Immune responses with DNA vaccines encoded different gene fragments of severe acute respiratory syndrome coronavirus in BALB/c mice

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Abstract

To analyze the immune responses of DNA vaccine encoded different gene fragments of severe acute respiratory syndrome coronavirus (SARS-Cov), SARS-Cov gene fragments of membrane (M), nucleocapsid (N), spike a (Sa), and spike b (Sb) proteins were cloned into pcDNA3.1 (Invitrogen) vector to form plasmids pcDNAM, pcDNAN, pcDNASa, and pcDNASb, respectively. After mice were immunized intramuscularly with pcDNAM, pcDNAN or pcDNASa–pcDNASb plasmid, blood was collected and serum was separated. Humoral immune response was detected with the enzyme-linked immunosorbent assay, and cellular immune response of SARS-Cov DNA vaccines was detected with lymphoproliferation assay and cytotoxic T lymphocyte assay. Results show that cellular and humoral immune responses can be detected after immunization with pcDNAM, pcDNAN or pcDNASa–pcDNASb plasmids in BALB/c mice. However, pcDNAM stimulated the highest cellular immune response than other plasmids, and pcDNASa–pcDNASb stimulated the highest humoral immune response in week 12. The present results not only suggest that DNA immunization with pcDNAM, pcDNAN or pcDNASa–pcDNASb could be used as potential DNA vaccination approaches to induce antibody in BALB/c mice, but also to illustrate that gene immunization with these SARS DNA vaccines different immune response characters.
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Since 2003, the outbreak of severe acute respiratory syndrome (SARS) coronaviruses has spread to several countries in the world, especially in People's Republic of China, Canada, the mortality rate appears to be ∼3% to 6%, and can be as high as 43–55% in the older people [1]. The RNA genome has been sequenced [1,2]. SARS coronavirus replicates in the cytoplasm of host cells, and distinguished by a single-stranded plus-sense RNA genome, it is about 30 kb in length. The viral proteins include the major proteins of spike (S), membrane (M), and nucleocapsid (N) proteins.

The development of a vaccine to prevent SARS acquired considerable interest. It is one of the methods to prevent the transmission of this kind of virus [3].

DNA-based vaccines have recently been shown to induce protective immune responses against several viral agents, such as human immunodeficiency virus [4], bovine and human herpesviruses [5], hepatitis B virus [6], influenza virus [7], rabies virus [8], hepatitis C virus [9], etc. Unlike conventional vaccines employing either killed virus or purified antigens, DNA vaccination efficiently

elicits cellular immune response including cytotoxic T lymphocyte (CTL) in addition to humoral immunity. DNA vaccine mediated cellular immunity provided protection. Usually, only live virus vaccines or virus-like particles were able to induce CTL response. The possession of such capacity appears to be particularly crucial for developing DNA vaccines to control viral diseases.

To compare different immunogenicities of SARS-Cov DNA vaccine encoding different main proteins (S, M, and N proteins), we have inserted S, M, and N protein genes into pcDNA3.1 DNA. Because S gene encoded a large protein with a molecular mass of 150 kDa, S protein can be structurally divided into N-terminal Sa and C-terminal Sb subunits. S protein gene was truncated into Sa and Sb genes into pcDNA3.1 DNA vector. Four plasmids pcD-NAM, pcDNAN, pcDNASa, and pcDNASb were successfully constructed, which code M (26372–27084), N (28102–29380), Sa (21460–23204), and Sb (23114–25301) genes of SARS-Cov genome, respectively. It is important to choose the appropriate gene from SARS-Cov genome for the feasibility of DNA vaccine trial. In this study, we evaluated the immunogenicity of four plasmid constructs expressing M protein, N protein, and Sa and Sb protein, respectively, in BALB/c mice.

Materials and methods

Plasmids

N (28102–29380), M (26372–27084), Sa (21460–23204), and Sb (23114–25301) genes were amplified by RT-PCR from SARS coronavirus (GenBank Accession No: NC_004718), and cloned into the pcDNA3.1 DNA vector (Invitrogen), and four plasmids pcDNAN, pcDNAM, pcDNASa, and pcDNASb were constructed. Briefly, SARS coronavirus RNA was extracted and reverse transcribed (RT) into cDNA. Then N, M, Sa, and Sb genes were amplified with PCR methods. PCR products were inserted into BamHI and EcoRI clone sites of pcDNA3.1 vector. Recombinant plasmids were confirmed by restriction enzyme mapping and DNA sequencing analysis. For DNA immunization, pcDNAN, pcDNAM, pcDNASa, and pcDNASb were transformed into Escherichia coli Top10, and cultured in LB culture medium for 24 h at 37 °C, plasmids were prepared using Qiagen Plasmid DNA Mid Kit (Qiagen), and the purified plasmids were dissolved in sterile phosphate-buffered saline (PBS) at a concentration of 1 mg ml^{-1}.

Animal experiments

Six- to eight-week-old female BALB/c mice were given free access to food and water, and divided into four groups (five mice each group), one group used as control. To evaluate the different abilities to elicit immune responses of SARS-Cov DNA vaccine expressing Sa + Sb, M, and N proteins, three groups of BALB/c mice (A–C) were vaccinated with plasmids by intramuscular injection in the hind leg muscles. Doses and components of plasmids are shown in Table 1. The time for vaccination is shown in Fig. 1.

Humoral immune response

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) was performed as follows: blood was collected

Table 1
The doses and plasmid DNA components used in DNA immunization experiments

Cage	Dose (μg)	DNA components
A	50	pcDNASa–pcDNASb (1:1)
B	50	pcDNAM
C	50	pcDNAN
D	0	Control

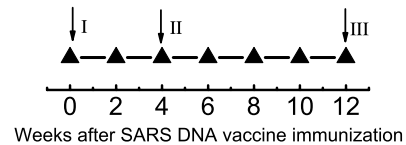BALB/c mice were divided into four cages A, B, C, and D. In each cage, there are five mice.

Fig. 1. The schedule of DNA immunization, (▲) indicated that bloods were sampled, and sera were separated at the indicated week; (I) indicated that mice were immunized; (II) means that the mice was boosted after four weeks of immunization; and (III) indicated that the mice were sacrificed in order to detect the lymphoproliferation and CTL response.

from the retro-orbital plexus using a capillary tube for antibody determination during each two weeks, all samples were incubated for 4 h at 37 °C, centrifuged for 10 min at 8000g, and the supernatants were transferred into new sterile tubes. For inactivation, sera were incubated for 30 min at 55 °C and stored at −20 °C, and 96-well microtiter plates were coated with 50 μl N (10 μg ml^{-1}), M (10 μg ml^{-1}) or 25 μl Sa (10 μg ml^{-1}) + 25 μl Sb (10 μg ml^{-1}), respectively. (Recombinant SARS-Cov N, M, Sa, and Sb proteins containing six-histidine tag were expressed in E. coli. as inclusion bodies). The proteins were purified by sonication and repeated washing, and then dissolved in Tris/HCl buffer with 8 M urea and further purified using Ni–NTA agarose (Invitrogen). Finally, the denatured proteins were refolded by dialysis in Tris/HCl buffer (pH 7.4). The purities of recombinant N, M, Sa, and Sb proteins were confirmed by Western blot experiments. The wells were rinsed with PBS supplemented with 0.05% Tween 20 and then blocked with 10% FBS in PBS supplemented with 0.05% Tween 20 for 1 h at 37 °C. Sera were appropriately diluted 100-fold, then 50 μl of diluted serum was added and plates were incubated for 2 h at 37 °C with 1% fetal bovine serum (FBS) in PBS supplemented with 0.05% Tween 20 and incubated for 2 h at 37 °C. After extensive washings, antibodies were detected by incubation with goat anti-mouse IgG antibodies conjugated with horseradish peroxidase for 2 h. After the substrate was added, the reaction was stopped with 2.0 M H$_2$SO$_4$, and the absorbance was measured at wavelengths of 450 nm with 600 nm for a reference value. The antibody titers were relatively expressed as the value of OD$_{450\ nm}$.

Cellular immune response

Lymphoproliferation assay. After separating spleen cells from the mice, washing three times with PBS, and resuspend at 4×10^5 ml^{-1} in complete growth media (RPMI 1640 containing 10% heat-inactivated fetal bovine serum), 500 μl cell suspension was dispensed into 12-well microtiter plates. 1 μg M, 1 μg N or 0.5 μg Sa + 0.5 μg Sb protein was added. Five micrograms of ConA was used as positive control and that without stimulate was used as negative control. The plates were propagated at 37 °C in 5% CO$_2$ for 70 h and then stained with 10 μl MTT (3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide). Reactions were stopped by 100 μl DMSO 4 h later. Proliferation

responses were calculated as means of triplicate wells. The optical density was measured by a microplate reader at a test wavelength of 550 nm and a reference wavelength of 690 nm.

Cytotoxic T lymphocyte assay. Spleen cells were isolated from the mice, and single-cell suspensions were obtained by teasing the tissue through a wire mesh in RPMI 1640 medium supplemented with 10% FBS, and dispended in the bottom of a 12-well tissue culture plate with 500 μl volume (4×10^6) cells ml^{-1}/well, and restimulated in vitro, and cultured for 6 days, supplemented with 25 IU IL-2 in each well in the last two days. Sa + Sb, N or M-pulsed P815 cells were labeled with Calcein AM (Molecular Probes) following the manufacturer's instructions. Fifty microliter target cells were added to assay the cytotoxicity activity, and the plates were incubated for 4 h in a humidified chamber at 37 °C with 5% CO_2. The percentage of specific cytotoxic activity was calculated as (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100%. Targeted cells incubated in medium alone and in medium supplemented with 5% Triton X-100 were used to determine spontaneous fluorescent release and maximum fluorescent release, respectively.

Results

Humoral immune response

Analysis of the antibody responses after immunization

N, M, and Sa + Sb-specific antibodies of SARS coronavirus in sera of mice were determined with ELISA analysis (Fig. 2). In our experiments, titers of anti-M antibody increased to the maximum 1.428 ± 0.299 in week 6 after immunized with SARS DNA vaccine pcDNAM; titers of anti-N antibody increased to the maximum 0.994 ± 0.207 in week 10 after mice were intramuscularly injected with SARS DNA vaccine pcDNAN; titers of anti-Sa–Sb antibody increased to the maximum 1.143 ± 0.201 week 12, after mice were immunized with pcDNASa–pcDNASb.

Among all humoral immune response experiments, results show that the pcDNAM plasmid DNA can stimulate stronger humoral immune in week 6, however, the humoral immune response of pcDNAM plasmid DNA showed a rapid decrease from average 1.428 (week 6) to 0.629 (week 12). Antibody titers' response of SARS Sa + Sb DNA vaccination is stable during week 8 to

week 12. Average antibody titers were changed between 1.111 and 1.143 from week 8 to week 12.

Among all DNA immunization groups, pcDNAM induced the highest humoral immune response at week 6 in BALB/c mice. However pcDNASa–pcDNASb induced the highest humoral immune response at week 12. Moreover, the antibody titers of anti-N, anti-M, and anti-Sa–Sb, were not detected in control mice in this experiment.

Several other groups showed that SARS-CoV S protein can effectively induce neutralizing antibodies, Zhou et al. [10] analyzed that a protein fragment of the spike protein might be a target for generation of therapeutic neutralizing antibodies against SARS CoV and for vaccine development to elicit protective humoral immunity. Zhang et al. [11] analyzed that SARS-CoV S protein has the potential for inducing neutralizing antibodies against SARS-CoV. Yang et al. [12] showed that the SARS-CoV S glycoprotein can mediate viral entry through pH-dependent endocytosis. Hsu et al. [13] showed that the SARS-CoV spike protein is a primary target for vaccine and drug development. Bisht et al. [14] constructed recombinant forms of the highly attenuated modified vaccinia virus Ankara (MVA) containing the gene encoding full-length SARS-CoV S produced serum antibodies that neutralized SARS-CoV in vitro. He et al. [15] show that a recombinant fusion protein containing fragments of SARS S protein and a human IgG1 Fc fragment can induce highly potent antibody responses in the immunized rabbits. Buchholz et al. [16] investigated the contributions of the S proteins of SARS-CoV to protective immunity by expressing into a recombinant parainfluenza virus type 3 vector (BHPIV3). The immunogenicity and protective efficacy of BHPIV3/SARS expressing S protein induced a high titer of antibodies than other proteins [16]. In our experiments, pcDNASa–pcDNASb can induce the strongest humoral immune response in week 12. These results show that SARS-CoV S protein is one of the important proteins to induce humoral immune response.

Cellular immune response

Lymphoproliferation assay

The results of the lymphocyte proliferation test are shown in Fig. 3. After lymphocytes were separated from mice, lymphocytes were re-stimulated with each respective S, N or M recombinant protein. The lymphoproliferative response was 0.124 ± 0.015 after pcDNAN immunization. The proliferation response was 0.257 ± 0.01 after pcDNAM immunization. The proliferation response was 0.07 ± 0.003 after pcDNASa–pcDNASb immunization.

In our experiments, pcDNAM stimulated the highest lymphoproliferation among all DNA immunization,
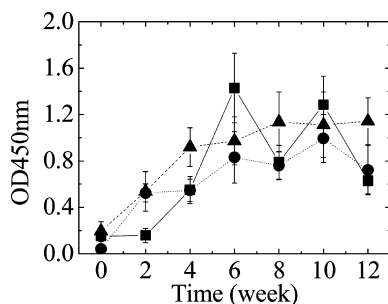
Fig. 2. Analysis of the anti-M (■), anti-N (●), and anti-Sa–Sb (▲) antibody titers after BALB/c mice were intramuscularly injected with pcDNAM, pcDNAN or pcDNASa–pcDNASb plasmids. Serum samples were collected for ELISA at a dilution of 1:100.
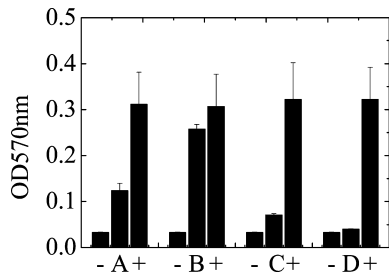
Fig. 3. The lymphoproliferative response of mice after SARS DNA vaccine immunization. (A) Immunization with pcDNAN; (B) immunization with pcDNAM; (C) immunization with pcDNASa–pcD-NASb; and (D) control; (−) without stimulation; (+) stimulation with ConA protein.

which is almost same as the positive control of ConA stimulation. pcDNASa–pcDNASb stimulated the lowest lymphoproliferation, which is almost same as the negative control. Results indicate that pcDNAM could induce stronger specific lymphoproliferative response in week 12 than other DNA constructs.

Cytotoxic T lymphocyte assay

Four weeks after the boast, M, N, and Sa + Sb-specific CTL responses were tested (Fig. 4). In our experiments, the ratio of effector to targeter is 50:1. Comparing with pcDNAN, pcDNAM, and pcDNASa–pcDNASb in the stimulating CTL, results show that pcDNAM developed the highest level specific lysis as $83.95 \pm 3.65\%$; pcDNAN developed specific lysis $60.87 \pm 3.19\%$; and pcDNASa–pcDNASb developed the lowest level specific lysis as $51.25 \pm 3.15\%$; the control mice have the lowest lysis as $11.3 \pm 1.2\%$.

Results show that pcDNAM stimulates the highest CTL response in BALB/c mice in week 12, however, elicits the lowest humoral immune response in 12w. pcDNASa–pcDNASb has the lowest CTL response in BALB/c mice in week 12, however, elicits the highest humoral immune response in week 12.
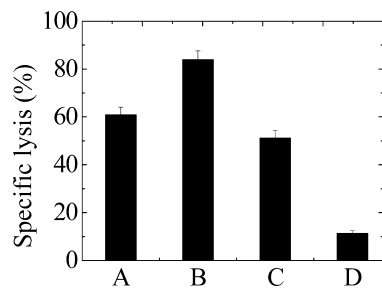

Fig. 4. Cytotoxic T lymphocyte assay. Lymphocytes were isolated from mice and cultured in 1640 10% FBS medium for 6 days in vitro after BALB/c mice were immunized with different SARS DNA vaccines. Effector to targeter cell ratio was 50:1. (A) immunization with pcDNAN; (B) immunization with pcDNAM; and (C) immunization with pcDNASa–pcDNASb; and (D) control.

Discussion

As a serious, infectious disease, SARS has a significant economic impact on China and the world [17]. In order to both prevent and control the transmission of this disease [3,18], several different methods have been recommended. The World Health Organization has recommended that health-care workers in all countries be immunized against influenza [19]. If SARS actually comes back we will be able to distinguish between SARS and influenza. Development of efficient specific antiviral drugs, such as human interferons [20], small interfering RNA [21], mucosal vaccine [22,23] or inactivated vaccine [24–26] is very important to protect the transmission of SARS-CoV.

DNA vaccination project was currently performed in our group. Plasmid DNA, which contain special foreign DNA, has shown great efficiencies and advantages. It has been demonstrated that immunizations of mice with plasmids regulated by the cytomegalovirus promoter induced humoral immune responses and cellular immunity. In our project, we want to compare the immune responses of DNA vaccine encoded different gene fragments of SARS-CoV in BALB/c mice.

Currently, there are several research articles about the experiment of SARS DNA vaccine with different gene fragments, for example S gene DNA vaccine experiment [27,28], N gene DNA vaccine experiments [29,30].

Zhu et al. [29] determine the ability of SARS-CoV N protein to induce antiviral immunity, results showing that the DNA vaccination induced both N protein-specific antibody and -specific CTL activity. SARS-CoV N gene may be a candidate gene for SARS DNA vaccination. Kim et al. [30] investigated that a DNA vaccine encoding calreticulin (CRT) linked to a SARS-CoV nucleocapsid (N) protein is capable of generating strong N-specific humoral and cellular immunity and may be potentially useful for control of infection with SARS-CoV.

Several groups concentrated on DNA vaccine encoding S protein of SARS-Cov. Yang et al. [27] reported that a DNA vaccine encoding the S protein of the SARS-CoV induces T cell and neutralizing antibody responses. Viral replication was reduced by more than six orders of magnitude in the lungs of mice vaccinated with the DNA vaccine. SARS-CoV DNA vaccine elicits effective immune responses in an animal model. Zeng et al. [28] analyzed the immunological characteristics of SARS-CoV S protein by administering mice with plasmids encoding various S gene fragments. Results showed that the DNA vaccines were capable of eliciting SARS-CoV-specific antibodies. These finding provide insights into understanding the immunological characteristics of spike protein in DNA vaccine developments. A combination delivery of SARS-Cov S, M, and N

genes into macaques using adenoviral vector elicited high titer of neutralizing antibodies [31]. These important results show that different gene fragments of SARS DNA vaccine have different immune response characteristics. Although there are several reports about SARS DNA vaccine experiments, there still has not been a report about the DNA vaccine experiment encoding different gene fragments of SARS-CoV.

In this study, a detailed comparison of the immunogenicity of SARS DNA vaccines encoding S, M, and N protein genes was performed. Our experiments may provide the important information for the design of SARS DNA vaccine. Although plasmids expressing N, M, and Sa–Sb proteins can elicit both humoral and cellular immunity in BALB/c mice, pcDNASa–pcDNASb stimulated the highest humoral immunity than other plasmids did in week 12. pcDNAM stimulated stronger cellular immunization in week 12. In summary, our results indicate that DNA immunization with different antigen gene constructs of SARS-Cov could induce different types of immune response. Further study with these DNA constructs with an appropriate adjuvant is essential to develop a DNA vaccine against SARS infection in people.

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