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Antiviral effects of liposome-encapsulated PolyICLC against Dengue virus in a mouse model

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ABSTRACT

This study presents the first investigation of the antiviral effects of the liposome-encapsulated PolyICLC (LE-PolyICLC) on Dengue virus (DENV) in a mouse model. *In vivo* efficacy studies showed that LE-PolyICLC acted to increase antiviral mechanisms mainly through promoting cytokine expression associated with innate immunity, such as IFN-γ. In addition, the pro-inflammatory cytokine TNF-α was also increased, while IL-6 level was decreased in serum. The titers of total antibodies against DENV2 in mice were also elevated. Administration of LE-PolyICLC not only alleviated the loss of body weight, degree of morbidity, and pathological damage in brains, but also reduced the viral titers and expression of viral E protein in the brain. Notably, the effectiveness of LE-PolyICLC was better than PolyICLC on the basis of the data presented in this study. These results, therefore, set a foundation for further development of LE-PolyICLC as an attractive candidate of antiviral agents to be used in both prophylactic and therapeutic settings in DENV diseases.

1. Introduction

Dengue is an acute febrile disease caused by the mosquito-borne Dengue viruses (DENVs). All four Dengue virus serotypes (DENV-1–4) circulate in over 100 countries, and are members of the *flaviviridae* family, genus *flavivirus*. The main vectors for Dengue viruses are *Aedes aegypti* and *Aedes albopictus* [1].

Human infection with DENV results in either asymptomatic or symptomatic disease, ranging from classical Dengue fever (DF) to more severe and often fatal Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS) [2]. The World Health Organization (WHO) estimates that 50–100 million Dengue infections occur annually, with approximately 500,000 people with Dengue hemorrhagic fever (DHF) requiring hospitalization, a large proportion being children [3]. Since 1978, Dengue fever has occurred endemically and epidemically every 4–7 years in China. The incidence of Dengue fever gradually decreased after 1997, but increased significantly in the most recent two years, especially in 2014, wherein a total of 44,894 cases were reported in Guangdong Province until November 2014 [4]. With the lack of suitable animal models and the antibody-dependent enhancement phenomenon, there is no DENV vaccine currently licensed [5]. In addition, no *anti*-DENV drugs are clinically available at present [6]. In the absence of available vaccines and antiviral drugs against DENV infection, specific treatment for Dengue patients primarily consists of supportive care including bed rest, antipyretics, and analgesics [7].

PolyICLC has been shown to be an effective prophylactic and therapeutic agent against a number of viral infections [8,9,10]. The antiviral activity of PolyICLC is the result of its immunomodulating ability to up-regulate the expression of α-, β-and γ-interferon *in vivo* [11]. Liposomes are microscopic lipid vesicles that have been wildly used as drug delivery systems. Liposome-encapsulated PolyICLC (LE-PolyICLC) has been shown to reduce the intrinsic toxicities of PolyICLC and enhance antiviral efficacy [10]. Both

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PolyICLC and LE-PolyICLC can provide almost 100% protection against lethal challenge with low pathogenic influenza A virus of subtypes H1N1 and H3N2 by administration prior to infection [10,12]. Moreover, LE-PolyICLC could be useful as a prophylactic and therapeutic agent, and as a vaccination adjuvant to combat highly pathogenic influenza infection [13].

In the present study, we investigated whether LE-PolyICLC could be utilized as an antiviral agent against Dengue 2 virus (DENV2) in a mouse model. The efficacy against DENV2 infection between LE-PolyICLC and PolyICLC were also compared. The data generated herein will be of significant interest for use in current strategies against Dengue virus infection.

2. Materials and methods

2.1. Virus

DENV-2 strain New Guinea C (NGC) obtained from Dr Jing An, Capital Medical University, China, was amplified once in C6/36 *Aedes albopictus* mosquito cells. The viral supernatant was harvested and stored in aliquots at −80 °C.

2.2. Liposome-encapsulated PolyICLC preparation

The liposome-encapsulated PolyICLC (LE-PolyICLC) was prepared as the method described previously [10].

2.3. Mice, drug treatment, and virus challenge

We obtained 5–6 week old female Balb/c mice (weight 15–17 g) from Vital River Laboratories (Beijing, China). Experimentation with animals was governed by the Regulations of Experimental Animals of Beijing Authority and approved by the Animal Ethics Committee of the China Agriculture University.

The animal experiment was carried out as outlined in Table S1.The mice were anesthetized with a mixture of ketamine-xylazine initially, and then challenged intracerebrally with 30 μl DENV2, which was approximately 4 LD_{50}, diluted in DMEM medium supplemented with 5% FCS. The mice were assessed and observed daily for changes in body weight, anorexia, paralysis, morbidity, and mortality for 21 days. The evaluation of different morbidity degrees in each group was performed using a scale ranging from 0 to 3 [14].

2.4. Samples collection

Mice were sacrificed at various time-points and a portion of the spleen, liver and brain from each mouse was collected and stored in liquid nitrogen until required. Sera were collected and stored at −20 °C until the analysis.

2.5. Histopathological analysis

Brains were removed from euthanized mice and fixed in 4% neutral formalin, embedding in paraffin, and then cut to 5 μm thickness. Each slide was stained with haematoxylin and eosin (H&E), and examined by light microscopy (Olympus BX41; Olympus, Tokyo, Japan).

2.6. Quantitative PCR (qPCR)

Total RNA was prepared from ~10 mg liver, spleen, and brain tissue in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. DNase-I-treated RNA (0.2 μg) was reverse-transcribed into cDNA using random primers. Real-time PCR was performed to amplify the *E* protein gene of DEN-2 virus using a real-time quantitative RT-PCR method with TaqMan probes to assess the RNA copy number of Dengue virus. The primers and probe primer were used in the PCR reactions were forward primer, 5′-AAATCCACTGTGTCGAACCCTAA-3′; reverse primer, 5′-CCTCTTGGAAAGGATATCTACATTTGT-3′, and the probe primer: ACCTAGGGACCCTCC. Data analysis was performed using the 7500 software (version 2.0; ABI, California, USA). The copy number of the *E* protein gene was calculated using an *E* gene-containing plasmid of known concentration as a standard. Real-time PCR was performed to amplify genes of tissues using the Power SYBR® Green PCR Master Mix kit (ABI, California, USA). The real-time PCR primer for β-actin, IFN-β, TNF-α, IFN-γ, and IL-6 are listed in Table S2. Gene expression was normalized to that of the control group using the $2^{-\Delta\Delta CT}$ method with β-actin as an internal standard.

2.7. Western blot analysis

The viral E protein in the infected animal brains was tested by Western blotting assay performed as previously described [15]. The primary antibodies were mouse monoclonal *anti*-DENV2 E protein antibody and mouse monoclonal *anti*-β-actin antibody. The secondary antibodies were both HRP-conjugated anti-mouse IgG. The β-actin protein was used as a normalizing control.

2.8. Measurement of cytokines concentrations

The concentration of cytokines (TNF-α, IFN-β, IFN-γ, IL-12p40, and IL-6) in serum were assayed by using commercial ELISA kits (R&D Systems, Minneapolis, manufactured by BD Pharmingen), according to the procedures supplied by the manufacturer. Results were showed as pg/mL. The detection limit of the ELISA assays was in the range of 4–8 pg/ml.

2.9. Statistical analysis

Statistical analysis was performed by one-way ANOVA using Graph Prism Software (version 4.0) and results with a $P < 0.05$ were considered statistically significant.

3. Results

3.1. Protection against Dengue 2 virus using LE-PolyICLC

To evaluate the potential of LE-PolyICLC against the Dengue 2 virus, a lethal Balb/c mouse model was established using NGC strain. In previously published animal studies, LE-PolyICLC treated by the intravenous route fared better than those treated *via* the intranasal route, as indicated by lack in body weight loss [10]. Therefore the protective efficiency of LE-PolyICLC by i.v. route was compared with s.c. and i.p. routes initially, and PolyICLC as the control drug. Results indicated that LE-PolyICLC induced varying levels of protection by the different administration routes. The two groups treated with LE-PolyICLC and PolyICLC by i.v. route presented 100% survival rate, while 57.14% of the mice in 0.9% NaCl-treated group survived viral challenge; however this difference was not statistically significant. Survival rate of the group treated with LE-PolyICLC by s.c. route was 85.71%, which was the same with i.p. route (Table S1). Although most of animals treated with LE-PolyICLC by different routes survived the viral challenge, they showed clinical signs of infection (Fig. 1). We observed that 29% of LE-PolyICLC-treated animals manifested severe neurological signs (grades 2 and 3) for both i.p. and s.c. routes, and only two mice in the LE-PolyICLC-i.v.-treated group had slight alteration of the spinal column with a small hump, without any clear signs of exacerbated disease. Therefore, the

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