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Biochemical and Biophysical Research Communications 315 (2004) 1134–1139
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Characterization of humoral responses in mice immunized with plasmid DNAs encoding SARS-CoV spike gene fragments

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Received 18 January 2004

Abstract

The immunological characteristics of SARS-CoV spike protein were investigated by administering mice with plasmids encoding various S gene fragments. We showed that the secreting forms of S1, S2 subunits and the N-terminus of S1 subunit (residues 18–495) were capable of eliciting SARS-CoV specific antibodies and the region immediate to N-terminus of matured S1 protein contained an important immunogenic determinant for elicitation of SARS-CoV specific antibodies. In addition, mice immunized with plasmids encoding S1 fragment developed a Th1-mediated antibody isotype switching. Another interesting finding was that mouse antibodies elicited separately by plasmids encoding S1 and S2 subunits cooperatively neutralized SARS-CoV but neither the S1 nor S2 specific antibodies did, suggesting the possible role of both S1 and S2 subunits in host cell docking and entry. These results provide insights into understanding the immunological characteristics of spike protein and the development of subunit vaccines against SARS-CoV.

Keywords: SARS; Coronavirus; Spike glycoprotein; DNA vaccine; Neutralizing antibody

Severe acute respiratory syndrome (SARS) is an infectious viral disease caused by a newly emerged coronavirus (SARS-CoV) from China Guangdong with unknown origin [1], with typical corona-like spikes on the surface of the virion [2], formed by oligomers of the largest viral glycoprotein, namely the spike protein (S protein) [3]. The spike protein could be structurally cleaved into N-terminal S1 and C-terminal S2 subunits after translation or functionally differentiated into the corresponding domains without cleavage [4]. The S1 subunit is the knob region of the spike, involved in viral attachment with the cellular receptors and hence determines the host cell tropism [5–7]. Previous studies in vaccine development against animal coronaviruses suggested that the S1 subunit contains neutralizing epitopes that conferred protection to animals upon viral challenge [8–13]. The S2 subunit is the stem region of the spike and its coiled-coil and transmembrane regions are involved in host cell entry and cell-to-cell fusion [14].

The S protein of SARS-CoV is a ~180-kDa glycoprotein [15] and the S2 subunit was defined by the high-score alignment with the amino acid sequences of other coronavirus S proteins (90.7% aligned to pfam01601, conserved domain of coronavirus S2 glycoprotein). The SARS-CoV S2 subunit shares several structural motifs with the well-studied gp41 protein of HIV-1, such as the N-terminal leucine/isoleucine heptad repeat (HR), the C-terminal HR, the loop between two HRs, the transmembrane domain, and the aromatic residue-rich motif [14,16]. However, the SARS-CoV S1 has a low sequence homology with the existing animal and human coronavirus S1 subunits, therefore no protein structure model could be utilized in helping the development of vaccine and drugs. Recently, the advances in molecular characterization of SARS-CoV S1 have been made. The genetic diversity of the S1 hypervariable region observed in animal coronaviruses was also reported in SARS-CoV [17]. A recent report has identified the ACE2 as a cellular receptor for the virus [18] and the binding domain has been defined between residues 318 and 510 of the S1 subunit [19].

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.01.166

Clinical observations in SARS patients imply both humoral and cell-mediated immune responses may be needed to prevent SARS-CoV infection. It was reported that an apparent depletion of T cells occurred in the early infection and a gradual increase to normal level was observed as the patients recovered [20]. SARS-CoV specific antibodies were detected at 7 days after the onset of symptoms and kept at high titer for at least 3 months in the recovered patients [21]. In addition, infusion of convalescent sera to patients had been shown to help disease recovery [Sung et al., unpublished data]. The seroconverted individuals with no illness in the epidemic region [22] and the inability of culturing viruses from RT-PCR positive specimens collected from the patients about 40 days after presentation [23] implied that the neutralizing antibodies could be stimulated rapidly and might restrict the virus propagation. Moreover, in preparation of this paper, Gao et al. [24] reported that the combination delivery of SARS-CoV S1, M, and N genes into macaques using adenoviral vector elicited high titer of neutralizing antibodies, suggesting the potential of using S gene as a vaccine candidate. In the present study, mice were immunized with plasmids encoding various S gene fragments and the humoral responses were investigated. The results showed that antibodies against SARS-CoV could be induced by both subunits and part of S1, and the mouse antisera against S1 and S2 could cooperatively neutralize SARS-CoV.

Materials and methods

Animal, immunization, and serum collection. BALB/c mice at the age 6–8 week were obtained from the Laboratory of Animal Unit and brought up in Animal House of Zoology Department, the University of Hong Kong. At day 1, 22, and 43 the mice were anesthetized and injected with 150 μg per dose of plasmid in 100 μl phosphate-buffered saline (PBS) through tibialis anterior (TA) muscle on each leg [25]. In the case of co-administration of two kinds of plasmids in the same mouse, each construct was dissolved in 50 μl PBS and injected into TA muscle of each leg. Mouse blood was regularly collected by tail bleeding at day 0, 14, 21, 28, 35, 42, 49, and 56, and the sera were kept at −20 °C until further use. At day 63 the seroconverted groups were terminally anesthetized and their sera were taken for the assays of virus specific neutralization, antibody isotypes, and geometric titer (GMT).

Construction of recombinant plasmid. SARS-CoV cDNA was reverse-transcribed from viral RNA extract of SARS-CoV HK-39 as described previously [26]. DNA fragments covering different regions of the S gene were amplified from the viral cDNA with primers at the proper sites. Fig. 1 showed a schematic diagram indicating the sizes and the locations of these fragments on S gene. The recombinant S fragments (S_{R1}, S_{R2}, and S_{R3}) were constructed by joining of short S fragments through overlapping extensions. The cloning sites, *Bam*HI and *Eco*RI, start and stop codons were incorporated into PCR fragments by synthesizing on primers when necessary. A Kozak sequence was also added to S_{Na} and S_{La} ahead of their signal sequence. An expression vector, pCI-SP_{pGH}, was reconstructed by adding 28 amino acid residues of pig growth hormone signal peptide sequence (SP_{pGH}) [27] at upstream of modified polyclone sites of pCI (Promega). All the PCR amplified fragments were cloned into pCR2.1-TOPO (Invitrogen) and sequenced as described [26] before they were cloned into pCI-SP_{pGH}, except S_{Na} and S_{La}, which possessed the signal sequence of S gene and hence were inserted to pCI. The fragments S_{Nb}, S_{Lb}, S_C, S_F, S_{R1}, S_{R2}, and S_{R3} were also cloned into another expression vector pcDNA3.1(+) (Invitrogen) without signal sequence. Plasmid DNA

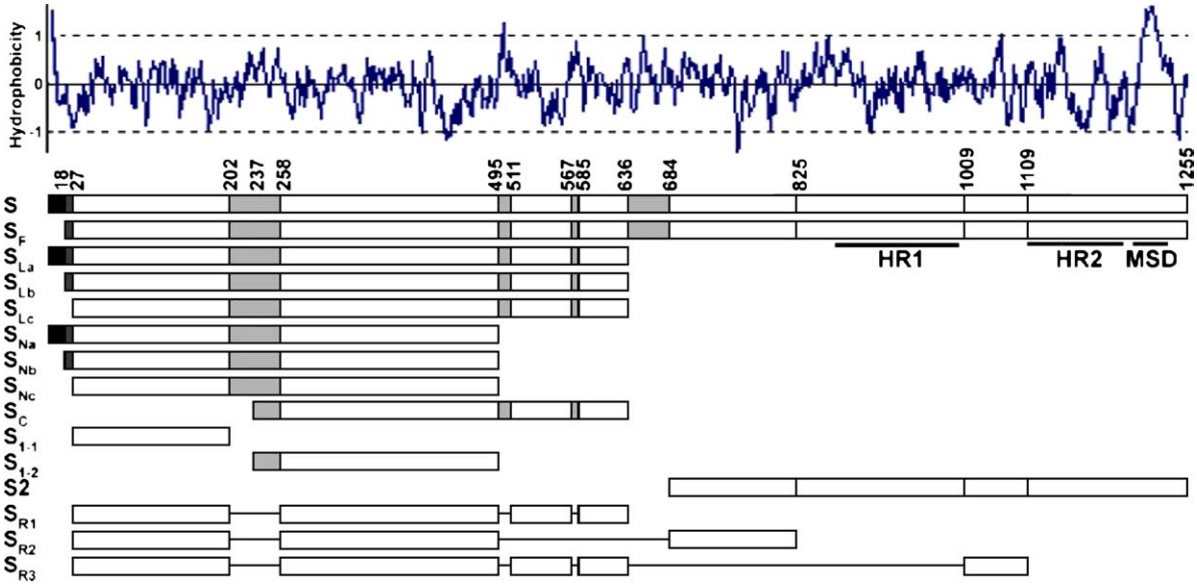

Fig. 1. Schematic diagram of full-length SARS-CoV S protein and its derived fragments used in the study. The full-length S gene is shown immediately under the hydrophobicity profile (upper). Signal sequence of S gene is marked as a solid black box and the putative highly hydrophobic regions of S1 are shown in solid box in light grey color. Heptad repeats (HR1 and HR1) and membrane spanning domain (MSD) are indicated as described elsewhere [16,26]. All the fragments including the recombinant fragments were cloned into mammalian expression vector pCI-SP_{pGH}, except for S_{La} and S_{Na} which were cloned in pCI. Fragments, S_{Nb}, S_{Lb}, S_C, S_F, S_{R1}, S_{R2}, and S_{R3} were inserted into pcDNA3.1 in addition. All the constructed plasmids were used for mice experiments. S_{R1-3} are different recombinants representing the putative hydrophilic regions.

used to immunize animals was prepared by Plasmid giga kit from Qiagen (Valencia, CA) according to manufacturer's instructions.

Expression and Western blot of spike protein. To test the expression of S gene and its derived fragments in mammalian cells, all the DNA fragments, as shown in Fig. 1, were cloned into pcDNA3.1-6×His-IRES-GFP through *Bam*HI and *Eco*RI sites. The vector was modified by adding the 6×His and IRES-GFP up- and downstream of the multiple cloning sites of pcDNA3.1(+), respectively. IRES-GFP was PCR amplified from the plasmid pBMN-I-GFP, which was a kind gift from Dr. G.P. Nolan (Stanford University School of Medicine). The recombinant plasmids were transfected into COS-7 cells cultured in 96-well microtiter plate in triplicate with DOSPER liposomal transfection reagent (Roche, Mannheim, Germany) following manufacturer's instruction. The expression was examined by direct observation using fluorescence microscopy. The expression of several selected fragments was further confirmed by Western blot. The transfected COS-7 cells were harvested in 48–72 h post-transfection (p.t.). The expressed protein was purified with Ni^{2+}-agarose (Amersham, UK) from clarified cell lysate and transferred onto PDVF membrane (Bio-Rad, Hercules, CA) by electroblotting after SDS–PAGE. Monoclonal mouse anti-his-tag antibody (Invitrogen) was utilized to detect expressed protein in Western blot.

ELISA tests of antibody titers. To detect SARS-CoV specific antibodies in mice immunized with the S gene DNA, human anti-SARS-CoV antibody (IgG) detecting ELISA kit (Beijing Huada GBI Biotechnology, Beijing, China) was used according to manufacturer's instructions, except that anti-human IgG was replaced with HRP-conjugated goat–anti-mouse IgG (H + L) antibody (Zymed, South San Francisco, CA) as the secondary antibody. According to the manufacturer, inactivated SARS-CoV viral particles were coated as antigen. Mice group and individual were defined as seroconverted if antibody titer was higher than 50 and 100, respectively. The GMT of the individual serum in the seroconverted groups was tested by end point dilution. SARS-CoV specific mouse IgG1 and IgG2a isotypes were detected with the same ELISA kit but either HRP-conjugated anti-IgG1 or IgG2a isotype monoclonal antibody (PharMingen) was used as the secondary antibody.

Virus neutralization assay. Microtiter plate (96-well) containing confluent FRhK4 cells in 0.1 ml maintenance medium (1% FCS in MEM) was prepared. Each of 2-fold diluted mouse serum (0.05 ml) starting from 1 in 10 was premixed with 0.05 ml of 200 $TCID_{50}$ SARS-CoV (strain HK-39) and incubated at 37 °C for 1.5 h. About 0.1 ml of the virus–serum mixture was then inoculated in wells seeded with FRhK4 cells in duplicate and further incubated at 37 °C. Cytopathic effect (CPE) was examined at 72 and 96 h after. Neutralization titer was determined as the highest dilution of serum which gives 50% CPE of cells.

Results

Cloning and expression of spike gene fragments

In order to investigate the antigenicity of S protein, especially the S1 subunit, S gene was segmented into S2, different S1 fragments ($S_{Na/b/c}$, $S_{La/b/c}$, S_C, S_{1-1}, and S_{1-2}), and recombinant S fragments (S_{R1}, S_{R2}, and S_{R3}). The recombinant S fragments were constructed by joining the various hydrophilic regions of S protein (Fig. 1), in an attempt to highly express the possible neutralizing epitopes on the exposing surface of S protein. All of the fragments were inserted in pcDNA3.1-6×His-IRES-GFP, including the full-length S gene, and expressed in COS-7 cells, as demonstrated by the observed fluorescence in the transfected cells (data not shown). In such expression system, the green fluorescence protein (GFP) was encoded by the same mRNA encoding S gene fragment under the control of CMV promoter, but translated in two independent open reading frames (ORF). Although the number of fluorescence emitting cells and the density of fluorescence were different among various S fragments transfected cells, it was difficult to establish a connection between GFP expression and antibody titers since the antibody response was not merely influenced by or directly related to the expression of antigen. In addition, the expression of S protein was also confirmed by Western blot (Fig. 2), and the expression of different S fragments in pcDNA 3.1-6×His-IRES-GFP was detected with expected size. However, we failed to detect the expression of S_F and other two small fragments, S_{1-1} and S_{1-2}, in the same experiment although their expression was proven at mRNA level through GFP co-expression.

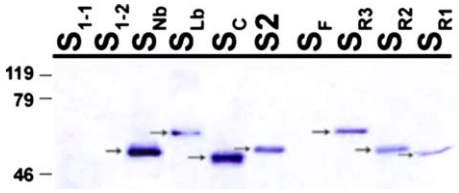

Fig. 2. The expressed S protein in Western blot. Expressed S protein by pcDNA 3.1-6×His-IRES-GFP was detected with monoclonal mouse anti-his-tag antibody. Arrow indicates the protein band with expected size, the lane with protein collected from cells transfected with control plasmid has no detectable band (not shown). Size marker is shown on the left (kDa).

Humoral responses of the S-gene DNA immunized mice

Grouped sera were prepared by pooling equal volume of individual serum of the same group. The SARS-CoV specific antibodies of the grouped sera were detected by ELISA and the humoral response profile of the seroconverted groups is shown in Fig. 3. The individual sera in possible seroconverted groups were titrated and are summarized in Table 1. In general, both S1 and S2 could elicit SARS-CoV specific antibodies. The immunogenic importance of the N-terminus of S1 subunit was revealed by comparing the SARS-CoV specific antibody titer of mouse groups immunized with plasmids containing S_{Na}, S_{Nb}, and S_{Nc}. These three fragments shared a common region of residues 27–495, but different only in their N-terminus (Fig. 1). A higher antibody titer and more seroconverted individuals were observed in S_{Nb} group than that of the S_{Na} group. The S_{Nc} group, however, showed no detectable SARS-CoV specific antibody response (Table 1), although it had the same leader sequence and most of the S1 amino acid residues as S_{Nb}. Another series of constructs, S_{La}, S_{Lb}, and S_{Lc},

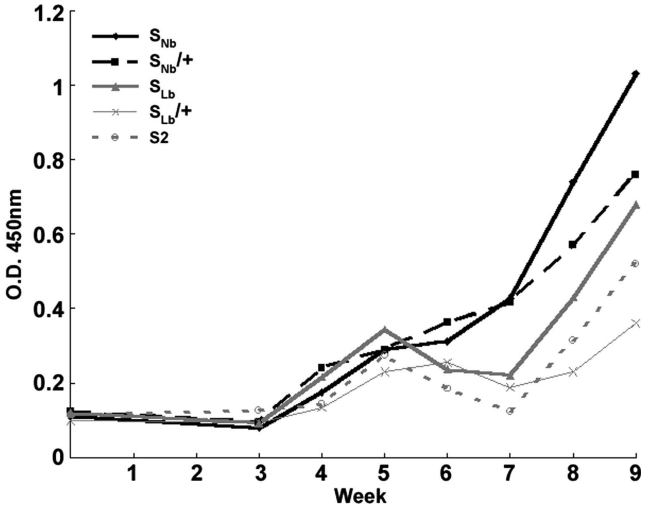

Fig. 3. Mouse anti-S protein IgG profile. Mice ($n = 5$ per group) were immunized with different spike gene fragments inserted in pCI-SP_{pGH} at day 1, 15, and 43. Serum samples were collected and grouped for ELISA assay at a dilution of 1:50.

showed a similar trend of antibody responses against SARS-CoV (Table 1). Groups immunized with pCI-SP_{pGH} containing S_F, S_{1-1}, and S_{1-2}, however, showed no detectable antibody response against the virus, while S_C, S_{R1}, S_{R2}, and S_{R3} groups showed a relatively low humoral response (Table 1). It was also noted that the groups immunized with pcDNA3.1 (without signal peptide) containing the fragments S_{Nb}, S_{Lb}, S_C, S_F, S_{R1}, S_{R2}, and S_{R3} showed no detectable antibody response (data not shown) although their expression was confirmed by Western blot (Fig. 2).

Antibody isotypes and Th1/Th2 response

The seroconverted sera were further tested for their antibody isotypes of IgG1 and IgG2a to estimate whether Th1 or Th2 was activated and mediated antibody isotype switching in DNA immunized mice. Several fragments (S_{Nb}, S_{Lb}, S_C, S_{R1}, and S_{R3}) inserted in pCI-SP_{pGH} were tested by a combined injection with S_{Lb} in pcDNA3.1 with no signal peptide attached to investigate if intracellular S1 could activate Th1 pathway leading to an IgG2a isotype switching. However, only S_{Nb} and S_{Lb} groups were seroconverted and hence only the antisera of these two groups were recruited for the study. The result showed that the secreting forms of S_{Nb} and S_{Lb} could elicit both IgG1 and IgG2a isotypes. Co-administration of an additional non-secreting S_{Lb} to both groups induced a higher IgG2a/IgG1 ratio in both groups (Table 1), although such non-secreting form of S protein seemed to have a negative effect on antibody elicitation, as a lower group's titer and a fewer number of seroconverted mice were observed in group S_{La}/+ and S_{Na}/+.

Antibody neutralization assay

Antisera from group of S_{Nb}, S_{Lb}, S_C, S_2, S_F, S_{R1}, S_{R2}, and S_{R3} were tested for their neutralizing antibody titer although S_C, S_{R1}, S_{R2}, and S_{R3} had only slightly group's antibody and even no detectable antibody response for

Table 1
Antibody and neutralizing antibody response against SARS-CoV

S fragment in pCI-SP_{pGH}	Serconverted mice/total mice	Group's titer	Titer (means ± SD)	Neutralizing antibody titer[d]	Titer of IgG1[d]	Titer of IgG2a[d]	IgG2a/ IgG1
S_{Na}	2/5	<50	N/A	N			
S_{Nb}	5/5	>50	219 ± 54.3	N	47	67	1.43
S_{Nb}/+[a]	3/5	>50	204 ± 55.1[c]	N	32	73	2.3
S_{Nc}	0/5	N	N/A	N/A			
S_{La}	0/5	N	N/A	N/A			
S_{Lb}	5/5	>50	181 ± 40.5	N	45	70	1.56
S_{Lb}/+[a]	2/5	<50	N/A	N	27	73	2.7
S_{Lc}	0/5	N	N/A	N/A			
S_C	1/5	<50	N/A	N			
S2	4/5	>50	168 ± 31.1[c]	N			
S_F	0/5	N	N/A	N			
S_{R1}	2/5	<50	N/A	N			
S_{R2}	3/5	<50	N/A	N			
S_{R3}	3/5	<50	N/A	N			
Control	0/5	N	N/A	N			
S_{Nb} /S2[b]				N			
S_{Lb} /S2[b]				80			

N, no detectable antibody response/neutralizing activity. N/A, not applicable.
[a] An additional 50 μg pcDNA3.1-S_{Lb} DNA was delivered in parallel for each mouse in each injection.
[b] By mixing equal volume of the two groups' mouse serum (for neutralizing assay).
[c] Titers were measured based on the seroconverted mice.
[d] Group sera (S_F and Control) or combined seroconverted sera in the group (the rest groups) were used.

S_F in the ELISA assay (Table 1). The result showed that only the antiserum combined with equal volume of sera from group S_{Lb} and S2 was capable of neutralizing SARS-CoV. No detectable neutralizing ability was observed by using other serum samples including the mixed S_{Nb} and S_2 group serum and the serum elicited by S_{R3} DNA which covered most hydrophilic regions of S gene (Table 1).

Discussion

Secreting S protein is necessary to induce antibody response

In the present study, different truncated fragments of SARS-CoV S gene were cloned into two mammalian expression vectors, with or without secreting signal sequence, and used to immunize mice. The non-secreting form of S protein could not elicit any detectable SARS-CoV specific antibody response while the secreting forms S1, S2, and the N-terminus of S1 (S_N) did. The inability of the non-secreting S protein to elicit SARS-CoV specific antibody may be explained by the lack of expressed antigens with correct conformation exposed to immune system for post-translational modification including complex folding, glycosylation, and oligomerization, which occurs during protein secretion and sorting. It was widely reported that the expression of viral membrane protein in different cellular locations (e.g., secreted, membrane bound, and non-secreted form) was the immune response of DNA immunization. Animals vaccinated with DNA encoding secreting form of E2 glycoprotein of classical swine fever virus (CSFV) showed both CSFV specific antibody response and protection upon viral challenge though the native E2 was anchor membrane protein ([28,29] and Zeng et al., unpublished data). In addition, a shift in T-helper cell response from Th1 to Th2 was induced by expression of secreting form of hemagglutinin glycoproteins [30]. The non-secreting form of S protein, however, might be useful in activation of Th1 response, as injection of secreting and non-secreting S1 into the same mouse enhanced Th1 response, as shown by the raised IgG2a to IgG1 ratio (Table 1). These preliminary data suggest that S1 may possess epitopes that could activate T-cell responses.

The antibody and neutralizing antibody elicited by S1 and S2

In the mice experiment, it was demonstrated that SARS-CoV specific antibodies could be induced by immunization with plasmids encoding S1, S2 and fragment of S1 subunit. Plasmid carrying S_{Nb} (residue 18–495), elicited the highest antibody titer among all the seroconverted groups (Table 1). Residues near the cleavage site of the signal peptide in matured S1 were very critical in terms of stimulating SARS-CoV specific antibodies, as demonstrated by two sets of fragments, $S_{Na/b/c}$ and $S_{La/b/c}$, which were expressed through the same plasmid vector.

As indicated in the virus neutralizing assay, none of the antisera from the SARS-CoV specific seroconverted groups can neutralize the virus. Instead, mixed antisera from seroconverted groups immunized with S1 (S_{Lb}) and S2 plasmids showed neutralizing activity, although the concentration of the antibodies was diluted one-half as compared to the parent sera. It was also noted that another set of antisera mixed sample from S_{Nb} and S2 showed no observable neutralizing activity as well, although the antibody titer of S_{Nb} was higher than that of S_{Lb} and the same S2 serum was used.

The obligatory cooperation of S1 and S2 specific antibodies in terms of virus neutralization, however, contradicts with previous researches on coronavirus. Several authors reported that immunization with recombinant S1 protein or plasmid encoding the S1 subunit of infectious bronchitis virus (IBV) could induce protective immune responses [8,12,13], although multiple inoculations were required [9]. The first paper on immunizing masques with structural genes of SARS-CoV, however, reported that co-delivery of three viral genes encoding S1, nucleocapsid (N), and membrane (M) protein could elicit a high titer of neutralizing antibody and T-cell response [24]. The authors stated that inoculation of S1 with N and or M could stimulate a strong antibody and T-cell response, in parallel with the previous experience on developing vaccine against transmissible gastroenteritis coronavirus (TGEV) [31]. From this, together with the experimental data from this report, it can be hypothesized that antibodies against the S1 glycoprotein are crucial in neutralizing SARS-CoV but the antibodies against other components of the virus may also play critical roles in virus neutralization. Such effects may become significant if the titer of the antibodies, including those against S1 protein, is low. The putative cooperative role of S2 specific polyclonal antibody to neutralize the virus may serve as an additional example which directly demonstrates the blockage of virus entry through the binding to the S2 subunit. Further experiments are desirable to define the exact roles S1 and S2 specific polyclonal antibodies play in neutralizing SARS-CoV and their protecting ability against virus challenge. Results described here are expected to speed up the design of subunit vaccine and the immunological research against the SARS-CoV.

Acknowledgment

This work was supported by Research Grant Council Grant HKU 7553/03M.

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