Elicitation of Immunity in Mice After Immunization with the S2 Subunit of the Severe Acute Respiratory Syndrome Coronavirus

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ABSTRACT

The S2 domain of the severe acute respiratory syndrome coronavirus (SARS-CoV) spike (S) protein is responsible for fusion between virus and target cell membranes, and is expected to be immungenic. In this study, we investigated the immune responses against the S2 subunit in BALB/c mice, which were vaccinated either with plasmid DNA encoding the S2 domain (residues 681–1120), the recombinant S2 fragment (residues 681–980) in incomplete Freund's adjuvant, or with inactivated SARS-CoV. The increased number of specific cytotoxic cells (CTLs) and the high titer of specific antibody showed stimulation of both arms of the immune system in these groups. The shift in cytokines suggested that Th1–polarized immune response was induced by plasmid pCoVS2, meanwhile the Th2-dominant response was induced by recombinant S2 fragment and inactivated vaccine. However, the titer of neutralizing antibodies was only detectable in mice immunized with inactivated virus, but not with pCoVS2 plasmid. Taken together, the S2 domain could induce specific cellular immune response and a high level of total IgG but little neutralizing antibodies against infection by SARS-CoV.

INTRODUCTION

SARS-CoV, A HIGHLY AGGRESSIVE PATHOGEN became evident in the Guangdong province of southern China in late 2002, and is the causative agent of an atypical, highly contagious respiratory disease that has occured in 32 countries in 2003, and has resulted in approximately 8400 cases and 900 deaths. The virus belongs to a diverse group of large (160–170 kDa), enveloped, positive-stranded RNA viruses that cause respiratory and enteric diseases in humans and other animals (Rota *et al*., 2003). Prior experience in infectious disease control suggests that vaccination will be one of the most effective measures to prevent future outbreaks of SARS (Batson, 1998; Chen *et al*., 1998, 2001; Rappuoli *et al*., 2002). An understanding of the immunology of coronavirus infections in mice and swine indicated that, of the large number of potential vaccine antigens encoded by the SARS-CoV genome, the partial or full S glyco- protein could potentially serve as the best antigen/immunogen (Fleming *et al*., 1983; Ontiveros *et al*., 2003). The spike (S) glycoprotein of the coronavirus is a large and multifunctional protein that plays crucial roles in mediating viral infection through receptor binding and specific membrane fusion. The glycoprotein has two functional regions. S1 is the receptor binding site and defines the host range of the virus, and S2 is a transmembrane subunit that mediates fusion between the viral and cellular membrane and has hydrophobic repeat (HR) domains that can form coiled-coil structure, which is thought to define the oligomeric structure of the spike protein in its native state and its fusogenic ability (Luo *et al*., 1999). The immunological and structural information on the S2 domain presented in several studies provide a possible method for the development of vaccines in which the S2 domain was used as antigen (Spiga *et al*., 2003; Hsu *et al*., 2004; Pang *et al*., 2004). In the present study, a plasmid DNA vaccine construct expressing S2 domain

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(residues 681–1120), recombinant S2 fragment (residues 681–980), and inactivated vaccine were prepared and inoculated into mice to characterize the cellular and humoral immune response to the S2 subunit.

MATERIALS AND METHODS

Virus, cell, and sera

The SARS-CoV BJ01 strain was isolated from a SARS patient from Beijing, China, by the Institute of Microbiology and Epidemiology, Academy of Military Medicine (Beijing, China).The vaccine-producing cell line, the Vero cell, was provided by the Chinese National Vaccine and Serum Institute (Beijing, China). This cell line met the relevant WHO requirements and standards for Biologics, People's Republic of China, 2000. Sera from the convalescence phase of SARS-infected patients was collected from Beijing Xiaotangshan Hospital, and inactivated by heating at 56° for 30 min.

Plasmid construction

General DNA manipulation was performed as described by Sambrook et al. (1989). The DNA encoding the S2 domain (residues 681–1200) of SARS-CoV was synthesized (according to the published sequence, GenBank accession number: AY278488) using modified codons and Kozak sequence (gc-caccatgg), flanking sequence of the ATG start codon, to optimize expression. For construction of the S2 domain expression vector, the S2 coding region with a tissue plasminogen activator (TPA) signal peptide sequence upstream of the initiation codon of S2 domain was cloned into the eukaryotic expression vector pcDNA3 generating the expression plasmid pCoVS2.

The prokaryotic expression construct, pET-S2, was constructed by cloning the PCR amplified S2 fragment (residues 681–980) into the NdeI /XhoI site of the pET-28a vector. The recombinant S2 fragment was expressed in a frame with a 6-histidine tag.

Expression of S2 fragment in Escherichia coli

Recombinant protein was induced with 0.1 mM IPTG (iso-propyl-beta-D-thiogalactopyranoside) in the E. coli BL21(DE3) strain. The S2 fragment, fused with a histidine tag, was expressed as an inclusion body and was then purified from the cell sonicate by affinity chromatography with Ni-NTA affinity resin (Qiagen Inc., Chatsworth, CA) after the process of denaturing and refolding. The purified S2 protein was then quantified by the Coomassie Brilliant Blue method. The recombinant S2 fragment was separated by SDS–PAGE on a 15% gel, and the separated proteins were either stained with 0.05% Coommassie Brilliant Blue or electrophoretically transferred onto nitrocellulose membrane. The membrane was blocked with 5% milk powder in phosphate-buffered saline (PBS) and subsequently probed with pooled sera from SARS patients for 1 h at 37°. Bound antibodies were detected by incubation for 1 h at 37° with goat antihuman horseradish peroxidase (HRP)-conjugated secondary antibody (Jingmei Biotech, Shenzhen, China) diluted 1:1000. Detection of secondary antibody binding was made with DAB substrate.

Transfection

The day before transfection, COS7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) plus 10% newborn calf serum in a six-well tissue plate until the cells reached approximately 60–80% confluence. Plasmid DNA transfection was performed with liposomes (Roche Molecular Biochemicals, Indianapolis, IN) as specified by the manufacturer. The transfection products were analyzed by ELISA, which was processed with pooled sera from patients infected with SARS-CoV.

Vaccine preparation

Plasmids used in this study were prepared by the alkaline lysis method followed by Triton X-114 treatment to remove endotoxin (Cotton et al., 1994). The plasmid DNA concentration was adjusted to 1 mg/ml with PBS. For protein vaccination, 50 μg of recombinant protein was formulated with 50 μl of Incomplete Freund's Adjuvant (IFA, Sigma-Aldrich, St. Louis, MO) in a total volume of 0.1 ml per dose. Inactivated vaccine was prepared as previously described (Tang et al., 2004). Briefly, Vero cells were infected with the SARS-CoV and incubated in a serum-free medium at 37°C for 36 h. When the infective titer reached 10^7 TCID$_{50}$, the cells were lysed by freezing and thawing at -20°C, followed by centrifugation at 6000 rpm for 20 min. The supernatant was harvested, added to 1:2000 diluted β-propiolactone (Sigma-Aldrich Inc.), and then shaken and incubated at 4°C for 24 h. After inactivation, the sample was kept at 37°C for 2 h to hydrolyze b-propiolactone. The inactivated virus suspension was centrifuged at 4°C, 6000 rpm for 30 min. The supernatant was harvested and concentrated with PEG20000 (Sigma-Aldrich Inc.) to the 1/20 of original volume, then further concentrated by centrifugation (30,000 $\times$ g, 20 min) using Cetriplus YM-100 (Millipore Corp., Bedford, MA), and then purified by Sepharose 4FF column chromatography (Amersham Biosciences Corp., Arlington Heights, IL). The harvested viruses were pooled and further concentrated by centrifugation using Centriplus-20 (Millipore Corp.). A stabilizer (1% human albumin) and an antiseptic (0.01% thimerosal) were added to the purified virus. The dosage of antigen was finally adjusted with PBS.

Immunization of mice

Sixty 8-week-old female BALB/c mice were randomly allocated into four groups, which were injected with the recombinant S2 fragment, inactivated virus, pCoVS2 plasmid and PBS, respectively. For DNA vaccination, mice were injected with pCoVS2 into both quadriceps with 2 $\times$ 50 μg DNA three times at 2-week intervals. For protein vaccination, mice were injected subcutaneously (s.c.) in the back with a 0.1-ml recombinant S2 fragment three times at 2-week intervals. Each mouse of the third group was inoculated with 1-μg inactivated vaccine via the intraperitoneal (i.p.) route twice at 3-week intervals. A group of mice inoculated with PBS served as the negative control.

Enzyme-linked immunosorbent assay

Sera for detection of IgG by ELISA was collected from the eye socket vein of mice weekly. Ninety-six-well plates (Sino-American Biotechnology Company) were coated with a 1-μg

recombinant S2 fragment or purified SARS-CoV per well diluted in carbonate buffer (100 μl per well, pH 9.6) overnight at 4°C. Plates were washed three times for 5 min with 200 μl per well of PBS containing 0.05% Tween 20 and blocked with 100 μl PBS containing 1% BSA for 2 h at 37°C. Serum samples, twofold diluted in PBS plus 1% BSA, were added and incubated for 1 h at 37°C, followed by the addition of antimouse IgG coupled to HRP (Jingmei Biotech), which was developed with 3,3-diaminobenzadine (DAB) as the chromogen substrate. Plates were then washed three times after each reaction step, and optical density readings at 450 nm were measured in an ELISA plate reader.

Evaluation of cytokine production *in vitro*

Single splenocyte suspensions from immunized mice were diluted in 10% bovine calf serum-supplemented RPMI 1640 to 5×10^6 cells/ml. One hundred microliter cell suspensions were placed in each well of a 96-well plate (Costar Inc., Cambridge, MA) and stimulated with a recombinant S2 fragment (10 μg/ml). After a 72-h incubation, cell-free supernatants were harvested and were screened for the presence of IFN-γ and IL-4 with an ELISA detection system (Jingmei, Biotech) according to the manufacturer's instructions.

Enzyme-linked immunospot assay

The enzyme-linked immunospot assay (ELISPOT) (Biosource Inc., Carmarillo, CA) was used to detect S2-specific T cells after stimulation with the S2 fragment. A 96-well ELISPOT plate was coated with 5 μg/ml of anti-interferon-gamma (IFN-γ) antibody at 4°C. The next day, the plate was washed with PBS/0.5% Tween-20 (Sigma Inc., St. Louis, MO) 10 times and blocked with 200 μl of postcoating solution (1% BSA in PBS) at 37°C for 1 h. One hundred microliters of a single splenocyte preparation (prestimulated with 10 μg/ml of recombinant S2 fragment for 40 h) was added at 10^6 cells/well for 5 h at 37°C in a humidified atmosphere containing 5% CO_2. Then, the wells were decanted and 200 μl of ice-cold deionized water was applied for 10 min on ice. After washing the wells 10 times with PBST, the plate was incubated for 1 h at room temperature with 100 ml of biotinylated anti-IFN-γ antibody (2.5 μg/ml) (Biosource Inc.). Fifty microlitres of ϕ-labeled antibiotin antibody solution (1.25 μg/ml) (Biosource Inc.) was added to the wells after washing the wells 10 times with PBST, and the plate was incubated for 1 h at 37°C. After washing with PBST 10 times, the plate was inverted and tap dried on an absorbent paper. Finally, the plate was incubated at room temperature in the dark for 30 min after the addition of activator solution (Biosource Inc.). When clear spots developed, the reaction was terminated with distilled water and the individual spots were counted. The spots represented T cells activated by the S2 fragment.

Neutralization of SARS-CoV infection

Neutralization of SARS-CoV infection was assessed as previously described (Tang *et al*., 2004). Briefly, the sera of mice were collected 1 week after the second immunization and doubly diluted with PBS. Two hundred microliters of the serial diluted antisera or control sera was mixed with an equal volume of 10 TCID50 SARS-CoV and preincubated at 20°C for 1 h.

Vero cells were plated in 24-well plate (DMEM containing 10% newborn calf serum, 37°C, CO_2 incubator). When the density of Vero cells reached more than 90%, the cells were washed with PBS. Four hundred microliters of the mixture (serum and virus) was added into each well, and kept at 37°C for 1 h. Then the mixture was replaced with culture medium (DMEM containing 10% newborn calf serum). Cytopathic effect (CPE) was observed 72 h later and the neutralization titer was calculated using the Reed and Muench method.

Statistical analysis

The statistical significance of differential findings between experimental groups was determined by the Student's *t*-test. Data were considered statistically significant at $P < 0.05$.

RESULTS

Expression of the S2 protein in eukaryotic cells

Prior to the use of pCoVS2 *in vivo*, we expressed the S2 protein in mammalian cells. COS7 cells were transfected with a pCoVS2 plasmid, and the expression of S2 was determined by ELISA using SARS-CoV infected human sera. The expressed S2 protein was positive at 1:2, 1:4, 1:8, and 1:16 dilutions compared to untransfected cell.

Expression of S2 protein in E. coli

Expression of the DNA encoding the S2 fragment (residues 681–980) from the pET-S2 vector in *E. coli* BL21(DE3) resulted in a fusion protein with a molecular mass of about 35 kDa after IPTG induction (Fig. 1). The recombinant S2 frag-
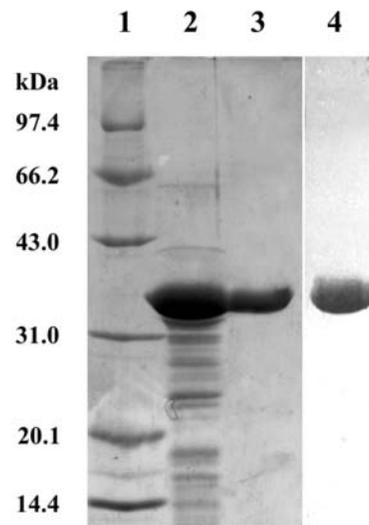
FIG. 1. SDS–PAGE and immunoblot of recombinant S2 protein. *E. coli* BL 21(DE3) was transformed with the recombinant plasmid pET-S2 and cultured with 0.1 mmol/l IPTG for induction of protein expression. Lane 1, molecular weight markers. Lane 2, inclusion body isolated from the bacteria lysates. Lane 3, the purified S2 fragment using Ni-NTA affinity resin. Lane 4, Western blot analysis of the S2 fusion protein with pooled sera from SARS patients.
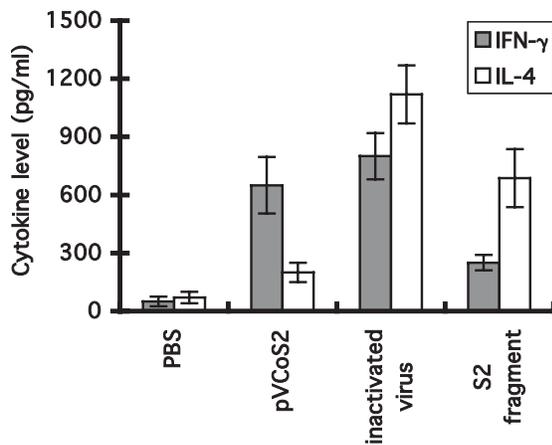
FIG. 2. Production of IFN-γ and IL-4 from splenocytes from different groups. Splenocytes obtained from the mice 6 weeks after the first immunization were stimulated with 10 μg/ml of recombinant S2 protein for 72 h. Samples were assayed in triplicate. Values and bars represent the mean of released cytokine concentrations and SD ($n = 8$).

ment could bind pooled sera from SARS patients. The result demonstrated that the recombinant protein expressed in prokaryotic system could be specifically recognized by sera from humans infected with SARS-CoV.

Antibody responses induced by vaccines

All immunized mice developed potent IgG antibody responses (titers $\geq$ 1:2300) against SARS-CoV as detected by ELISA using the purified virus as a coating antigen while the sera from the control mice did not react with the coated virus. To determine antibody responses specific for the S2 protein in immunized mice, a recombinant S2 fragment was used as an antigen in ELISA. The data also indicated that all the vaccinated mice developed high titers of S2–specific IgG antibodies (titers $\geq$ 1:1600).

Cytokine production by splenocytes from vaccinated mice

We examined the cytokine production profiles since Th1 cytokines (IL-2, IFN-γ) and Th2 cytokines (IL-4, IL-5, and IL-10) are major parameters for our understanding of the polarization of immune responses. As demonstrated in Figure 2, compared with the control group, all vaccinated mice showed significantly increased IFN-γ and IL-4 levels ($P < 0.01$). A significant trend towards IFN-γ response was observed in the group of mice immunized with pCoVS2, while the dominant cytokine observed in recombinant protein and inactivated virus groups were IL-4. The data indicates that the immune response induced by the DNA vaccine was Th1 biased, whereas a Th2-biased response was induced by protein and inactivated virus.

Number of specific IFN-γ releasing T cells

The induction of CTLs by vaccination was tested by an ELISPOT assay in our study. The number of S2-specific CTLs was established in splenocytes of vaccinated mice by stimulation with the recombinant S2 fragment (10 μg/ml for 40 h). As depicted in Figure 3, specific CTL responses induced by vaccines were statistically different from those in mice vaccinated with PBS ($P < 0.01$). The number of S2 fragment specific CTLs in vaccinated groups were all significantly higher ($P < 0.01$) than in the control group.

Antiviral activity of immune sera

The virus-neutralizing activity assay was performed to analyze the ability of antisera to neutralize SARS-CoV infection *in vitro*. SARS-CoV was incubated with serial dilutions of antisera. The result indicated that mice immunized with inactivated virus exhibited a potent neutralizing antibody (mean endpoint titer of antisera was 1820), whereas little virus-neutralizing activity was detected in the sera of mice immunized with the pCoVS2 plasmid.

DISCUSSION

The spike glycoprotein, a membrane component of SARS-CoV, is anticipated to be an important component for candidate vaccines against the SARS virus. Several laboratories are currently testing naked DNA or viral vector systems as a means of delivering the S gene for vaccination (Bisht *et al.*, 2004; Bukreyev *et al.*, 2004; Yang *et al.*, 2004).

However, as He *et al.* (2004a) reported, the viral functional domain, i.e., the receptor-binding domain (RBD), could induce
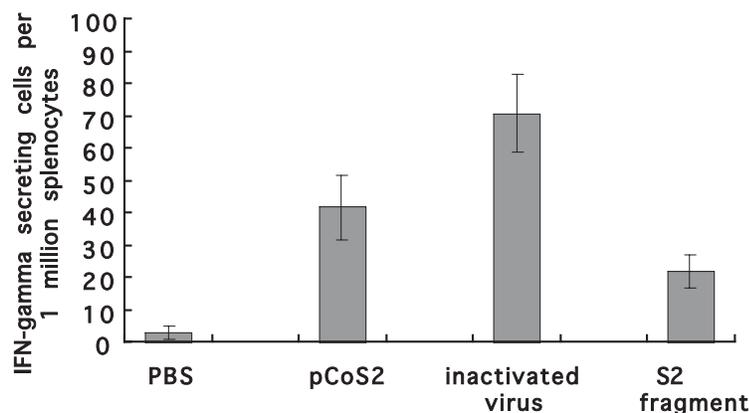
FIG. 3. Induction of CTLs by different vaccines. Six weeks after the first immunization, the splenocytes from each group were stimulated with recombinant S2 fragment (10 μg/ml) for 40 h. CD8 specificity of IFN-γ release was detected by enzyme-linked immunospot assay. The number of specific IFN-γ secreting T cells was determined by counting the individual spots, which represented T cells activated by the S2 fragment. The experiments were performed in triplicate. Values and bars represent the mean and SD ($n = 8$).

more potent neutralizing antibodies than full-length S protein. A possible reason why full-length S protein induced relatively lower titres of neutralizing antibodies than the functional domain is that the former contains nonneutralizing epitopes that may elicit nonneutralizing or even enhancing antibodies, which will facilitate subsequent natural infection (Jiang *et al*., 1991; Takada *et al*., 2001; Takada and Kawaoka, 2003; Geisbert *et al*., 2002; Hebben *et al*., 2004). It has been demonstrated that the S1 domain at the N-terminus of the S protein is responsible for virus binding to the receptor on the target cells and immunodominant sites located in it can induce protective humoral responses to virus infection (Nakanaga *et al*., 1986; Wesseling *et al*., 1993; Pang *et al*., 2004). However, whether the S2 domain can induce neutralizing antibodies remains unclear. On the other hand, as we have known, antigenic drift can be a problem for vaccination use against some virus strains with distinct genotypes and phenotypes. The analysis of the mutation distribution among SARS-CoV proteins could be important to predict antigenic drift. This has been done by aligning the 15 S protein sequences present in the NCBI database for this virus, and 11 mutation sites were identified (from the NCBI). Nine of these mutations are in the S1 subunit and only two mutations are in the S2 subunit (Spiga *et al*., 2003). This means that the vaccines expressing the S2 subunit may avoid the possible problem of antigenic drift.

To investigate the immunization effects and neutralizing antibody-inducing ability of the S2 domain, we prepared a DNA vaccine encoding the S2 domain, recombinant S2 fragment, and inactivated vaccine to inoculate BALB/c mice. The result of cytokine-production profiles demonstrated that IFN-γ responses was dominant in the group of mice immunized with pCoVS2, while the cytokines observed in recombinant protein and inactivated virus groups were IL-4 biased; the ELISPOT assay showed that specific CTL response were induced in all vaccinated mice. Although the pCoVS2 plasmid induced high levels of S2-specific IgG, little virus-neutralizing antibodies were detected. The negative result may be explained by a shielding effect caused by the S1 domain, which is the peripheral fragment of the viral envelope glycoprotein and preferentially exposed to the immune system. Most functional sites of the S2 domain, especially the membrane fusion sites, may be buried in the S1 peripheral region in the native state. Therefore, it may not be accessible to immune effector cells (He *et al*., 2004b).

Studies of the immune response to coronaviruses suggest that both cell-mediated and humoral immunity contribute to long-term protection (Sestak *et al*., 1999). Moreover, the HLA-A2-restricted T-cell epitopes in the S2 domain of SARS-CoV could elicit T-cell immune response in donors who had fully recovered from SARS-CoV infection (Wang *et al*., 2004). This means that although the S2 subunit makes few contributions to inducing virus-neutralizing antibodies, we should not absolutely give up using it as a full or partial candidate antigen before obtaining protection results.

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