted with a single band of ∼55 kDa, suggesting that we had successfully purified recombinant E1 proteins. Serum from mice immunized with whole inactivated virus vaccine showed strong binding to the recombinant E1 protein in an ELISA indicating that antibodies elicited by E1 expressed by whole virus also reacted with recombinant E1. The recombinant E1 antigen hence could be useful as an antigen for screening of serum levels of specific antibodies in immunized or infected animals. In addition, the antigen-based ELISA could be the basis of developing a sensitive serum diagnostic to monitor WEE outbreaks. Purified recombinant E1 protein was stored at three different temperatures (4, −20 and −80 °C) and stability was demonstrated by direct ELISA after 2, 4, 6 and 8 weeks (data not shown). The results demonstrated that the recombinant protein was very stable when stored at three different temperatures.

The immunogenicity of recombinant E1 protein was tested prior to live virus challenge studies. Both humoral and cell-mediated responses were monitored. Humoral response was demonstrated to be robust by antibody titers on solid phase antigen ELISA. All mice in the test groups elicited strong humoral immune responses after immunization with E1. CMI responses were also demonstrated by an antigen-specific cell proliferation assay. It was apparent that recombinant E1 protein could induce humoral and CMI responses in mice suggesting that the antigen has both B-cell and T-cell epitopes. Based on this encouraging result, we initiated a prophylaxis study. The recombinant E1 protein was used to immunize mice that were subsequently challenged with the two strains of the WEE virus. A subset of recombinant E1-vaccinated mice survived the challenge with WEE strain 71V-1658. However, no survival was observed among the recombinant E1-vaccinated group challenged with the WEE Fleming strain. When mice were vaccinated with chemically inactivated virus as a positive control, complete protection was observed with WEE strain 71V-1658 and 83% survival was observed in WEE Fleming challenge group. The marginal protective responses elicited by recombinant E1 alone suggest it might be imperative to include other viral antigens such as E2 to elicit better protection. In addition, the *E. coli*-derived recombinant E1 is not glycosylated. The E1 gene was cloned from 71V-1658 strain of the WEE virus and it is interesting that little or no protection was seen upon challenge with this strain. Our data indicate a requirement to develop effective WEE vaccines broadly protective to several strains of the virus. Plans are underway to express the viral glycoprotein genes in a mammalian expression system, which will provide proper glycosylation for further testing.

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