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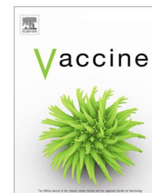

Expression of interleukin-6 by a recombinant rabies virus enhances its immunogenicity as a potential vaccine

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Several studies have confirmed that interleukin-6 (IL6) mediates multiple biological effects that enhance immune responses when used as an adjuvant. In the present study, recombinant rabies virus (RABV) expressing canine IL6 (rHEP-CaIL6) was rescued and its pathogenicity and immunogenicity were investigated in mice. We demonstrated that mice received a single intramuscular immunization with rHEP-CaIL6 showed an earlier increase and higher maximum titres of virus-neutralizing antibody (VNA) as well as anti-RABV antibodies compared with mice immunized with the parent strain. Moreover, survival rates of mice immunized with rHEP-CaIL6 were higher compared with mice immunized with parent HEP-Flury according to the challenge assay. Flow cytometry further confirmed that immunization with rHEP-CaIL6 induced the strong recruitment of mature B cells and $CD8^+$ T cells to lymph nodes, which may partially explain the high levels of VNA and enhanced cellular immunity. Quantitative real-time PCR indicated that rHEP-CaIL6 induced stronger inflammatory and immune responses in the central nervous system, which might have allowed virus clearance in the early infection phase. Furthermore, mice infected intranasally with rHEP-CaIL6 developed no clinical symptoms while mice infected with HEP-Flury showed piloerection. In summary, these data indicate that rHEP-CaIL6 induces a strong, protective immune response with a good safety profile. Therefore, a recombinant RABV strain expressing canine IL6 may aid the development of an effective, safe attenuated rabies vaccine.

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1. Introduction

Rabies is an ancient zoonosis and a central nervous system (CNS) disease, causing mortality in more than 55,000 humans annually, with most cases occurring in the developing regions of Asia and Africa, where canine rabies is the main source of infection [1]. Several developed countries have successfully eradicated human rabies by implementing programs to control canine rabies [1]. Although rabies is associated with a high mortality rate in both humans and dogs, rabies vaccines can effectively be used to control the disease. Although many rabies vaccines, most of which are inactivated, have been approved for use in dogs, the costs of these vaccines limit their use in developing countries. Therefore, attenuated live rabies vaccines are appropriate because of their low cost and effectiveness.

Foreign proteins have been expressed between nucleoprotein (N) and phosphoprotein (P), P and matrix protein (M) as well as between glycoprotein (G) and RNA-dependent RNA polymerase (L) in rabies virus (RABV) [2–5]. Furthermore, previous studies reported that a pseudogene sequence between G and L showed no detectable effect on the transcription and growth of RABV [6–8]. Several studies have confirmed the feasibility of enhancing immunogenicity and the viral attenuation of recombinant RABV (rRABV) strains by inserting and expressing genes encoding for immunomodulatory molecules [9–11]. In this context, interleukin-6 (IL6) has been shown to mediate various effects in the immune system. IL6 stimulates the differentiation and maturation of B cells to antibody-producing plasma cells [12], enhances mucosal immune responses [13], and stimulates T cell proliferation [14]. Accordingly, IL6 has been used as a molecular adjuvant expressed in eukaryotic expression vectors to enhance immune responses. For instance, mice challenged with influenza virus after receiving an immunization with an influenza virus DNA vaccine showed better protection if IL6 was used as an adjuvant [15,16]. It was also confirmed that antibodies against porcine circovirus

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type 2 (PCV2) and percentages of $CD3^{+}CD8^{+}$ peripheral blood T lymphocytes were significantly higher in mice immunized with a PCV2 DNA vaccine and IL6 than in animals that did not receive IL6 [17]. Furthermore, levels of antibodies against classical swine fever virus, IgG, IgA, IFN-γ, and $CD8^{+}$ T cells in peripheral blood were higher if the vaccine used to immunize piglets was combined with IL6 [18]. Previous research implied that IL6 might also fulfil its function in heterologous tissues and animals of other species [14,15].

In this study, the effects of rRABV carrying canine IL6 (rHEP-CaIL6) between G and L was investigated in mice. Our results indicate that rHEP-CaIL6 enhances the immunogenicity of RABV but has a good safety profile when compared with the parent strain HEP-Flury. In addition, HEP-Flury was previously shown to induce an immune response in dogs [19]. Therefore, rHEP-CaIL6 might be a rabies vaccine candidate.

2. Materials and methods

2.1. Viruses and animals

rHEP-CaIL6 was rescued based on the HEP-Flury strain and confirmed (Supplementary material 2). HEP-Flury and rHEP-CaIL6 were propagated in BHK-21 cells. CVS-24 was propagated in suckling mouse brains. Female Kunming (KM) and BALB/c mice were purchased from the Center for Laboratory Animal Science of the Southern Medical University (Guangzhou, China). Mice were housed in the Laboratory Animal Center of the South China Agricultural University. All animal experiments were carried out in compliance with specific pathogen-free requirements that were previously approved by the ethics committee for animal experiments of the South China Agricultural University. All possible efforts were made to minimize the suffering of laboratory animals. Procedures were based on the national standard Laboratory Animal Requirements of Environment and Housing Facilities (CALAS, GB 14925-2001) as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Investigation of body weight changes and clinical symptoms in mice

BALB/c mice (6–7 weeks of age) were infected intracerebrally (i.c.) with 3.3×10^{4} FFU of HEP-Flury, rHEP-CaIL6 or with 30 μl medium (mock infection). Each group consisted of eight mice. Body weight and clinical symptoms were recorded daily for 31 days. Changes in body weight are presented as the mean value ± standard error (SE). Regarding clinical symptoms observed in mice, scores were assigned as follows: 0, no clinical symptoms observed; 1, loss of initial body weight > 5%; and 2, piloerection.

2.3. Immunization and in vivo challenge

Groups of 10 KM mice (6–7 weeks of age) were immunized by the intramuscular injection of 1.0×10^{3} FFU, 1.0×10^{4} FFU, 1.0×10^{5} FFU HEP-Flury or rHEP-CaIL6, respectively. Medium was used for mock infections. Serum was obtained at days 7, 14, and 21 after immunization and used to determine VNA levels by means of fluorescent antibody virus neutralization (FAVN) tests as described previously [20]. Concentrations/titres of anti-RABV antibodies were assessed by ELISA (Synbiotics, USA) according to the manufacture's protocols. For challenge experiments, 23 days after immunization, mice were i.c. challenged with 50 mouse intracerebral lethal doses 50 ($MILD_{50}$) of CVS-24. The number of survivors was recorded daily for 3 weeks.

2.4. Flow cytometry

KM mice of 6–7 weeks of age were infected intramuscularly with 100 μl (equivalent to 10^{6} FFU/ml) of rHEP-CaIL6, HEP-Flury or DMEM, respectively. Mouse inguinal lymph nodes were harvested at days 4, 7, and 10 post-infection. Single-cell suspensions were obtained as described previously [10], and stained with antibodies against markers of T cells (CD3e-FITC, CD4-PE, CD8a-PerCP-Cy5.5) or B cells (CD19-FITC, CD40-PE) (all antibodies purchased from Affymetrix eBioscience, USA) by incubation for 30 min on ice. A minimum of 100,000 events were counted using a Beckman FC 500 flow cytometer (Beckman Coulter, USA). Data were analyzed using FCS Express 4 flow cytometry (De Novo Software, USA).

2.5. Quantitative real-time PCR

IL6-induced gene expression at days 6, 9, and 12 after infection in CNS tissues was investigated as described in Supplementary material 2. Primers used to amplify target and reference genes are listed in Supplementary material 2.

2.6. Statistical analysis

Experiments were carried out in triplicate. Data were analyzed using GraphPad Prism 6 software (GraphPad Software, USA). The statistical significance was determined using the Student's *t*-test or Log-rank (Mantel-Cox) Test. $P < 0.05$ was considered to indicate statistically significant differences.

3. Results

3.1. Safety of rHEP-CaIL6 in mice

The safety of rHEP-CaIL6 was determined in BALB/c mice. As shown in Fig. 1A, the body weight of mice infected with rHEP-CaIL6 recovered faster compared with HEP-Flury and regained their initial body weight at day 9 post-infection. Clinical symptoms observed are shown in Fig. 1B. Mice infected with either rHEP-CaIL6 or HEP-Flury showed similar clinical symptoms during the early stages of infection. However, at day 10 post-infection, mice infected with HEP-Flury started to develop more severe symptoms that persisted for a longer period ($P < 0.05$ at several time points) than those mice infected with rHEP-CaIL6. In summary, mice infected with rHEP-CaIL6 showed an earlier onset of recovery (increased body weights) compared with HEP-Flury.

3.2. Immunogenicity of rHEP-CaIL6

KM mice were immunized intramuscularly with different doses of rHEP-CaIL6 or HEP-Flury. Increasing inoculating doses of vaccine were designed to investigate whether the low dose vaccine induced high antibody titres when IL6 was over expressed and the lowest dose of rHEP-CaIL6 that would provide sufficient protection from a lethal challenge. Peripheral blood samples were collected at 7, 14, and 21 days post-immunization (dpi) and serum was used to determine the concentrations of anti-RABV antibodies and VNA. As shown in Fig. 2A, at 7 dpi, levels of anti-RABV antibodies were <0.6 EU, independent of the strain and dose administered. After two or three weeks, the anti-RABV antibody concentrations had increased to >0.6 EU, which is considered protective against RABV infection [21]. Interestingly, at 14 and 21 dpi, significantly higher levels of anti-RABV antibodies were detected in mice immunized with rHEP-CaIL6 compared with HEP-Flury group. VNA levels >0.5 international units (IU) are considered protective against RABV infection. As shown in Fig. 2B, concentrations of VNA in mice

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