Immune Responses and Histopathological Changes in Rabbits Immunized with Inactivated SARS Coronavirus[*]

Chuan-hai ZHANG[1,2], Xin-jian LIU[1,3], Yi-fei WANG[1**], Jia-hai LU[4], Huan-ying ZHENG[5], Sheng XIONG[1], Mei-ying ZHANG[1] and Qiu-ying LIU[1]

(1. Biomedicine Research & Development Center, Jinan University, Guangzhou 510630, China; 2. Department of Chemistry and Biology, West Anhui University, Lu-an 237012, China; 3. The First People's Hospital, Shanghai Jiaotong University, Shanghai 200080, China; 4. School of Public Health, Sun Yat-sen University, Guangzhou 510080, China; 5. Center for Diseases Control and Prevention of Guangdong Province, Guangzhou 510300, China)

Abstract: To evaluate the immunogenicity of inactivated SARS coronavirus (SARS-CoV), three groups of rabbits were immunized three times at 2-week intervals with inactivated vaccine + adjuvant, adjuvant, and normal saline respectively. Eight batchs of serum were sampled from the auricular vein at day 7 to day 51, and specific IgG antibody titers and neutralizing antibody titers were detected by indirect ELISA and micro-cytopathic effect neutralizing test. Antibody specificity was identified by proteinchip assay. Histopathological changes were detected by H&E staining. The results showed that, rabbits in the experimental group immunized with inactivated SARS-CoV all generated specific IgG antibodies with neutralizing activity, which suggested the inactivated SARS-CoV could preserve its antigenicity well and elicit an effective humoral immune responses. The peak titer value of specific IgG antibody and neutralizing antibody reached 1:40960 and 1:2560 respectively. In the experimental group, no obvious histopathological changes was detected in the H&E stained slides of heart, spleen, kidney and testis samples, but the livers had slight histopathological changes, and the lungs presented remarkable histopathological changes. These findings are of importance for SARS-CoV inactivated vaccine development.

Key words: SARS coronavirus(SARS-CoV); Humoral immune response; Histopathological change

Severe acute respiratory syndrome (SARS) is a novel viral epidemic which spread to more than thirty countries in 2003 and severely threatened worldwide public health. The clinical symptom of SARS is characterized by fever, dyspnoea, lymphopenia, and rapidly progressing changes observed by radiography. Its pathogen has been affirmed to be a novel coronavirus, SARS coronavirus (SARS-CoV), which

established a solid bases for effective control and ultimate eradication of the disease (3,4,8,9). The genomes of SARS-CoV has been mapped and some important structural and functional proteins have been determined, but many features regarding the noso-genesis, the antigenicity and the immuogenicity of SARS-CoV still remain indistinct (2,7,10,17).

Vaccines play a crucial role in the control of infectious diseases. Previous experiences on human antiviral vaccines indicated that inactivated vaccines are much easier to develop and hold more potential. To investigate a SARS inactivated vaccine, effec-tiveness and safety of vaccine are two important aspects. Our efforts have focused on the investigation of the immunogenicity and safety of inactivated SARS-CoV on animals such as mice, rabbits, pigs, and horses (15,16). This paper reports the specific antibody kinetics and the histopathological changes in rabbits immunized with inactivated SARS-CoV, which a view to gaining some insight towards the develop-ment of SARS inactivated vaccine.

MATERIALS AND METHODS

SARS-CoV strain

SARS-CoV F69 strain (GenBank AY313906) was isolated from the samples of a onset of SARS patient in Guangdong province, China in 2003, and was screened out as the vaccine strain (5,18). Vero E6 cells were cultivated routinely with MEM medium con-taining no bovine serum, followed by infection with F69 strain virus. After the cytopathic effect (CPE) reached more than 75%, the cell suspension was frozen and thawed three times, and stored at $-70\,℃$. The titer of virus suspension was $10^{6.7}$ TCID$_{50}$/mL determined with micro-plate CPE method.

Vaccine preparation

Large-scale cultivated F69 virus suspension was inactivated with 0.4% formaldehyde (v/v) for 24 h at $37\,℃$, and the inactivation efficiency (100%) was precisely identified (6). After centrifugation at $4\,000\times g$ for 30 min to remove precipitate, the virus supernatant was collected and purified by concentrating and gel permeation chromatography, then stored at $-70\,℃$ as the immunogen for animal immunization. The protein concentration of the prepared vaccine was 0.8 mg/mL.

Animal immunization

Adult New Zealand rabbits (male, 2.0-2.5 kg) were randomly divided into 3 groups (n=4): the experi-mental group (G1, immunized with vaccine + adjuvant), the adjuvant group (G2, immunized just with adjuvant), and the control group (G3, injected with 0.9% NaCl sulution). Rabbits all were inoculated 3 times. The immune protocol was shown in Table 1. Eight batches of blood were sampled from the auricular vein at day 7, 14, 21, 28, 35, 38, 42 and 51. Serum was separated by centrifugation at $2\,500\times g$ for 10 min and stored at $-20\,℃$.

Enzyme-linked immunosorbent assay

The titer of serum specific IgG antibody was measured

Table 1. Rabbit immunization protocol

Group (n=4)	Immunization (day, inoculation way)		
	1st (day 0, subcutaneously)	2nd (day 14, subcutaneously)	3rd (day 28, auricular vein)
G1	1 mL Vaccine + 1 mL FCA	1 mL Vaccine + 1 mL FIA	1 mL Vaccine
G2	1 mL FCA + 1 mL NS	1 mL FIA + 1 mL NS	1 mL NS
G3	2 mL NS	2 mL NS	2 mL NS

Vaccine: purified inactivated SARS-CoV; FCA: Freund's complete adjuvant; FIA: Freund's incomplete adjuvant; NS: normal saline.

by indirect enzyme-linked immunosorbent assay (EL-ISA). A 96-wells microtiter plate was coated with inactivated SARS-CoV dilution (containing 1.0 µg/ mL total virus proteins). Then 2-fold serial serum dilutions were added (100 µL/well) and incubated at 37℃ for 60 min. The plate was washed with PBST, HRP-conjugated goat-anti-rabbit IgG antibodies (1:1000 dillution, Sino-American Biotech) were added (100 µL/well), and incubated at 37℃ for 60 min. The plate was washed, then OPD substrate (*O*-pheny- lendiamine, Sigma) was added (100 µL/well) and incubated at 37℃ for 20 min. The reaction was stopped with 2.0 mol/L sulfuric acid and the absorbance at 490 nm (A_{490}) was measured by a microplate reader. 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.

Neutralizing test

Neutralizing antibody titer was determined by the micro-cytopathic effect (CPE) neutralizing test according to the modified protocol for polio antibodies (13). The serum was diluted into two-fold serial dilutions with MEM maintenance medium, then mixed with an equal volume of 100 $TCID_{50}$ active SARS-CoV and incubated at 37℃ for 60 min. After neutralization, the pretreated mixtures were successively added into Vero E6 cell monolayers in a microtiter plate (100 µL/well); wells for normal cell control were added into 100 µL maintenance medium, and wells for virus control were added into unneutralized virus. The plate was incubated at 37℃ in 5% CO_2 incubator, and cell status was monitored by SARS-CoV CPE every 24 h, until all wells of the virus control showed CPE but the cell control remained normal. Neutralizing antibody titer was defined as the highest dilution of serum which protected 50% of the cultures against CPE.

Proteinchip assay for antibody specificity

Antiserum samples were sent to Shanghai Health-Digit Biotech Ltd (11) for antibody specificity determination with a special SARS protein chip system based on specific antigen-antibody interactions. Recombinant antigenic SARS-CoV proteins-M, N, E, 3CL and four segments of S protein were immobilized on a solid matrix. Diluted mice serum was added into the chip and incubated for 30 min at 37℃. After washing, specific enzyme-conjugated antibodies and tracer-marked substrates were added and incubated at 37℃. Finally, the developed chemiluminescent light signals were captured, digitized and analyzed by a chip reader.

Histopathological detection

On day 51 after the first vaccination, all the rabbits were sacrificed, then autopsied to gain the lung, liver, heart, spleen, kidney and testis. Tissues of the sampled organs were fixed in 10% neutral-buffered formalin and routinely processed for paraffin embedding. Serial 4 µm sections were cut from each block. The tissue slices were stained with hemotoxylin and eosin dyes (H&E), and detected with photomicrography.

RESULTS

Kinetics of specific IgG antibody titers

Antibody titers of eight batches of serum samples from day 7 to day 51 were measured by indirect ELISA (Fig.1). The specific IgG antibodies in experimental group (G1) were detectable even on day

7, and rapidly reached a peak value of 1:40 960 on day 35. After day 35, the antibody levels had a gradual decline. On day 51, the specific IgG antibody titers remained at about 1:12 800. In the adjuvant group (G2) and control group (G3), the sera all were negative.

Variation of neutralizing antibody titers

The inactivated SARS-CoV antigen elicited potent neutralizing antibody responses (Fig. 2). On day 14, neutralizing antibody was detected in the experimental
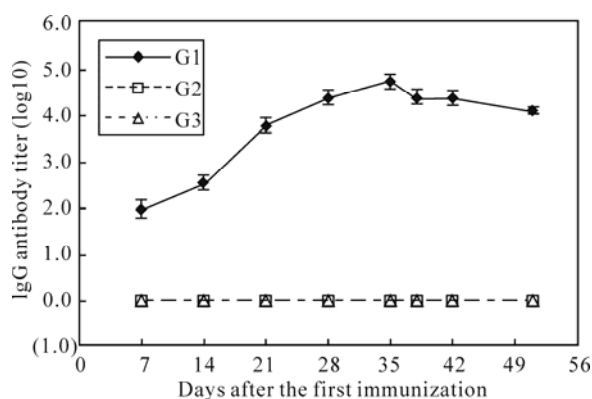

Fig. 1. Kinetics of the titers of specific anti-SARS-CoV IgG antibodies. G1, G2, G3 represent three test groups with different immunization protocols shown in Table 1. Eight batches of rabbit serum from day 7 to day 51 were collected and measured with indirect ELISA. Antibody titers are shown on a logarithmic scale
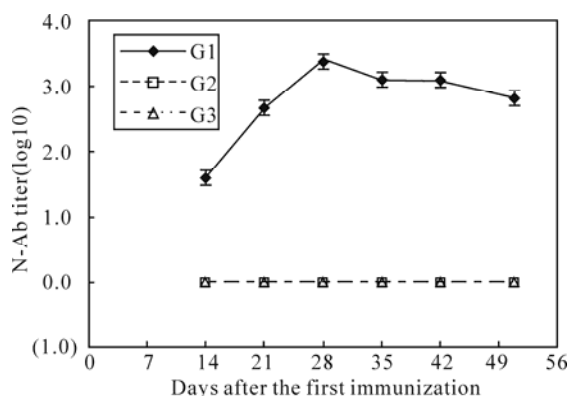

Fig. 2. The dynamic variation of neutralizing antibody (N-Ab) titers. Six batches of rabbit serum were sampled from day 14 to day 51 in three test groups (G1, G2, G3). Neutralizing antibody titers were determined by micro-cytopathic effect neutralizing test and are shown on a logarithmic scale.

group, then followed a rapid increase. Four weeks after the first vaccination, neutralizing titer reached a peak value of 1:2 560. In the subsequent four weeks, neutralizing antibody titers remained or declined slightly despite a third vaccination being administered at this stage, but it was negative in the adjuvant group and control group.

Specificity identification of the antiserum

Two sets of antiserum samples from experimental group and one normal serum were analyzed on day 33 with the SARS protein chip. The representative profiles are shown in Fig. 3. Antibodies of the 2 stocks of antiserum showed specific interactions with the known recombinant SARS-CoV proteins, including the N, 3CL and four S protein segments. The normal serum showed a negative result.

Histopathological changes of rabbit tissues

In experimental group (G1), no obvious histopathological changes were detected in H&E stained tissue slides of the heart, spleen, kidney, testis, but the liver tissues had slight histopathological changes, while the
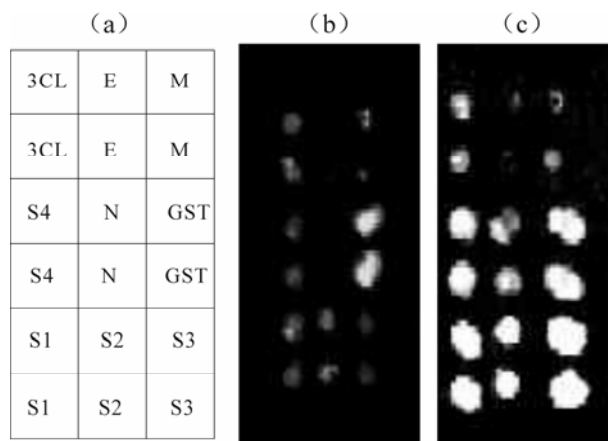

Fig.3. SARS-CoV proteinchip assay. (a) Layout of the recombinant SARS-CoV proteins-M, N, E, 3CL, and four segments of S protein (S1, S2, S3 and S4) on the chip. (b) Chemiluminescent signals of normal serum. The inner control (GST) correctly showed positive. (c) Interaction signals of antiserum. Positive interactions are shown.
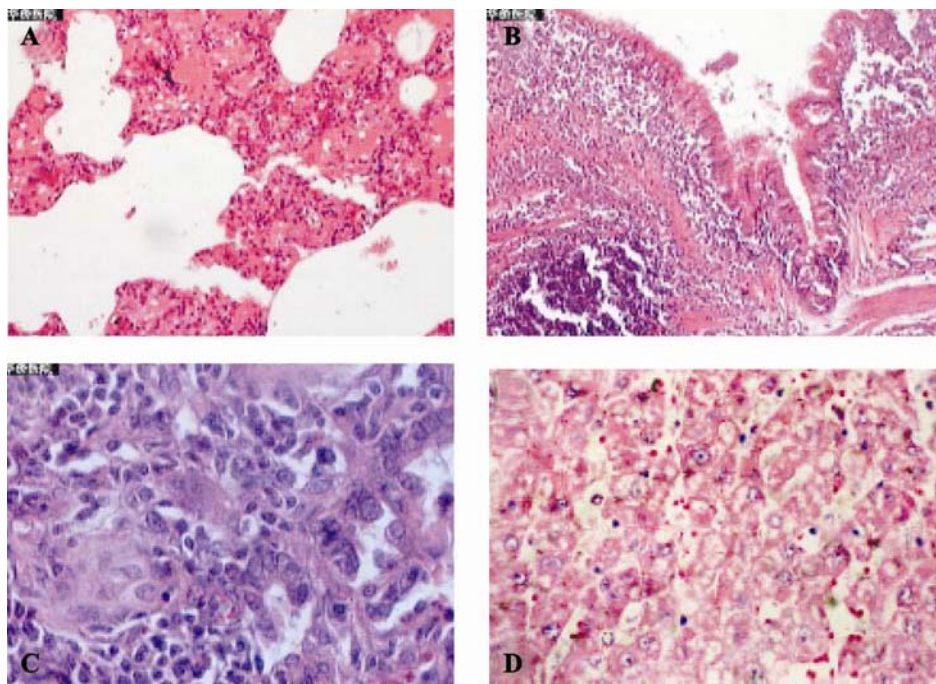
Fig. 4. Immunization-induced tissue histopathological changes in rabbit. **A:** Diffuse alveolar damages in lung tissue: some alveolar narrow replacing other alveolar compensatory hyperinflation, alveolar wall thickening, desquamation of pneumocytes in alveolar spaces. **B:** Interstitial mononuclear inflammatory infiltrates, focal necrotic inflammatory debris on bronchiole wall. **C:** Interstitial mononuclear inflammatory infiltrates mainly included lymphocytes and plasmacyte (H&E ×400). **D:** Some liver cell degeneration, slight ballooning degeneration in rarefied cytoplasm.

lungs showed marked histopathological changes. The representative profiles and relevant notation are shown in Fig. 4.

DISCUSSION

Immunogenicity evaluation is a basis for viral inactivated vaccine development. In this study, SARS-CoV was 100% inactivated by treating with 0.4% formaldehyde (v/v) for 24 h, which ensured the antigenicity was maintained (7). Three sets of immunizations at 2-week intervals were administrated. The results showed that the SARS-CoV F69 strain inactivated vaccine could elicit a potent humoral immune responses in rabbits, and the specific antibodies including neutralizing antibodies, remained at high levels for a extended period. Our other studies on the evaluation of inactivated SARS-CoV in mice

(15,16), pigs and horses (results not shown) also showed positive results which demonstrated the strong immunogenicity of this virus.

Bioinformatics analyses of the SARS-CoV genome may provide valuable information for vaccine development. In this study, rabbit antiserum was detected with a SARS specific protein chip. From the profiles in Fig. 3, the positive interaction signals fully identified the specificity of the antiserum antibodies. Furthermore, the different signal intensity (data not shown) indicated different antigenicities for the tested SARS-CoV proteins, the S protein (comprising 4 separate segments on the chip) was much stronger compared with the N, 3CL, or M proteins.

It is of interest that rabbits immunized with inactivated SARS-CoV exhibited interstitial pneumonitis and liver cell degeneration which were not present in

control group. We are certain such histopathological changes were not because of incomplete inactivation since the inactivation efficiency to cultured SRAS-CoV was precisely identified (5, 6, 16). The possible causes of this immunization-induced tissue damages may be the anti-SARS-CoV antibody cross- reacting with cytokine and thus modulating cytokine responses (1), the induction of autoantibodies (12), or virus-induced cell-mediated immuopathological responses (14). Based on the primary observation above, further investigations are worthwhile to confirm these histopathological changes, detect the principal mechanism, and to find the possible relationship with immunization dosages.

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