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Immune responses in Balb/c mice induced by a candidate SARS-CoV inactivated vaccine prepared from F69 strain

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Abstract

The immunogenicity of a candidate-inactivated vaccine prepared from SARS-CoV F69 strain was evaluated in Balb/c mice. Potent humoral immune responses were induced under the elicitation of three times of immunizations at 2-week intervals with this vaccine, combined with three types of adjuvants (Freund's adjuvant, $Al(OH)_3$ adjuvant and CpG adjuvant). Titers of specific IgG antibodies in three test groups all peaked in the sixth week after first vaccination, but significant differences existed in the kinetics of specific IgG antibody levels. The strong neutralizing capacity exhibited in micro-cytopathic effect neutralization tests indicated the specific antibodies are protective. Western blot assay further demonstrated the specificity of the induced serum antibodies.

Keywords: SARS coronavirus (SARS-CoV); Inactivated vaccine; Immunogenicity; Balb/c mouse

1. Introduction

Severe acute respiratory syndrome (SARS) is the first severe viral epidemic we encountered this century. It once spread in more than 30 countries and regions in 2003, severely threatened worldwide public health. The causative agent of SARS was confirmed and formally announced to be a novel coronavirus by the World Health Organization in April 2003, which established solid bases for us to effectively control and finally eradicate this disease [1–6].

SARS coronavirus (SARS-CoV) is novel, mutative and highly infectious compared to other known coronaviruses. More than 40 genomic sequences of different SARS-CoV isolates have been determined, and some important structural and functional proteins have been unscrambled [7–11]. The first outbreak of SARS has been controlled worldwide, but many things regarding the nosogenesis, the antigenicity and the immuogenicity of SARS-CoV still remain unclear, and no specific drugs have been developed by now. In the long term, safe and efficient SARS vaccines will play im-

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portant roles in the prevention of SARS from possible future outbreaks.

The present study was performed with the objective of determining the immunogenicity of a candidate-inactivated SARS-CoV vaccine made from F69 strain in Balb/c mice.

2. Materials and methods

2.1. SARS-CoV strain

SARS-CoV F69 strain (NCBI/Genbank AY313906) was isolated from the samples of an onset SARS patient in the Guangdong province, China in 2003, and was screened out as the vaccine strain [12,13]. Vero E6 cells were cultivated routinely with MEM medium containing no bovine serum, followed by infection with F69 strain virus. When the cytopathic effect (CPE) reached above 75%, the cell suspension was frozen and thawed three times, and stored at −70 °C. The titer of virus suspension was measured at about $10^{6.7}$ $TCID_{50}$/ml.

2.2. Preparation of inactivated vaccine

Large-scale cultivated F69 strain virus was inactivated by treatment with 0.4% formaldehyde (v/v) for 24 h, and the inactivation efficiency (100%) and antigenicity of the inactivated virus were strictly identified [14]. After centrifugating at 4000 × *g* for 30 min, the virus supernatant was collected and purified (concentration and gel permeation chromatography) as the immunogen for animal immunization. Three adjuvants were employed, namely, Freund's adjuvant (including Freund's complete adjuvant and Freund's incomplete adjuvant, Sigma), CpG adjuvant (100 μg/ml, provided by the Molecular Immunology Institute of the First Military Medical University, China), and $Al(OH)_3$ adjuvant (self-prepared, 1.0 mg/ml). An equal volume of adjuvant was mixed with the prepared immunogen before immunization.

2.3. Animal immunization

The 6-week-old Balb/c mice were fed in groups in a negative pressure isolator. These mice were divided into three test groups according to the adjuvant: group A (immunized with Freund's adjuvant vaccine, $n = 4$), group B ($Al(OH)_3$ adjuvant vaccine, $n = 4$), and group C (CpG adjuvant vaccine, $n = 4$). Simultaneously, adjuvant controls (injected with adjuvant alone) were set. Three times of immunizations were given at intervals of 2 weeks (on D0, D14 and D28). The first immunization was performed subcutaneously with a dose of 0.2 ml (the virus protein concentration was 0.8 mg/ml before mixing with adjuvant) each mouse. The second and the third vaccination were carried out in celiac way with a dose of 0.1 ml, but the vaccine for the third immunization contained no adjuvant. After the first vaccination, 11 batches of blood samples were collected by tail bleeding from the initial day (D0) to day 64 (D64). Serum was separated by centrifugation at 2500 × *g* for 10 min and stored at −20 °C.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates were coated with inactivated SARS-CoV dilution (containing 1.0 μg/ml total virus proteins) overnight at 4 °C. After blocking with 15% bovine serum in PBST (phosphate-buffered saline containing 0.1% Tween-20) at 37 °C for 60 min, the plates were washed five times with PBST. Then two-fold serial serum dilutions were added (100 μl/well) and incubated at 37 °C for 60 min. Washing the plates, HRP (horseradish peroxidase)-conjugated antibodies (1:1000 goat anti-mouse IgG, or 1:10,000 goat anti-mouse IgM, Sino-American Biotech) were added (100 μl/well), and incubated at 37 °C for 60 min. Washing the plates with PBST, then OPD substrate (*O*-phenylendiamine, Sigma) was added (100 μl/well) and incubated at 37 °C for 20 min. The reaction was stopped by 2.0 M sulfuric acid, and the absorbance at 490 nm (A_{490}) was measured by a microplate reader (BioRad, Model 550). In this assay, normal serum was used as negative control, and a positive antiserum was included in each plate as an inter-plate variability control. Antibody titer was defined as the highest dilution of serum at which the A_{490} ratio (A_{490} of sample/A_{490} of negative control) was greater than 2.0.

2.5. Neutralization test

Neutralizing antibody titer was measured according to the modified protocol for polio antibodies [15]. Each serum sample was diluted into two-fold serial dilutions with MEM maintenance medium, then mixed with an equal volume of 100 $TCID_{50}$ active virus and incubated at 37 °C for 60 min. After neutralization, the mixtures were successively added (100 μl/well) into Vero E6 cell monolayers in microtiter plates; but wells for normal cell control were added into 100 μl maintenance medium, and wells for virus control were added into unneutralized virus instead. After that, the plates were incubated at 37 °C in 5% CO_2 incubator, and cell status was monitored by SARS-CoV CPE every 24 h, until all wells of virus control showed CPE but the cell control remained normal. Neutralizing antibody titer was defined as the highest dilution of serum, which protects 50% of the cultures against CPE. Photographs were taken by a phase-contrast microscope.

2.6. Western blot assay

Western blot assay was carried out according to the Protocol in Molecular Cloning [16]. First, SARS-CoV proteins were separated by SDS-PAGE on a 5% stacking polyacrylamide gel in combination with a 12% separating gel in a mini-apparatus (Bio-Rad, Mini Protean 3 Well). The separated proteins in the gel were then transferred to a nitrocellulose membrane in a small apparatus (Bio-Rad, Mini Trans-Blot) with 0.65 mA/cm^2 current for 2.5 h. After blocking with