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DNA Vaccine of SARS-Cov S Gene Induces Antibody Response in Mice

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Abstract The spike (S) protein, a main surface antigen of SARS-coronavirus (SARS-CoV), is one of the most important antigen candidates for vaccine design. In the present study, three fragments of the truncated S protein were expressed in *E. coli*, and analyzed with pooled sera of convalescence phase of SARS patients. The full length S gene DNA vaccine was constructed and used to immunize BALB/c mice. The mouse serum IgG antibody against SARS-CoV was measured by ELISA with *E. coli* expressed truncated S protein or SARS-CoV lysate as diagnostic antigen. The results showed that all the three fragments of S protein expressed by *E. coli* was able to react with sera of SARS patients and the S gene DNA candidate vaccine could induce the production of specific IgG antibody against SARS-CoV efficiently in mice with seroconversion ratio of 75% after 3 times of immunization. These findings lay some foundations for further understanding the immunology of SARS-CoV and developing SARS vaccines.

Key words severe acute respiratory syndrome-coronavirus (SARS-CoV); spike protein; DNA vaccine; antibody

The severe acute respiratory syndrome (SARS), also named infectious atypical pneumonia, is a newly described and highly contagious respiratory infection that first occurred in late 2002 in Guangdong Province, China, and spread to more than 30 countries in early 2003. It has been identified that the etiological agent of SARS is a novel coronavirus, named as SARS-CoV [1]. The spike (S) protein of coronavirus is the major envelope component, constituting the spike projecting from the virion surface, which mediates many of the biological properties of the virus, such as attachment to cell receptors, penetration, and spread by virus-induced cell to cell fusion. The S protein also plays an important role in the immune responses against the virus since neutralizing antibody, passive antibody protection, and cellular immunity have been related to the protein. Therefore, the spike protein may act as the most potential antigen for SARS vaccine design [2]. The S gene of SARS-CoV encodes a surface glycoprotein precursor predicted to be 1255 amino acids in length. It belongs to type I membrane protein and has low level of similarity (20%–27% amino acid identity) with other coronaviruses [3]. In the present study, the truncated fragments of SARS-CoV S protein were expressed in *E. coli* and their antigenicity were analyzed, and furthermore, a candidate DNA vaccine containing full-length S gene was constructed and its immunogenicity was evaluated in mice.

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Materials and Methods

Materials

The DNA fragment SI which encodes nucleotide (nt) 1–1650 of SARS-CoV S gene was synthesized by Shanghai BioAsia Bio-Tech Company. Two other DNA fragments, SII encoding nt 1626–2934 and SIII encoding nt 2766–3768 of SARS-CoV S gene, were kindly provided by Prof. Zheng-Hong YUAN (Molecular Virology Lab, Fudan University). The nucleotide sequences of the three DNA fragments are identical to the published data (GenBank accession number AY278554). Expand long template PCR system was from Roche. Prokaryotic expression plasmid

pPROEX HTa was from Life Technologies. Prokaryotic expression plasmid pGEX-4T-1 and *E. coli* strain BL21 (DE3) were the products of Amersham Biosciences. Ni-NTA affinity resin was from Qiagen. Sera of convalescence phase of SARS patients were collected from Beijing Xiaotangshan Hospital, and inactivated by heating at 56 ℃ for 30 min. ELISA kits for IgG or IgM antibodies to SARS-CoV were from Beijing BGI-GBI Biotech Co., Ltd.. Eukaryotic expression plasmid pCIDN, was constructed from the vector pS2S/IL-2, in which the S2S gene of HBV and the murine IL-2 gene was replaced by synthetic rabbit beta-globin intron [4]. Six-week old female BALB/c mice were purchased from Xiper-Bikai Experimental Animal Co., LTD, Shanghai. HRP conjugated goat-anti-human IgG and goat-anti-mouse IgG were from Sigma.

Construction of prokaryotic expression plasmid containing the fragments of S gene

DNA fragment S301, encoding amino acid 14–314 of SARS-CoV S protein, was amplified using the following primers: SAF, 5′-GAA TTC ATG AGT GAC CTT GAC CGG TGC ACC-3′, and SAR, 5′-GAA TTC TTA ACC TCG AGT AAC ATC TCC TGA GGG AAC AAC C-3′. The fragment SI was used as the template to amplify S301, which was then inserted into pMD18-T vector and subcloned into *Eco*RI site of pPROEX HTa. The expression plasmid pHT-S301 was identified by *Eco*RI and *Pst*I digestion, respectively. DNA fragment S338, encoding aa 557–894 of SARS-CoV S protein, was amplified by the following primers: SBF, 5′-GAA TTC ATG GAT TTC ACT GAT TCC GTT CGA GAT CC-3′, and SBR, 5′-GCG GCC GCT TAG GTA ACT CCA ATG CCA TTG AAC C-3′. Fragment SII was used as the template for amplifying S338. The amplified fragment was inserted into pMD18-T vector for the construction of the expression plasmid pGEX-S338, obtained by subcloning the fragment S338 into pGEX-4T-1 with *Eco*RI/*Not*I digestion. DNA fragment S324, encoding a.a. 867–1190 of SARS-CoV S protein, was amplified by the following primers: SCF, 5′-GAA TTC ATG GGA TGG ACA TTT GGT GCT GGC GCT G-3′, and SCR, 5′-GCG GCC GCT TAT TGC TCA TAT TTT CCC AAT TCT TG-3′. Fragments SII and SIII were used as the templates for amplifying S324 by SOE-PCR methods [5]. The product was cloned into pMD18-T vector and further inserted into the *Eco*RI/*Not*I sites of pGEX-4T-1for the expression plasmid pGEX-S324.

Expression of truncated SARS-CoV S proteins in *E. coli* and identification of their antigenicity

E. coli BL21 (DE3) was transformed with plasmid pHT-S301, pGEX-S338 or pGEX-S324, respectively. The positive colonies were chosen and cultured overnight in LB medium containing 100 mg/L ampicillin. The cultures were diluted 100 times with 2×YT medium and incubated at 37 ℃ until A_{600} reached 0.6. Then IPTG was added to a final concentration of 0.6 mmol/L for the induction of expression. The bacteria were collected after 3 hours and lysed in 2×SDS loading buffer for SDS-PAGE analysis. The antigenicity of these expressed proteins was determined by Western blot, using 200 fold diluted pooled sera of convalescence phase of two SARS patients as the primary antibody, and 100 fold diluted HRP conjugated goat-anti-human IgG as the second antibody.

Construction of SARS-CoV S gene DNA vaccine

A DNA fragment encoding nt 1–2058 of S gene was amplified with primers: NF, 5′-GAA TTC GCT AGC CAC CAT GTT TAT TTT CTT ATT ATT TC-3′, and NR, 5′-GGA TCC TCT AGA TTA TGA ACT ATC AGC ACC TAA AGA C-3′ from the fragment SI and SII by SOE-PCR. The amplified fragment was inserted into pMD18-T vector, and then subcloned into *Eco*RI/*Bam*HI sites of the eukaryotic expression plasmid pCIDN. The resulting plasmid was named pCIDN-SA. Another DNA fragment, covering nt 1913–3768 of S gene was amplified with primers: CF, 5′-GAG CTG AGC ATG TCG AC-3′, and CR, 5′-GGA TCC TCT AGA TTA TGT GTA ATG TAA TTT GAC ACC-3′ using the template SII and SIII. After cloning it into pMD18-T vector and sequenced, the amplified DNA fragment was fused with pCIDN-SA at *Sal*I/*Bam*HI site. The eukaryotic expression plasmid pCIDN-FS, containing the full-length S gene of SARS CoV was obtained.

Immunization of mice [6]

Plasmid pCIDN-FS and pCIDN were prepared with Mega plasmid preparation kit (Qiagen) and dissolved in 0.01 mol/L PBS to a final concentration 2 g/L. Sixteen BALB/c mice were randomly divided into two groups (eight mice in each group). The mice in the experimental group were injected with 200 μg of pCIDN-FS in both tibialis anterior muscles. Mice were boosted twice with the same dosage at 2 week intervals. The mice in control group received the same amount of pCIDN vector with identical route and frequency.

Detection of anti-SARS-CoV IgG in mice

The recombinant S301 protein was purified by Ni-NTA affinity resin according to the manufacture's directions. Microtiter plates were coated with the purified S301 protein (50 mg/L in carbonate buffer) to detect the antibody against SARS-CoV in mouse sera. In addition, the microtiter plates coated with SARS-CoV lysate was also used for the detection. Mouse sera were collected at 0, 2, 4, 6 and 8 week after the first immunization and diluted

with PBS (pH 7.4) containing 12% goat sera and 0.5% Tween-20. The detection antibody was HRP conjugated goat-anti-mouse IgG (1:10,000 dilution). TMB/H_2O_2 was used as the substrate. The absorbance at 450/630 nm was measured on an ELISA reader (Bio-Rad). It was considered positive when the A_{450}/A_{630} of the mice in the experimental group is larger than or equal to 2.1-time of the average value in the control group.

Statistical analysis

All data were analyzed with Student's *t*-test for the significance of the difference.

Results

Expression of truncated S proteins in *E. coli*

The plasmid pHT-S301 encodes a fusion protein about 37 kD with 6 histidines at its N-terminus. The plasmids pGEX-S338 and pGEX-S324 encode two fragments of truncated S protein fused with GST, and the molecular weights are 64 kD and 62 kD, respectively. In SDS-PAGE, recombinant BL21(DE3) expressed proteins could be visualized and the molecular weights were the same as predicted (Fig. 1).

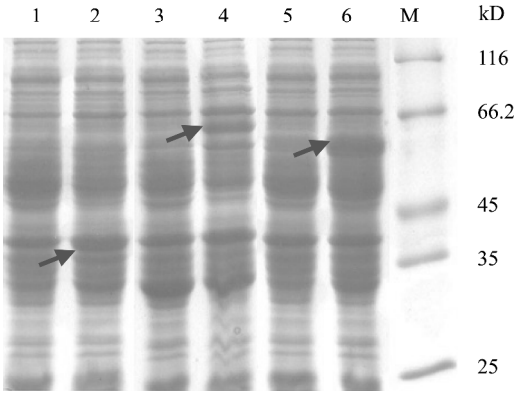

Fig. 1 SDS-PAGE analysis of expressed S protein from recombinant *E. coli* BL21(DE3)

1, BL21(DE3)/pHT-S301 without IPTG induction; 2, BL21(DE3)/pHT-S301 with IPTG induction; 3, BL21(DE3)/pGEX-S338 without IPTG induction; 4, BL21(DE3)/ pGEX-S338 with IPTG induction; 5, BL21(DE3)/pGEX-S324 without IPTG induction; 6, BL21(DE3)/pGEX-S324 with IPTG induction; M, molecular weight markers. Arrows indicate the bands of target proteins.

Antigenicity analysis of S protein fragments

Antigenicity of the S protein fragments expressed in *E. coli* was determined by anti-SARS-CoV IgG positive sera of SARS patients in Western blot. As shown in Fig. 2, all of the three truncated S proteins could react with the pooled sera of SARS patients, and the reactivity of S310 and S338 protein is stronger than that of S324.

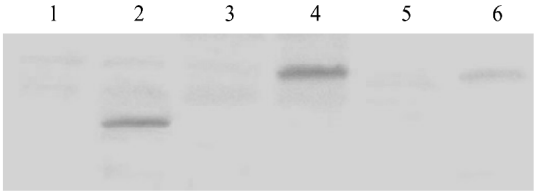

Fig. 2 Western blot analysis of expressed S protein from recombinant *E. coli* BL21(DE3)

1, BL21(DE3)/pHT-S301 without IPTG induction; 2, BL21(DE3)/pHT-S301 with IPTG induction; 3, BL21(DE3)/pGEX-S338 without IPTG induction; 4, BL21(DE3)/pGEX-S338 with IPTG induction; 5, BL21(DE3)/pGEX-S324 without IPTG induction; 6, BL21(DE3)/pGEX-S324 with IPTG induction.

Structure and sequence analysis of DNA vaccine containing full-length S gene

The structure of DNA vaccine pCIDN-FS is shown in Fig. 3, in which "P_{CMV}" is early promoter/enhancer sequence of CMV, "intron" is rabbit beta-globin intron, "S" represents the full-length S gene of SARS-CoV and "BGH pA" is transcription terminal signal of bovine growth hormone gene, "DHFR" is dihydrofolate reductase gene and "NEO" is neomycin phosphotransferase gene. Three nucleotide mutations were found in the S gene of pCIDN-FS compared with the parental sequence (GenBank accession number AY278554). The first one occurred at nt 822 (T → C), which is a nonsense mutation. The second one at nt 1189 (A → G) resulting in amino acid Ile → Val mutation, and the last one at nt 2032 (A → T) leading to amino acid Thr → Ser mutation. There is a restriction endonuclease *Pst*I site at nt 750 and a S*al*I site at nt 1924 in the S gene of SARS-CoV.

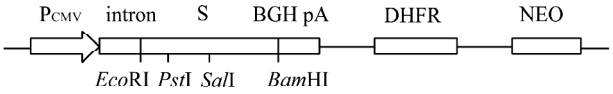

Fig. 3 The structure of the DNA vaccine pCIDN-FS

Antibody responses induced by full length S gene DNA vaccine

The S301 protein was used as the diagnostic antigen in ELISA for the detection of IgG antibodies against SARS-CoV in mouse sera. Positive ratio was found to be 0%, 0%, 25%, 50% and 50% at various time courses of 0, 2, 4, 6 and 8 weeks after the first immunization with pCIDN-FS. The positive ratio increased to 0%, 12.5%, 50%,

62.5% and 75% when serum samples were tested using the virion lysate of SARS-CoV as the diagnostic antigen. All sera positive in S301 protein based ELISA were also positive in virion lysate based ELISA. Significant difference was observed by comparing the values of A_{450}/A_{630} of ELISA detection between the pCIDN-FS immunized group and pCIDN group. The difference developed at week 4 after the first immunization, and increased gradually at the 6th and 8th weeks (Table 1, Table 2).

Discussion

The S protein is important for the infectivity and pathogenicity of coronavirus. Mutations in S gene have previously been correlated with altered pathogenesis, virulence and tropism in other coronaviruses [7]. The S proteins of some coronaviruses are cleaved by a host cell trypsin-like protease into S1 involved in receptor binding and S2 involved in cell fusion. The S proteins are the major targets of the neutralizing antibodies in some animal coronaviruses and can also induce cytotoxic T lymphocytes. For example, the S proteins of mouse hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV) could elicit neutralizing antibodies which protect animals from virulent virus challenge. In addition, the immune protection of S1 subunit at N-terminus of S protein is more potent than that of S2 subunit at C-terminus [8,9]. However, it is not the case in feline infectious peritonitis (FIPV), in which the antibodies against the S protein of FIPV are not protective. It has been found that the antibodies against FIPV S protein enhance the disease progression, this is the same as the antibody-dependent enhancement (ADE) of infection effects of Dengue Virus, in which virus-antibody immune complexes bind to monocytes or macrophages via Fc receptors for immunoglobulin G or complement receptors on the cell surface [10]. The target cells of SARS-CoV are not monocytes or macrophages and convalescence sera of SARS patients were effective in clinical treatment of SARS-CoV infection, therefore, the ADE effect may not occur in SARS-CoV.

In the present study, fragments of S protein of SARS-CoV were expressed in *E. coli* and the antigenicity of these recombinant proteins were determined with the convalescence sera of SARS patients. The antigenicity of S301 and S338 were found to be stronger than that of S324, which is consistent with other reports that major antigenic domains were located at the N-terminus of S protein and the N-terminus of S2 subunits in some coronaviruses [11,12]. At present, the above result may not reliably represent the distribution of epitopes in SARS-CoV S protein, because only two serum samples were used, and the recombinant proteins were non-glycosylated and denatured.

DNA vaccine pCIDN-FS containing full-length S gene was used to immunize BALB/c mice and significant difference of antibody response was developed between the DNA vaccine group and the mock plasmid control group. The difference was further increased after boosts, indicating that the DNA vaccine can induce specific humoral immune responses against S protein of SARS-CoV. In the DNA vaccine group, one mouse generated specific antibodies right after the first immunization. Using the virion lysate as diagnostic antigen for detecting antibodies against SARS-CoV S protein, 62.5% of the DNA vaccine-immunized mice was positive at the 2nd week after the last immunization and 75% was positive at the 4th week after the last immunization. The reactivity of SARS-CoV virion lysate is more sensitive than that of recombinant S301

Table 1 Detection of anti-SARS-CoV IgG in sera of DNA immunized mice using recombinant S301 antigen coated microplate (1∶50 diluted sera)

Time (week)	0	2	4	6	8
pCIDN	0.09±0.04	0.12±0.03	0.13±0.03	0.15±0.04	0.16±0.03
pCIDN-FS	0.07±0.04	0.13±0.05	0.2±0.07	0.34±0.13	0.42±0.17
P	> 0.05	> 0.05	< 0.05	< 0.01	< 0.01

Table 2 Detection of anti-SARS-CoV IgG in sera of DNA immunized mice using SARS-CoV viron lysate coated microplate (1∶50 diluted sera)

Time (week)	0	2	4	6	8
pCIDN	0.14±0.05	0.16±0.05	0.16±0.04	0.18±0.04	0.17±0.03
pCIDN-FS	0.12±0.04	0.19±0.07	0.37±0.16	0.43±0.18	0.49±0.19
P	> 0.05	> 0.05	< 0.05	< 0.01	< 0.001

protein in the detection of specific antibodies, probably because S301 protein is non-glycosylated nature and covers only partial epitopes of the complete S protein. In addition, the quantity and structure of the coated antigen may also influence the sensitivity of ELISA. The detective specificity of the recombinant S301 protein was the same as virion lysate, indicating that the antibodies induced by the DNA vaccine are specific for S protein. The S gene of SARS-CoV encodes type I membrane glycoprotein, the elimination of the transmembrane domain at its carboxyl terminus may make the DNA vaccine expressing secretive protein and inducing stronger immune response.

In most cases, DNA vaccines were less effective in larger species than in small animals, so it is not used very much on human. Antigens are produced in host cells in DNA immunization, and the mechanisms are basically similar to recombinant or inactivated vaccines, in addition to the immune stimulation of vector DNA itself [13]. So, our results also uphold that recombinant S protein may be a good candidate antigen for SARS vaccine design. The DNA vaccine pCIDN-FS contains dihydrofolate reductase (DHFR) gene and neomycin phosphotransferase (Neo) gene. These two markers can be used to select the transfected cells and amplify the target gene, so this DNA construct can be used to express S protein of SARS-CoV in CHO/*dhfr*$^-$ cell expression system to prepare recombinant SARS vaccine.

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