Modulation of immune responses by the antimicrobial peptide, epinecidin (Epi)-1, and establishment of an Epi-1-based inactivated vaccine

Han-Ning Huang, Chieh-Yu Pan, Venugopal Rajanbabu, Yi-Lin Chan, Chang-Jer Wu, Jyh-Yih Chen

ABSTRACT

Current efforts to improve the effectiveness of vaccines include incorporating antimicrobial peptides mixed with a virus. The antimicrobial peptide, epinecidin (Epi)-1, was reported to have an antiviral function, and an Epi-1-based inactivated vaccine was postulated as a model and discussed. In this report, we demonstrated modulation of immune responses by Epi-1 and an Epi-1-based Japanese encephalitis virus (JEV)-inactivated vaccine against JEV infection in mice. Under in vitro conditions, Epi-1 prevented JEV infection-mediated loss of cell viability in BHK-21 cells. When Epi-1 and JEV were co-injected into mice and mice were re-challenged with JEV after 14 days, all mice survived. In addition, Epi-1 modulated the expressions of immune-responsive genes like interleukin (IL)-6, IL-10, MCP-1, tumor necrosis factor-α, interferon-γ and IL-12, and elevated the levels of anti-JEV-neutralizing antibodies in the serum. The presence of Epi-1 suppressed the multiplication of JEV in brain sections at 4 days after an injection. Mice immunized with the developed vaccine showed complete survival against JEV infection, and it was superior to the traditional formalin-based JEV-inactivated vaccine. This study demonstrates the use of Epi-1 to develop an inactivated vaccine can provide guidelines for the future design of Epi-1-virus formulations for various in vivo applications.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Even though prophylactic vaccinations are hailed as a miracle of modern medicine [1], the efficacy of a given vaccine varies with the formulated antigenic context it contains. Unlike small-molecule drugs, vaccines are made of diverse materials, including proteins, polysaccharides, DNA, viruses, virus-like particles, irradiated live cells, synthetic peptides, and attenuated live organisms (viruses, bacteria, or parasites) [2]. In general, vaccines composed of antigenic proteins or their subunits are less immunogenic than live viruses, or parasites) [2]. In general, vaccines composed of antigenic proteins or their subunits are less immunogenic than live vaccines are made of diverse materials, including proteins, polysaccharides, DNA, viruses, virus-like particles, irradiated live cells, synthetic peptides, and attenuated live organisms (viruses, bacteria, or parasites) [2]. In general, vaccines composed of antigenic proteins or their subunits are less immunogenic than live organisms (viruses, bacteria, or parasites) [2]. In general, vaccines composed of antigenic proteins or their subunits are less immunogenic than small-molecule drugs, such as aluminum, oligonucleotides (CpG DNA sequences), oil emulsions (MF59), and saponin-based mixtures (QS-21 and ISCOMATRIX) [3]. The clinical use of some adjuvants, for example aluminum, has however raised some safety concerns [4]. Safer and more-effective adjuvants are constantly being searched for in contemporary vaccine development.

Antimicrobial peptides (AMPs) are a family of short cationic peptides synthesized and released by a wide variety of organisms [5]. Recently, a synthetic adjuvant termed IC31 (composed of the antimicrobial peptide, KLKL5KLK, and deoxy-inosine/deoxy-cytosine (ODN1a)) was revealed to be able to sufficiently induce antigen-specific Th1 cellular and humoral immune responses [6,7]. Plus, a DNA vaccine in conjunction with the synthetic KLKL5KLK AMP was also reported to have good efficacy against Mycobacterium tuberculosis infections [8]. Although several hundred AMPs have been identified, only a few of their roles in host immunity have been studied [9–12].

To establish an epinecidin-1 (Epi-1)-based inactivated vaccine, we used Japanese encephalitis virus (JEV) as a model to study viral infections in general. The Japanese encephalitis virus (JEV), a member of the family Flaviviridae, causes deadly encephalitis in humans and animals [13,14]. JEV is composed of three virion proteins, capsid (C), premembrane/membrane (prM/M), and envelope (E) proteins [15]. The E-protein was found to be the most...
protection antigen that elicits a considerable number of neutralizing antibodies [16]. To date, three kinds of JEV vaccines exist, all of which possess some adverse effects as reported from time to time [17–22]. For example, an imbalance in the immune system can be provoked when formalin-inactivated vaccines are used against measles [23] or respiratory syncytial virus [24]. Incomplete inactivation by formaldehyde was condemned for being incapable of controlling outbreaks of Venezuelan equine encephalitis [17] and foot and mouth disease [21]. Hence, suitable and effective inactivating agents are urgently needed to avoid repeating such unfortunate incidences.

Epi-1, derived from grouper (Epinephelus coioides), was characterized as being an AMP, and also possesses other properties such as antimutum, antimicrobial, and antiviral activities [25–28]. We investigated whether this host-friendly AMP could possibly be substituted for formalin in JEV inactivation in addition to being an adjuvant. In this paper, we characterized Epi-1’s function in JEV infection; evaluated the performance of Epi-1 applied to an Epi-1-based JEV-inactivated vaccine against JEV infection in mice; elucidated the function of Epi-1 mixed with virus in mice after JEV infection, and demonstrated the immune-related gene responses by microarray and real-time polymerase chain reaction (PCR) experiments. Moreover, isotypes of immunoglobulin G (IgG) were also determined in Epi-1–induced immunity against JEV infection.

2. Materials and methods

2.1. Mice, cells, and the virus

Baby hamster kidney (BHK)-21 cells were purchased and maintained as per American Type Culture Collection (ATCC no. CCL10, Manassas, VA, USA) instructions. Female C3H/HeN mice, purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and BioLASCO (Taipei, Taiwan), were housed at the Laboratory Animal Facility, National Taiwan Ocean University (NTOU; Keelung, Taiwan). Mice were maintained in pathogen-free sterile isolators according to institutional guidelines, and all food, water, caging, and bedding were sterilized before use. All procedures were approved by the Animal Care and Use Committee of NTOU. The 50% lethal dose (LD50) of JEV in female C3H/HeN mice was determined [29]. The Beijing J-1 vaccine strain was maintained in suckling mouse brains as previously described [30].

2.2. Cell proliferation assay

BHK-21 cells in 48–well plates were assayed with CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA) following the vendor’s instructions. Epi-1 was administered together with JEV as co-treatment; Epi-1 was administered 24 h prior to (prophylaxis) or after (curative) the JEV inoculation. After blank value subtraction, the percentage survival against control cells was calculated. The percentage of JEV infection or cell inhibition was obtained by subtracting the cell viability percentage from 100.

2.3. Viral challenge and Epi-1 treatments

Six-week-old adult C3H/HeN mice were randomly divided into five groups of 10 mice each. Individuals in each group of mice were intraperitoneally (i.p.) injected with 100 μL of PBS containing 50–500 pfu of JEV in the presence or absence of 50, 100, or 200 μg/ml Epi-1 or phosphate-buffered saline (PBS). After 14 days, a re-challenge was carried out on surviving mice with 50–500 pfu of JEV. Mouse survival and activity were monitored on a daily basis for up to 25 days after the primary injection. For serological studies, serum was collected on days 4, 7, and 21 after treatment. A similar approach was followed to test immunized mice.

2.4. Anti-JEV-E protein antibody assay

Serum was collected by tail bleeding, and an anti-E protein antibody assay was conducted as described previously with some modifications [29]. Briefly, 10-fold-diluted standard serum was serially diluted 2-fold. JEV-antigen-coated 96-well plates were treated with blocking buffer, and 50 μL of standard serum dilutions was loaded into each well in triplicate for 1 h at 37°C. After aspiration and washing steps, a 1:1000-diluted goat horseradish peroxidase (HRP)-conjugated mouse IgG antibody (ICN,Cappel, Aurora, OH, USA) was added to each well for 2 h at 37°C. Next, aliquots were aspirated and washed, and 50 μL of 100 μmol of 4-phenylenediamine dihydrochloride (OPD) (Sigma–Aldrich, St. Louis, MO, USA) was added. The reaction mixture was incubated in the dark for 30 min at room temperature, and the peroxidase activity was measured as an absorbance of OD450. From the standard curve between the serial dilution and absorbance values, the absorbance values reached a plateau referred to as the ODmax. Then the reciprocal of the dilution extent corresponding to 1/2 of the ODmax was referred to as one unit (U) of anti-JEV-E neutralization. Readings of unknown samples were compared with standard serum, and results are expressed as arbitrary units/ml. A biotin-conjugated rat anti-mouse IgG1 or IgG2a (Pharmingen, San Diego, CA, USA) detector was used to measure the respective IgG isotypes. Then Avidin HRP was added, and color development was recorded.

2.5. Plaque-reduction neutralization test (PRNT)

Neutralization tests were carried out using BHK-21 cells with the 50% plaque-reduction technique. A 2-fold dilution of serum with PBS (containing 5% fetal bovine serum (PBS) (Invitrogen, San Diego, CA, USA)) was incubated at 36°C for 30 min to inactivate the complement. The solution was then mixed with an equal volume of JEV solution in minimum essential medium (MEM; Invitrogen) containing 5% FBS to yield approximately 1000 plaque-forming units (pfu) of a virus solution (virus/ml). The virus-antibody mixtures were incubated at 4°C overnight, and then added to triplicate wells of 6-well plates. Each well contained a confluent monolayer of BHK-21 cells, which was incubated at 37°C for 1 h with gentle shaking every 15 min. The wells were then overlaid with 2 ml of 1.5% agarose (SeaPlaque, FMC, USA) in MEM containing 2% FBS. The plates were incubated for 3 days at 37°C in a 5% CO2 atmosphere. Cells then were fixed with formalin and stained with 1% crystal violet. Plaque numbers were counted. Neutralizing antibody titers were calculated as the reciprocal of the highest dilution factor, which resulted in a 50% reduction in numbers of plaques compared to the control virus (that was mixed with normal sera from naive C3H/HeN mice of the same age).

2.6. Cytokine enzyme-linked immunosorbent assay (ELISA)

Cytokine levels were quantified by an ELISA according to the manufacturer’s instructions. Interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12, MCP-1, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, were assessed using paired antibodies from the mouse cytokine ELISA set, BD OptEIA™ (BD Bioscience, San Diego, CA, USA). Briefly, the 96-well plates (Nunc, Denmark) were coated with capture antibody overnight at 4°C. Wells were washed and blocked with 1% bovine serum albumin (BSA) in PBS at 1 h room temperature. A sample or standard was added for 2 h at 37°C, and bound cytokines were detected using a biotinylated anti-cytokine antibody, avidin HRP, and tetramethylbenzidine. Color development was stopped with 2 M H2SO4, and optical densities were read at 450 nm. The concentrations of cytokine were determined from the standard curve.

2.7. Microarray analysis

RNA was extracted from different treatment groups of mouse brain with the Trizol reagent, and two independent replicates were separated for each sample. All of these treated groups were compared to the PBS control. Isolated total RNA was quantified on a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA). Complementary (c) RNA from 1.5 μg of total RNA from each sample was amplified and labeled with cyanine-3- or cyanine-5-dCTP according to the 1-color labeling protocol of a low-RNA input linear amplification kit (Agilent Technologies, Santa Clara, CA, USA). The resulting Cy3- or Cy5-labeled samples were purified using RNeasy mini kits (Qiagen, Valencia, CA, USA), then the cRNA concentration and dye incorporation were quantified using a Nanodrop spectrophotometer in order to determine the labeling efficiency. cDNA samples were hybridized to 4 × 44-k mouse oligo microarrays using Agilent reagents and protocols. After hybridization, the arrays were washed, scanned, and analyzed following Agilent protocols. All data were entered into Agilent GeneSpring GX7 (Agilent Technologies) and Pathway Studio software (Ariadne, Genomics, Rockville, MD, USA) for analysis and data mining. Genes considered to be significantly regulated were based on an average expression level change of ≥2-fold (average ratio of biological duplicates of ≥2 or ≤0.5). The gene list was converted to a log2 ratio, and analyzed using Pathway Studio 6.2 software. Genes which showed a direct relationship with the entities based on protein expression, transcription, and regulation were mapped using Adriane ontology. Gene symbols were color-coded red for upregulated and green for downregulated conditions and are shown in a graphical representation.

2.8. Isolation of messenger (m)RNA and real-time PCR

Total RNA was isolated from brain tissues and purified using a Qagen RNeasy kit. Reverse transcription into cDNA was performed with iScript cDNA Synthesis Kits (BIO-RAD, USA) according to the manufacturer’s recommendations. A real-time polymerase chain reaction (PCR) analysis was used to analyze the gene expressions in Supplementary Table 1, according to the manufacturer’s instructions. The Qi™ SYBR® Green Supermix (BIO-RAD) and specific primer pairs were used for selected genes, and a primer pair for GAPDH was used as the reference gene. A quantitative PCR was performed according to the following conditions: 40 cycles of 1 min at 95°C, 30 s at 55°C, and 1 min at 72°C. Using 0.5 μL of cDNA, 2× SYBR Green PCR Supermix, and 500 nm of the forward and reverse primers, the threshold cycle number (Cq) was calculated with BIO-RAD software. Relative transcript quantities

Please cite this article in press as: Huang H-N, et al., Modulation of immune responses by the antimicrobial peptide, epinecidin (Epi)-1, and establishment of an Epi-1–based inactivated vaccine, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.01.061
were calculated using the $\Delta Ct$ method with GAPDH as the reference gene which was amplified from the same samples. $\Delta Ct$ is the difference in the threshold cycles of messenger (m)RNA for selected genes relative to those of GAPDH mRNA. The real-time PCR was performed in triplicate for each experimental group.

2.9. Immunofluorescence study

Tissue sections on slides were fixed with 3.7% paraformaldehyde (PFA), and washed three times in PBS. The fixed sample was permeabilized with a 0.2% Triton X-100 solution for 15 min at 37°C, and washed with PBS. Blocking was performed using 5% normal bovine serum in PBS for 30 min at 37°C. Then the section was incubated with an anti-JEV-E primary antibody[29] overnight at 4°C, and rinsed three times with PBS for 5 min. Sections were next treated with a goat anti-mouse-conjugated FITC secondary antibody (Cappel, Organon Teknika, Veedijk, Belgium) for 45 min at room temperature (RT). After washing three times with PBS, the stained brain tissues were observed under a fluorescence microscope (BX-51, Olympus, Tokyo, Japan) equipped with a digital camera.

2.10. Western blotting

Proteins were extracted from mouse brain samples as previously described with some modifications [43]. Mouse brain regions were homogenized in urea buffer consisting of 125 mM Tris-Cl (pH 6.8), 2.2% sodium dodecylsulfate (SDS), 5% β-mercaptoethanol, 10% glycerol, 8 M urea, and a protease inhibitor (Boehringer

![Figure 1](image-url)

Fig. 1. Epinecidin (Epi)-1 controls Japanese encephalitis virus (JEV) infection. (a) Different concentrations of Epi-1 were used to treat BHK-21 cells with JEV at a multiplicity of infection (MOI) of 0.1 by three methods. For prophylaxis treatment, Epi-1 was given first, and cells were infected with JEV 1 h later. For co-treatment, Epi-1 and JEV were given at the same time, while Epi-1 was injected 1 h later than JEV for curative treatment. The percentage of cells infected by JEV was calculated by measuring cell inhibition. Cell inhibition values of samples infected with 0.1 MOI JEV alone (C+V) were converted to 100%, and other treatment values (0.1 MOI JEV + Epi-1) were normalized to that. *p < 0.05. Error bars, SEM (n = 4–7). (b) Survival curves of C3H/HeN mice treated with JEV and Epi-1. Adult mice were injected with 200 μg/ml of Epi-1, JEV, or JEV co-treated with different doses (50, 100, and 200 μg/ml) of Epi-1. Surviving mice were re-challenged with JEV on the 14th day. Survival of treated mice was monitored on a daily basis for up to 25 days (n = 10–13).

Please cite this article in press as: Huang H-N, et al., Modulation of immune responses by the antimicrobial peptide, epinecidin (Epi)-1, and establishment of an Epi-1-based inactivated vaccine, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.01.061
Mannheim, Germany). The mixture was centrifuged, and the insoluble materials were removed. After quantification, 10 μg of denatured proteins was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose membrane. The membrane was blocked with 3% milk powder and 2% BSA in TBS/Tween-20 at RT for 1 h, and incubated with a 1:1000-diluted anti-JEV-E primary antibody, followed by a goat anti-mouse HRP-conjugated secondary antibody (Amersham, Little Chalfont, UK). The antibody was detected by a chemiluminescent Western blot detection system (Amersham) over x-ray film. Data were analyzed by UVP Vision Works Software (vers. 5.5.3) (UVP, Upland, CA, USA).

2.11. Immunization of neonate mice by inactivated vaccines

One-week-old C3H/HeN neonate mice were separated into four groups of 13 mice each. One group was separately intraperitoneally (i.p.) injected with 1.5 × 10⁷ plaque-forming units (pfu) of JEV alone, formalin-inactivated JEV vaccine (10 μg/mice), Epi-1-JEV-inactivated vaccine (10 μg/mice), or PBS alone. Booster immunizations were performed at 14 (booster 1) and 28 (booster 2) days after the primary immunization. Serum was collected on the 4th day after challenge (35th day) for the serological analysis, and mouse survival was monitored on a daily basis for up to 53 days.

2.12. Statistical analyses

Experiments were conducted in triplicate, and repeated three times. Univariate analysis of variance (ANOVA) in SPSS software (Chicago, IL, USA) was used to analyze the significance among treatments. Error bars represent the standard deviation or standard error of the mean (SEM). The survival of tested animals was depicted using Kaplan–Meier curves, and the corresponding analyses were performed by a log-rank test.

3. Results

3.1. Effects of Epi-1 treatments in the in vitro study

We first studied the effects of Epi-1 on JEV in BHK-21 cells. In cell proliferation assays, Epi-1 at various concentrations up to 1 μg/ml did not affect cell viability (Supplementary Fig. 1). Then, overnight-cultured BHK-21 cells were infected with JEV at a multiplicity of infection (MOI) of 0.1 (equivalent to 5000 pfu/well) with or without adding Epi-1. At 48 h, cell viability of JEV infection was determined (Fig. 1a). The pre- (prophylactic) and post-treatments (curative) with Epi-1 failed to prevent JEV infection. When cells were co-treated with 0.5 or 1 μg/ml Epi-1 plus JEV, infection rates dropped by 40% and 50%, respectively, compared to that of the control. Thus, Epi-1 likely played a critical role in JEV inactivation in cells.

3.2. Epi-1 performance on JEV infection

In an animal study, mice (n = 10) were first i.p. injected with 200 μg/ml of Epi-1; all mice survived and behaved normally. No toxic effects were noted after Epi-1 inoculation. Epi-1 treatment against mice i.p. injected with 50 × the LD₅₀ of JEV (1.5 × 10⁷ pfu in 500 μl) was then performed. JEV was co-injected with or without Epi-1 at various dosages (50, 100, and 200 μg/ml) into adult mice and subsequently boosted (a second JEV challenge without Epi-1) on day 14. The survival rate of mice was recorded for 25 days. Without the Epi-1 co-injection, all mice (n = 10) died within 1 week; mice (n = 10) that had received Epi-1 died only after JEV re-challenge (Fig. 1b). Ten mice that received JEV plus 200 μg/ml of Epi-1 surprisingly survived even after the second JEV challenge; they otherwise all behaved normally (Fig. 1b). Although one and four mice treated with JEV plus 100 and 50 μg/ml of Epi-1, respectively, died within 1 week, and they all behaved normally (n = 10; Fig. 1b). An ideal dosage against JEV infection in this given model system may be 200 μg/ml of Epi-1. Co-injection of Epi-1 and JEV may induce some desirable adaptive immunity against JEV re-challenge.

3.3. Effects of Epi-1 on the production of neutralizing antibodies

To evaluate whether co-injection of Epi-1 and JEV can induce neutralizing antibodies and serve as a vaccine against JEV infection, serum was collected on the 4th, 7th, and 21st days from surviving mice injected with 200 μg/ml of Epi-1, JEV, or JEV co-treated with different doses (50, 100, and 200 μg/ml) of Epi-1 and re-challenged JEV on the 14th day. The anti-JEV titer was determined and cytokine assays were performed using an ELISA. On the 4th day, mice co-injected with Epi-1 and JEV showed higher anti-JEV titers than JEV alone, and this further significantly increased after re-challenge (Fig. 2). The production of anti-JEV antibodies against JEV was through T-helper (Th) cells, Th1 and/or Th2 [8]. Co-injection of Epi-1 with JEV produced increased IgG1 antibodies, which suggests that Epi-1 activates Th₂ cells in response to JEV (Fig. 3). Th₂ cell activation resulting in the production of IgG₁ is well documented as a humoral response [1,3,8]. As shown in Fig. 3b, Th₂ cytokine levels (IL-4 and IL-10) were higher than Th₁ cytokine levels (IL-2 and INF-γ) when Epi-1 was co-injected with JEV. Therefore, co-injection of Epi-1 and JEV can induce a humoral response against JEV infection.

Then, the anti-JEV neutralization ability of serum from C3H/HeN mice injected with JEV with or without Epi-1 was examined by a plaque-reduction neutralization test. Sera were collected on the
7th day, and the JEV-E-neutralizing antibody titers were assayed. As shown in Table 1, no neutralizing antibody titer was detected (<1/10) in the group only treated with 0, 50, 100, and 200 μg/ml Epi-1. Surprisingly, a low neutralizing antibody titer was detected (<1/40) in the group treated with JEV alone. The group treated with JEV plus 200 μg/ml of Epi-1 showed the highest neutralizing antibody titer (>1/1280). The groups treated with JEV plus 50 and 100 μg/ml of Epi-1 respectively revealed >1/160 and >1/320 neutralizing antibody titers. These results showed that the co-injection of Epi-1 and JEV may be used as an inactivated vaccine, and it provided significant immune protection against JEV infection.

3.4. Effects of Epi-1 on the expressions of JEV-dependent genes in mice

To profile expressions of antiviral genes modulated by Epi-1 in JEV-infected mice, cDNAs from brain sections of mice post-injected...
on days 4 or 14 were analyzed using a mouse 44 k oligo microarray. Genes expressed under [Epi-JEV] vs. [JEV] conditions were analyzed to understand the overall modulation of JEV-induced genes by Epi-1. Such immune-responsive, antiviral and inflammatory genes such as STAT1, STAT2, IL-6, IFNB1, MyD88, MX1, TLR3, Casp4, TLR-1, TLR7, IL7R, and IFNA5 were found to be regulated by Epi-1 (see Supplementary Data 1 and Supplementary Fig. 2 online). In addition, genes associated with mitogen-activated protein kinase (MAPK) and calcium-dependent calmodulin kinase (CaMK) were also affected by Epi-1 in the microarray. The expression levels of given genes selected from those above were further investigated by real-time PCR (Supplementary Table 1). cDNAs of mouse brain only injected with Epi-1 served as a control. Similar to the microarray results, expression levels of the genes, STAT1, STAT2, Bax, IFN-A7, IFN-β, IFN-γ, MX-1, TLR-1, MyD88, IL-2, and Atf3, were down-regulated in mice injected with Epi-1 and JEV on day 4 (Fig. 4a, b). In contrast, the antiapoptosis and anti-inflammatory genes, Bcl-2, SOCS-3, and IL-4, were upregulated (Fig. 4b). Since all mice infected with JEV died within a week, the expression levels of the given genes in mice co-injected with Epi-1 and JEV were compared to those of the control (the day 14 sample with Epi-1 treatment). Expressions of these given genes were generally low over 14 days (Fig. 4c, d). As a result, the expression levels of JEV immune-responsive and -induced antiviral genes were ascertained to have been downregulated by Epi-1, which is consistent with the results of the microarray and quantitative PCR (Fig. 4c).

3.5. Effects of Epi-1 on JEV-associated proinflammatory cytokines

To understand the effects of Epi-1 on proinflammatory cytokine release in mice infected by JEV, we measured levels of IL-6, IL-10, IL-12p70, MCP-1, TNF, and IFN-γ from 4-day-treated samples using a BD OptEIA®/C212 with an ELISA kit (BD Bioscience). The JEV-mediated

### Table 1

<table>
<thead>
<tr>
<th>Mode</th>
<th>Neutralizing antibodies titer[^a^]</th>
<th>Neutralizing antibodies titer[^b^]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JEV</td>
<td>Epi-1 + JEV</td>
</tr>
<tr>
<td>Epi-1 (0 µg/ml)</td>
<td>&lt; 1/10</td>
<td>&lt; 1/10</td>
</tr>
<tr>
<td>Epi-1 (50 µg/ml)</td>
<td>&lt; 1/10</td>
<td>&gt; 1/160</td>
</tr>
<tr>
<td>Epi-1 (100 µg/ml)</td>
<td>&lt; 1/10</td>
<td>&gt; 1/320</td>
</tr>
<tr>
<td>Epi-1 (200 µg/ml)</td>
<td>&lt; 1/10</td>
<td>&gt; 1/1280</td>
</tr>
</tbody>
</table>

[^a^] C3H/HeN mice were co-injected JEV with or without Epi-1 at various dosages (50, 100, and 200 µg/ml).

[^b^] JEV-neutralizing antibodies titer in serum collected 7th day after treatment expressed as the reciprocals of the serum dilution yielding a 50% reduction in plaque numbers.

![Fig. 4](image-url). Expression patterns of selective antiviral and immune response genes in epinecidin (Epi)-1 and/or Japanese encephalitis virus (JEV)-injected mice at 4 and 14 days. Mice were i.p. injected with 50% lethal dose (LD₅₀) of JEV in the presence or absence of 200 µg/ml Epi-1 (Epi-1 + JEV and JEV respectively) or 200 µg/ml Epi-1 alone (Epi-1), and cDNA isolated from the brain at 4 and 14 days post-injection and JEV-responsive gene expressions in the presence of Epi-1 were detected by a real-time PCR analysis. Gene expressions were normalized to GAPDH, and converted to multiples of change over the untreated control. *p < 0.05. Error bars, s.d. (n = 3).
release of IL-6, IL-10, IL-12p70, MCP-1, TNF, and IFN-γ was found to be suppressed by Epi-1 (Fig. 5), as manifested in gene and protein expression levels (Fig. 4).

3.6. Epi-1 performance on JEV-infected mouse brain

Next, we studied whether Epi-1 directly acts as an antiviral agent against JEV. JEV mixed with 50, 100, or 200 μg/ml of Epi-1 for 15 min at room temperature (RT) was fixed on a slide for transmission electron microscopic (TEM) inspection. Viral particles were found to be subject to lysis and destruction in the presence of Epi-1 (Supplementary Fig. 3). To study the antiviral activity, adult mice were injected with either 50× the 50% lethal dose (LD50) of JEV alone or JEV and Epi-1 (50, 100, or 200 μg/ml). On day 4 after treatment, brain specimens were treated first with an anti-JEV-E primary antibody and then a goat anti-mouse-conjugated FITC secondary antibody. Samples were then subjected to a fluorescence microscopic examination (with an FITC filter). Bright fluorescent dots, representing virus particles associated with anti-JEV-E, were clearly observed in the JEV-alone samples, but there were dramatically fewer spots in samples co-injected with Epi-1 and JEV (Fig. 6a). Total proteins from brain samples were further subjected to a Western blot analysis, using an anti-JEV-E antibody (Fig. 6b). The 52-kDa JEV-E protein was clearly noted in mice samples injected with JEV, while it was barely seen in samples with Epi-1. Differences in relative intensities were up to 6-fold between samples co-injected with Epi-1 (200 μg) and those injected with JEV alone (Fig. 6c). These results show that Epi-1 directly acted as an antiviral agent against JEV infection.

3.7. Effects of Epi-1-inactivated vaccine on neonate mice

Mouse brain-derived, formalin-killed vaccines are currently used in countries with vaccine-preventable infections, including Taiwan. The limitations of formalin-inactivated JE vaccines include the high cost of manufacture, a lack of long-term immunity, the need for multiple doses, and the risk of allergic reactions [19,31]. To determine whether Epi-1-inactivated JEV can serve as a vaccine in neonate mice, we formulated a solution of Epi-1 (200 μg/ml) plus 50× the LD50 of JEV (1.5 × 10⁷ pfu). In parallel, a formalin-inactivated JEV vaccine was prepared according to standard procedures to serve as a positive control. A primary immunization was performed in 7-day-old neonate mice; boosters were given on days 14 and 28. Anti-JEV antibodies were then measured by an ELISA. The anti-JEV-E titer activity was poor after the primary immunization in both tested groups (Fig. 8a). After the second immunization, the anti-JEV-E titers significantly increased in mice vaccinated with either vaccine, but that in the group with Epi-1 was slightly higher. After the third immunization, levels of the anti-JEV-E antibody were determined to be about 150 units in serum of vaccinated mice, but again that of the group with Epi-1 was slightly higher (Fig. 7a). With JEV challenge, the anti-JEV-E titers dramatically increased to 200 units on day 4 in both vaccinated groups, while the group with Epi-1 was higher (Fig. 7b). On the other hand, the survival rates of mice re-challenged with 50× the LD50 of JEV on the 35th day showed the following results: all Epi-1-JEV-immunized mice (n = 13) survived (Fig. 8); all but one of the formalin-JEV-immunized mice survived, as opposed to all controls that died within 1 week. As a result, the Epi-1-based JEV-inactivated vaccine performed the same as or slightly better than the conventional vaccine in preventing JEV infection in neonate mice.

4. Discussion

The antiviral and host defense functions of Epi-1 against JEV infection were determined both in vitro and in vivo, so that Epi-1 is an antiviral against JEV and is able to modulate some immune-related genes in the mouse. Epi-1 showed no detectable cytotoxicity toward
BHK-21 cells (Supplementary Fig. 1) or in adult mice, although several AMPs were reported to be cytotoxic at high levels [9]. In addition, Epi-1 was not reported to exhibit any cytotoxicity in previous reports [28]. This information suggests that Epi-1 acts as a host-friendly peptide and can be used for advanced studies.

In vivo studies demonstrated that 200 μg/ml of Epi-1 is an appropriate quantity to achieve desired immune responses. The induction of IgG1 was correlated with activation of Th2 cells as well as a humoral immune response in the given conditions [32,33]. The increased production of the anti-E-neutralizing antibody in Epi-1/JEV-co-treated mice was ascribed to an induction of adaptive immunity (Figs. 2 and 3; Table 1). Epi-1/JEV-co-treated mice induced higher Th2 cytokine levels (IL-4 and IL-10) than Th1 cytokine levels (IL-2 and INF-γ). Antibody isotyping revealed that the induction of IgG1 by Epi-1 was through Th2 cells, so it was a humoral response (Fig. 3). In Fig. 4, the IL-7 receptor (IL7R) plays a critical Th2 role in V(D)J recombination during lymphocyte development which was also activated [34].

The microarray and real-time PCR confirmed that Epi-1 modulates gene expressions during JEV infection (Fig. 4). In Epi-1/JEV-co-treated mice, virus-induced STAT1 and STAT2 were downregulated, and the expressions of downstream genes were reduced, including IFN-A7, IFN-β, and IFN-γ (Fig. 4) [35]. IFN-γ is normally induced by Th1 cells, but Th1 cells were not induced by Epi-1, which is consistent with our observations. JEV can induce caspase-3 activation of mitochondrion-mediated apoptosis and increase inflammatory cytokine secretion [36]. Interestingly, co-injection of Epi-1 with JEV-induced Bcl-2 but suppressed Bax-2 expression (Fig. 4), so that Epi-1 possesses pro-survival and proapoptotic functions [37]. The Mx protein is responsible for a specific antiviral state against virus infection [38]. Activation of Mx-1 was also mediated by Epi-1. Therefore, Epi-1 may prevent JEV-induced host-defense mechanism-mediated cell death by directly damaging viral particles. Toll-like receptor (TLR) 1 is a member of the TLR family of pattern-recognition receptors of the innate immune system, and its downstream adapter, MyD88, can activate the transcription factor, nuclear factor (NF)-κB,

Fig. 6. Epinecidin (Epi)-1 controls Japanese encephalitis virus (JEV) multiplication in the mouse brain. (a) Processed brain sections from 4-day post-treated mice were incubated with a primary anti-JEV-envelope (E) protein antibody and a secondary goat anti-mouse-conjugated FITC antibody. Samples were visualized under a fluorescence microscope using an FITC filter. (b) Total proteins from brain samples of mice injected with 10 μg from control (C), 50% the 50% lethal dose (LD50) of JEV, and JEV with Epi-1 at 50 (E50), 100 (E100), and 200 (E200) μg/ml were subjected to a Western blot analysis using an anti-JEV-E primary antibody. (c) The intensity of the JEV-E protein expression in Western blots was measured by a densitometer and normalized to the untreated control (C). *p < 0.05. Error bars, s.d. (n = 3).

Please cite this article in press as: Huang H-N, et al., Modulation of immune responses by the antimicrobial peptide, epinecidin (Epi)-1, and establishment of an Epi-1-based inactivated vaccine, Biomaterials (2011), doi:10.1016/j.biomaterials.2011.01.061
to stimulate cytokines (such as IL-2 and IL-6 which are involved in the activation of macrophages, and B and T cells) [29,30]. However, these genes were downregulated on day 4 in mice injected with Epi-1 and JEV (Fig. 4b). Atf3 prevents immune pathologies associated with uncontrolled proinflammatory cytokine production [39]. The downregulation of proinflammatory cytokines such as TNF-α, IL-12, IL-6, and MCP-1 by Epi-1 (Fig. 5) may prevent an autoimmune disorder from uncontrolled induction of these cytokines. The expression profiles of major immune-responsive genes (Fig. 4) in the study were in agreement with this inference.

The immunofluorescence experiments and Western blot analysis demonstrated that Epi-1 has an antiviral function (Fig. 6). An electron microscopic examination provided further support that Epi-1 directly interacts with JEV (Supplementary Fig. 2). The idea of using Epi-1 to develop a JEV-inactivated vaccine therefore is workable. The much-increased anti-E antibody demonstrated that an Epi-1-based vaccine was superior to the traditional formalin-inactivated vaccine. Previous studies showed that the clinical efficiency of a formalin-based inactivated vaccine was around 91% [19,31], while the Epi-1-inactivated JEV vaccine-immunized mice reached 100% survival in this study. We consider Epi-1 to be very promising as a substitute for formalin in vaccine development, although additional clinical studies are required for its subsequent development. Nevertheless, the new use of Epi-1 demonstrated herein should pave the way for the next generation of JEV vaccines.

5. Conclusions

In this study, an antimicrobial peptide of Epi-1 was synthesized and evaluated for an Epi-1-based inactivated vaccine with JEV by in vitro and in vivo assays as an example. The Epi-1 has an antiviral function and inhibited JEV activity by a co-treatment method in which it functioned as an inactivated vaccine. Epi-1 modulated the expressions of immune-responsive genes such as IL-6, IL-10, MCP-1, TNF-α, INF-γ and IL-12, and elevated the levels of anti-JEV-neutralizing antibodies in the serum. The ability of these two types of adjuvants of formalin and Epi-1 to protect neonate mice by immunization with inactivated vaccines in the in vivo test suggests that the approach of using Epi-1 for vaccine modification is a promising strategy and could replace the role of formalin. In particular, the addition of Epi-1 to other viruses presents excellent opportunities for the development of new vaccines as well as applications in general.
Acknowledgments

Research funding was obtained from the Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, Jiauhsi, Iilan, Taiwan. We would like to thank the core facility (Miss Ching-Chun Lin), the Institute of Cellular and Organismic Biology, Academia Sinica, for assistance with the analysis of the microarray data.

Appendix

Figure with essential colour discrimination. A figure in this article, particularly Fig. 6, is difficult to interpret in black and white. The full colour image can be found in the online version, at doi:10.1016/j.biomaterials.2011.01.061.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.01.061.

References