Chinese Science Bulletin 2003 Vol. 48 No. 23 2621—2625

Preparation, characterization and preliminary *in vivo* studies of inactivated SARS-CoV vaccine

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Abstract **A large quantity of SARS-CoV virus was proliferated in Vero cells, inactivated with β-propiolactone, then purified by Sepharose 4FF column chromatography to prepare inactivated vaccine. The vaccine was identified by Western blot, mass spectrographic analysis, ELISA and electron microscopy. The vaccine with or without aluminum hydroxide adjuvant was inoculated into female BALB/c mice at different dosages. The result showed that the antibodies to SARS-CoV were induced in the mice. The antibody levels induced by the vaccine with aluminum hydroxide were higher than those without aluminum hydroxide.**

Keywords: SARS, coronavirus, inactivated vaccine.

DOI: 10.1360/03wc464

Severe acute respiratory syndrome (SARS) is one of the most serious emerging diseases in China during the past several decades. It is caused by a novel coronavirus and widely spread in common population. The symptoms of the disease are severe, and the mortality of patients is as high as 6%—7%. At present, research showed that the disease was mainly transmitted by the droplet in respiratory tract and mucosa[1—5]. According to the experience in controlling infectious disease, vaccination would be the most effective measure to prevent the epidemic of SARS[6—9]. Our study began with developing inactivated SARS vaccine. This report describes a procedure for preparing inactivated vaccine by the cultivation of SARS virus in Vero cells. Besides this, the immunity of the vaccine in mice is reported.

1 Materials and methods

(ⅰ) Virus and cells. SARS-CoV BJ01 strain was isolated from a patient in Beijing by the Institute of Microbiology and Epidemiology, Academy of Military Medicine[10]. The whole genome of the strain was sequenced, and the sequence has been submitted to GenBank (Accession: NCBIAY 278488). Vero cells were provided by the National Vaccine and Serum Institute and specially used for vaccine production. The cells meet the relevant WHO requirements and Requirements for Biologics, People's Republic of China, 2000.

(ⅱ) Preparation of vaccine. Vero cells were infected with SARS-CoV BJ01 strain and incubated in serum-free medium, 5 mL per bottle, at 37℃ for 36 h. When the infective titer reached $10^7 TCID_{50}$, the cells were lysed by freezing and thawing at −20℃, then centrifuged at 6000 r/min (Beckman, 25R) for 20 min. The supernatant was harvested, added with 1∶2000 diluted β-propiolactone (Sigma), shaken up and incubated at 4℃ for 24—72 h. After inactivation, the sample stood at 37℃ for 2 h to hydrolyze β-propiolactone. Inactivated virus suspension was centrifuged at 4℃, 6000 r/min (Beckman, 25R) for 30 min. The supernatant was harvested and concentrated with PEG20000 (Sigma) to the 1/20 of original volume, further concentrated by centrifugation (30000 *g*, 20 min), using Cetriplus YM-100 (Millipore), then purified by Sepharose 4FF column chromatography (2.6 cm in diameter, 95 cm in length, at a flow rate of 0.8 mL/min). Each fraction was analyzed by SDS-PAGE (data not shown). The harvested viruses were pooled and further concentrated by centrifugation using Centriplus-20. The total protein content of the sample before and after chromatography was measured by Lowry method. 1% human albumin as a stabilizer and 0.01% thimerosal as an antiseptic were added to the purified virus. Samples taken from the purified virus were examined by electron microscopy, Western blotting, ELISA and mass spectrographic analysis. The dosage of antigen was finally adjusted with PBS. To prepare the pure antigen containing aluminum hydroxide adjuvant, the following procedures were adopted: aluminum hydroxide (National Vaccine and Serum Institute) was added to antigen solution to a final concentration of 0.6 mg/mL, shaken for 1 h at 4℃ to make the antigen adsorbed onto the adjuvant completely.

(ⅲ) Immunity test in animals. Six-week-old female BALB/c mice were purchased from Beijing Weitonglihua Ltd. Co. of Laboratory Animal Technique. The mice were divided into several groups and inoculated with different dosages of the inactivated vaccine or vaccine plus aluminum hydroxide adjuvant, respectively. Each mouse was injected i.p. twice at an interval of three weeks. Sera for detection of IgG by ELISA were collected from orbit of mice three weeks after the first injection, and from

heart one week after the second injection. ELISA was performed sequentially from the solid phase, polystyrene plate, with a ladder of reagents consisting of first, purified SARS-CoV virus; second, two-fold diluted serum; third, biotin-labeled goat anti-mouse IgG (γ-chain specific, Southern Biotechnology Associates, Inc. USA); fourth, streptavidin-conjugated with alkaline phosphatase (Southern Biotechnology Associates, Inc. USA); and finally, p-nitrophenyl-phosphate. The chromogen product was measured for absorbance at 414 and 405 nm with the aid of an autoreader (Labsystems Muliskan Ascent, Thermo Bioanalysis Company, Finland).

2 Result

(ⅰ) Preparation of inactivated SARS-CoV vaccine. After amplifying in Vero cells, SARS-CoV was harvested and inactivated with 1∶2000 diluted β-propiolactone. The sample was concentrated with PEG20000, further by Centriplus YM 100 centrifugation, and then purified by Sepharose 4FF column chromatography. As shown in Fig. 1, two peaks appeared on Sepharose 4FF chromatogram. By electron-microscopic inspection viral particles of about 100 nm with typical corona spikes could be observed in elute of the first peak (Fig. 2) while there were not similar particles in elute of the second. Therefore SARS-CoV appeared in the first peak and other macro-proteins in the second peak. All fractions containing SARS virus were pooled and condensed with Centriplus-20 for the preparation of inactivated vaccine of SARS-COV.

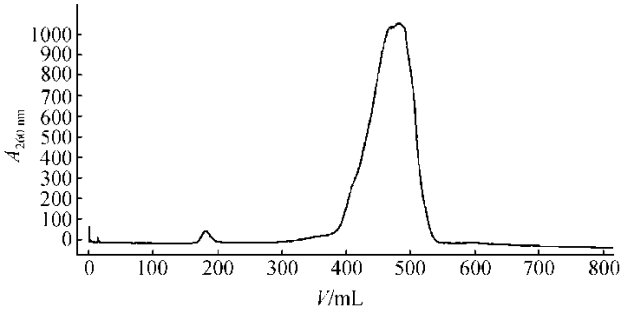

Fig. 1. The gel filtration chromatogram of inactivated SARS-CoV via Sepharose 4FF. The supernatant of β-propiolactone inactivated SARS virus was concentrated by PEG 20000, condensed with Centriplus YM-100, and applied to the Sepharose 4FF column (2.6 cm × 95 cm) in PBS at the flow rate of 0.8 mL/min. The changes of absorbance under 260 nm were checked on AKTA purifier. Each fraction was collected in 5 mL size.

(ⅱ) Detection of residual live virus in the inactivated vaccine. β-propiolactone was employed to inactivate the virus at 4℃ with a ratio of 1∶2000 or 1∶4000. Samples were taken after 20, 24, 28, 42, 48, 66 and 72 h of inactivation respectively and put at 37℃ for 2 h to hydrolyze β-propiolactone. The infectivity of residual virus was examined by inoculating each sample into Vero cells for three blind passages. The result showed that the virus could be completely inactivated with β-propiolactone, at dilution of 1∶2000 for 24 h, or 1∶4000 for 28 h.

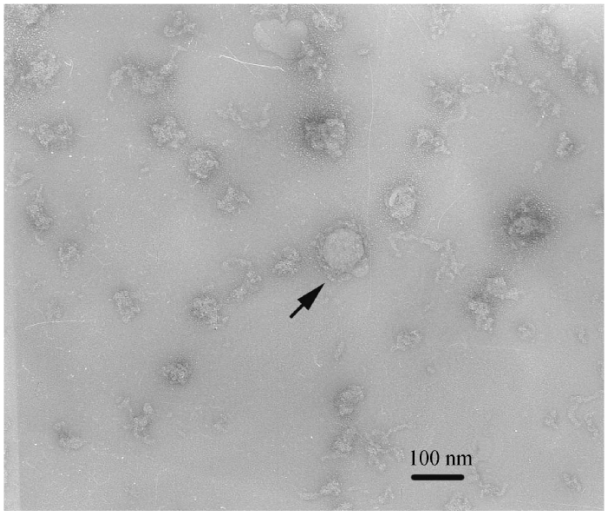

Fig. 2. Electron micrograph of inactivated vaccine (virus particles).

(ⅲ) SDS-PAGE and Western blot identification. The purified virus particles were identified by SDS-PAGE (Fig. 3) and Western blotting (Fig. 4) using the sera from the SARS patients in Beijing as the first antibody, and AP-labeled goat anti-human IgG as the second antibody. The result showed that in Fig. 4, the

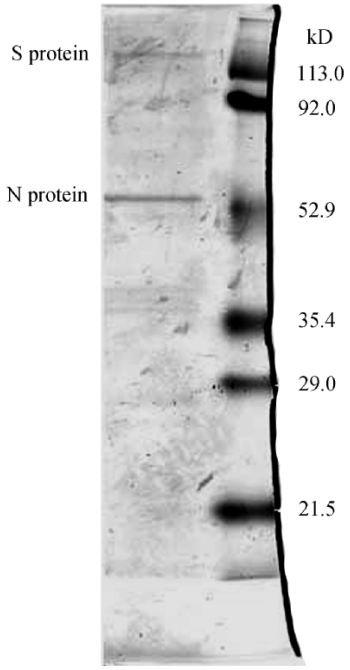

Fig. 3. SDS-PAGE profile of S and N proteins in inactivated vaccine.

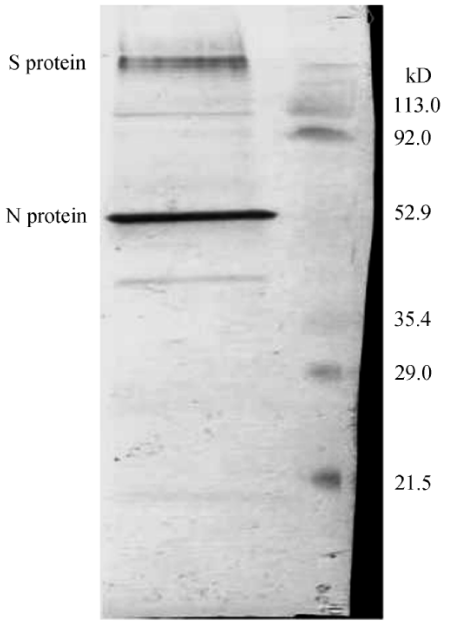

Fig. 4. Western blot profile of S and N proteins in inactivated vaccine. Purified SARS virus was subjected to 12% SDS-PAGE (see Fig. 3) under reducing conditions and was transferred onto a Millipore Immobilon-P PVDF membrane. The membrane was blocked in TTBS containing 5% nonfat milk powder at 37℃ for 30 min before incubation with the diluted serum of anti-SARS positive patient (from Beijing region, TTBS, 1∶1000, 4℃ overnight). Bound antibodies were detected using appropriate alkaline phosphatase-conjugated goat anti-human IgG (TTBS, 1∶1000), followed by nitro-blue tetrazolium chloride (NBT) and 5-bromo-4chloro-3′-indolyphosphate p-toluidine salt (BCIP) for development.

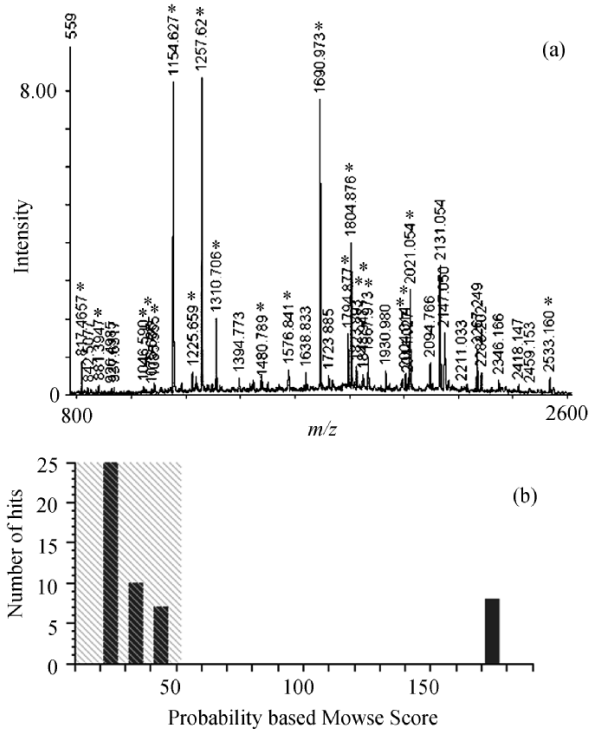

Fig. 5. (a) Fingerprint of S protein peptide. The peaks marked with "*" are from tryptic peptides of S protein. (b) Mass spectrographic analysis of fingerprint of S protein peptide by Mascot. Protein scores greater than 52 are significant (P<0.05). Accession: 1. qi30173397; Mass: 141260; Score: 174; Description: E2 glycoprotein precursor (Spike glycoprotein) (Peplomer protein).

anti-SARS positive sera reacted mainly at about 50 and 140 kD bands, representing N and S proteins respectively.

(ⅳ) Mass spectrographic analysis. The bands of N and S proteins were cut from SDS-PAGE gel (Fig. 3) and used for MALDI-TOF analysis. As shown in Figs. 5 and 6, the two bands were identified as N and S proteins of SARS-CoV BJ01 according to the MS data of SARS-CoV BJ01 provided by NCBI.

(ⅴ) ELISA. The inactivated vaccine was further confirmed by ELISA method using a kit for SARS detection (Beijing Huadajibiai Ltd. Co. of Biotech). The prepared vaccine was subjected to ultrasonic dissolution using 7 mol/L urea, used for coating a 96-well ELISA plate. As suggested by manufacture, we used sera from SARS patients or from normal human as first antibody. Strong positive result was observed by using SARS sera compared with control sera from normal human.

(ⅵ) Immunization protocol. 140 mice were divided into 7 groups, 20 mice per group. Of the 7 groups, 3 were inoculated with the vaccine without adjuvant, and another 3 groups with vaccine containing aluminum hydroxide adjuvant, both at the dosages of 0.1, 1 and 3 μg respectively. The rest group was set up as a blank control (Tables 1 and 2). Each mouse was inoculated with two doses at an interval of three weeks. Mice sera were collected three weeks after the first dose and one week after the second dose for detecting serum antibody.

(ⅶ) Immune response. The immune responses in the mice inoculated with the inactivated vaccine (with or without aluminum hydroxide adjuvant) at various dosages were shown in Tables 1 and 2. The results were summarized as follows: ⅰ) the antibody level induced by two doses of vaccine was significantly higher than that by one dose; ⅱ) the larger the dosage of vaccine was, the higher the antibody titer induced was; and ⅲ) the antibody level induced by the vaccine with aluminum hydroxide adjuvant was slightly higher than that without adjuvant.

3 Discussion

A method for the inactivation and purification of SARS-CoV virus was reported in this paper. Our study proved that the inactivated vaccine, prepared by the proliferation of SARS-CoV virus in Vero cells, inactivation with β-propiolactone and purification by Sepharose 4FF column chromatography, induced antibodies in mice. It provided a practical basis for developing an effective vaccine. The immunization protocol, production procedure and adjuvant of the vaccine are discussed as follows.

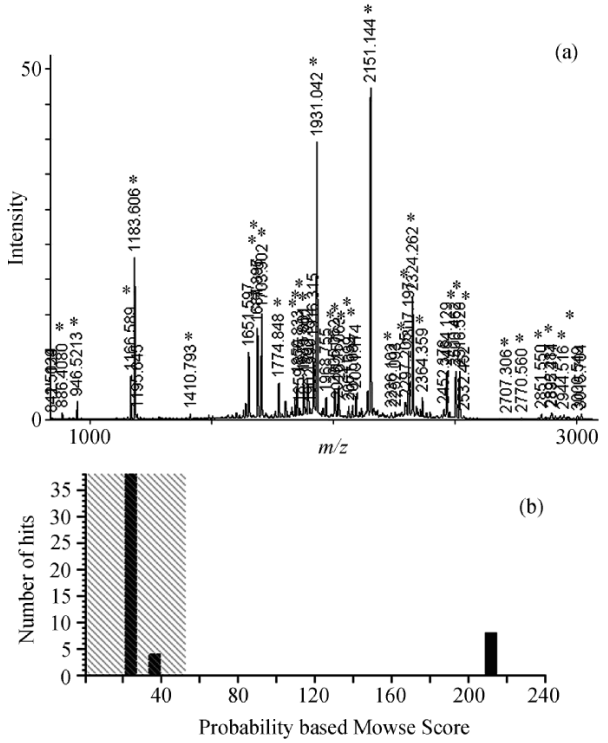

Fig. 6. (a) Fingerprint of N protein peptide. The peaks marked with * are from tryptic peptides of N protein. (b) Mass spectrographic analysis of fingerprint of N protein peptide by Mascot. Protein scores greater than 52 are significant (P<0.05). Accession: 1. qi30173007; Mass: 45997; Score: 212; Description: Nucleocapsid protein.

Table 1 ELISA titers of anti-SARS-CoV IgG of mice 3 weeks after primary immunization[a)]

Inactivated vaccine dose (μg/mouse) immunization	Serum anti-SARS-CoV IgG titer (2^n)	
primary	without alum	with alum
0.1	6±0	5.7±0.6
1	9.7±0.6	11.3±0.6
3	9±0	12.75±1.5

a) Values represent mean ± SD in each group of mice.

Table 2 ELISA titers of serum IgG of mice one week after booster immunization[a)]

Inactivated vaccine dose (μg/mouse) immunization		Serum anti-SARS-CoV IgG titer (2^n)	
primary	secondary	without alum	with alum
0.1	0.1	9.5±1.0	10.25±1.3
1	1	12.25±1.7	13.4±1.8
3	3	13±0	16±0

a) Values represent mean ± SD in each group of mice.

(ⅰ) Immunization protocol. Since this is the first paper reporting the research of inactivated SARS-COV vaccine, and SARS is a new viral disease, there is no immunization schedule for reference. According to our experience in developing influenza vaccine, we set up a schedule of inoculation with two doses at an interval of three weeks[11,12]. The mice were bled three weeks after the first dose and one week after the second dose for detecting serum antibody. The result showed that either one dose or two doses of the vaccine induced good immune response. At present, a two-dose schedule is adopted in the immunization with inactivated influenza vaccine and shows good effect. However, the protective effect——at least partially protective effect——of one dose of influenza vaccine, has been reported recently. On the basis of experience in developing vaccines against other respiratory diseases, we consider that the immune effect of two doses of vaccine is satisfactory. During the epidemic period or in an emergency condition, whether a single dose of inactivated SARS-COV vaccine can provide protective or partially protective effect is to be further studied in clinical trial. However, the result of clinical trial shall be determined after two epidemic periods.

(ⅱ) Production procedure for inactivated vaccine. A procedure for the production of inactivated SARS vaccine was primarily established in this paper. We selected β-propiolactone to inactivate virus because it was a kind of alkylating agent which could react with nucleic acid. β -propiolactone could break the structure of nucleic acid through its reaction with purine bases (mainly guanine). The alkylating agent could either form gaps inside DNA or cause the cross linking of double stranded DNA, so it has been widely used for the inactivation of DNA and RNA viruses[13—16]. We could not detect the residual live virus in the vaccine after inactivation, proving that β-propiolactone was suitable for the preparation of inactivated SARS-COV vaccine. After inactivation, we selected Sepharose 4FF column chromatography to purify virus particles. The antibody was under detective level in the mice immunized with crude antigen (data not shown). We considered that it might be due to the influence of interference of hetero-proteins on the normal immune response of animals. The result of our study showed that the production and purification procedure established here met the requirements of animal test. The procedure suitable for large-scale production is to be further studied.

(ⅲ) Adjuvant. Aluminum hydroxide was selected in our study because it was a safe adjuvant recommended by WHO and has been widely used for various viral and bacterial vaccines including DTP[17—19]. Aluminum hydroxide is an insoluble colloid precipitate with the particle size of 0.1—1 μm, so antigen is bound to the colloid by static electricity. After being adsorbed onto aluminum hydroxide, antigen forms storage pool at inoculation site and releases slowly. It may cause local inflammatory reactions to attract active lymphocytes and enhance immune response. It can also activate the complement system. Our study showed that the antibody level induced by the vaccine with aluminum hydroxide adjuvant was higher than that without adjuvant. However, whether the SARS-COV vaccine in future contains aluminum hydroxide adjuvant needs to be further studied.

(iv) Prospect. Our study showed that it might be successful to develop effective vaccines for the prevention of SARS-CoV. There is still a lot of work to do in this field. Perhaps some scientists have objections to our purification method, even to the inactivated vaccine. They may be anxious about the safety of the inactivated vaccine. They wonder if RNA strand can be completely broken by β-propiolactone. In case individual virus particle contains an incompletely broken RNA strand, a problem in safety may occur. Some scientists may suggest developing subunit vaccine. The subunit vaccine shall be technically feasible, and the production cost is not very expensive. However, unlike the antigen determinants of inactivated vaccine in a natural state, the development of recombinant immunogens that mimic the oligomeric presentation of proteins found in virus particles may be difficult. Meanwhile, whether a single gene or multiple genes shall be selected to prepare the subunit vaccine is to be verified by further study. Another problem faced to us is whether the current BJ01 strain has individual representativeness or not. If SARS-CoV is prevalent again this winter, whether it will be widely varied is still unclear. The field for production is also a problem to the large-scale manufacturing of inactivated SARS-CoV vaccine, since the proliferation of a large quantity of virus can only be performed in P3 laboratory. If an attenuated strain is selected and used for the production of vaccine instead of the current SARS-CoV strain, the above-mentioned problems are hopeful to be solved. In fact, the work to solve the problems is already in progress. This paper describes the inactivation and purification of SARS virus, the preparation of inactivated vaccine and the immunogenicity of the vaccine. It is beneficial to the scientists engaging in the research of SARS vaccine.

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(Received September 15, 2003; accepted November 12, 2003)