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Epitope mapping and biological function analysis of antibodies produced by immunization of mice with an inactivated Chinese isolate of severe acute respiratory syndrome-associated coronavirus (SARS-CoV)

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Abstract

Inactivated severe acute respiratory syndrome-associated coronavirus (SARS-CoV) has been tested as a candidate vaccine against the re-emergence of SARS. In order to understand the efficacy and safety of this approach, it is important to know the antibody specificities generated with inactivated SARS-CoV. In the current study, a panel of twelve monoclonal antibodies (mAbs) was established by immunizing Balb/c mice with the inactivated BJ01 strain of SARS-CoV isolated from the lung tissue of a SARS-infected Chinese patient. These mAbs could recognize SARS-CoV-infected cells by immunofluorescence analysis (IFA). Seven of them were mapped to the specific segments of recombinant spike (S) protein: six on S1 subunit (aa 12–798) and one on S2 subunit (aa 797–1192). High neutralizing titers against SARS-CoV were detected with two mAbs (1A5 and 2C5) targeting at a subdomain of S protein (aa 310–535), consistent with the previous report that this segment of S protein contains the major neutralizing domain. Some of these S-specific mAbs were able to recognize cleaved products of S protein in SARS-CoV-infected Vero E6 cells. None of the remaining five mAbs could recognize either of the recombinant S, N, M, or E antigens by ELISA. This study demonstrated that the inactivated SARS-CoV was able to preserve the immunogenicity of S protein including its major neutralizing domain. The relative ease with which these mAbs were generated against SARS-CoV virions further supports that subunit vaccination with S constructs may also be able to protect animals and perhaps humans. It is somewhat unexpected that no N-specific mAbs were identified albeit anti-N IgG was easily identified in SARS-CoV-infected patients. The availability of this panel of mAbs also provided potentially useful agents with applications in therapy, diagnosis, and basic research of SARS-CoV.
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Keywords: SARS-CoV; Monoclonal antibody; Epitope mapping; Inactivated vaccine

Introduction

Severe acute respiratory syndrome (SARS), a highly virulent emerging infectious disease, can spread rapidly among large human populations as demonstrated in the first half of 2003. The etiological agent of SARS was identified as a new human coronavirus, SARS-CoV (Ksiazek et al., 2003; Marra et al., 2003; Rota et al., 2003). While the first SARS epidemic was successfully contained with the collaborative efforts organized by the World Health Organization, SARS remains a potential threat due to the mysterious source of its initial infection and highly transmittable nature of this virus (Callow et al., 1990; Holmes, 2001; Kraaijeveld et al., 1980). Its apparent presence in animal reservoirs provided the possibility of reemergence, including in forms with increased infectivity. Because the symptoms of SARS can be confused clinically with many respiratory diseases caused by other

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etiological agents, a simple and highly specific diagnostic test applicable at the early onset of SARS still needs to be developed. The current medical strategies mainly relying on non-specific anti-viral and supportive treatment are not sufficient. While questions remain whether natural SARS infection will ever return in the form seen in 2003, it is important to test various candidate SARS-CoV vaccines and to produce tools which can be used to diagnose and treat this infection when it re-emerges.

The SARS-CoV is an enveloped positive-sense single-stranded RNA virus of 29,700 nucleotides that has been completely sequenced. Open reading frames (ORFs) analysis by analogy with other known coronaviruses indicated that four structural proteins might play important functions associated with SARS-CoV infection, including the surface spike protein S (1,255 aa), which has an N-terminal receptor binding domain to mediate attachment to cellular receptors (Lewicki and Gallagher, 2002; Wong et al., 2004) and C-terminal heptad repeats (HRs) to promote virus entry by fusion with cell membranes (Bosch et al., 2003; Luo and Weiss, 1998; Marra et al., 2003; Rota et al., 2003; Spiga et al., 2003). The nucleocapsid N protein (422 aa) is a highly basic structural protein, which is usually known to bind viral RNA to form the helical core structure in coronavirus (Davies et al., 1981) and to promote viral packaging and viral core formation (He et al., 2004a; Hiscox et al., 2001). The matrix membrane glycoprotein M (221 aa) is an integral membrane protein involved in budding. There is a small envelope protein E (76 aa), a critical component of the virus responsible for virion envelope morphogenesis (Arbely et al., 2004) which acts as a scaffold protein to trigger assembly. SARS-CoV lacks the envelope-associated hemagglutinin-esterase glycoprotein that is encoded by some coronaviruses. There are additional genome fragments which code for RNA-dependent RNA-polymerase (1a/b) and eight other non-structure proteins (Marra et al., 2003; Rota et al., 2003).

Since the discovery of SARS-CoV as the cause of SARS, inactivated SARS-CoV has been proposed as one of the prophylactic vaccination approaches against SARS. Animal and early phase human studies have been started (Darnell et al., 2004; He et al., 2004b; Marshall and Enserink, 2004; Takasuka et al., 2004; Tang et al., 2004; Xiong et al., 2004). However, the breadth and specificity of antibody responses in animals immunized with inactivated SARS-CoV have not been well characterized. At the same time, a number of studies have mapped the major neutralizing domain on SARS-CoV to the S protein between amino acids 261 and 672 (Sui et al., 2004). This epitope coincides with the angiotensin-converting enzyme 2 (ACE2) receptor binding site identified for S protein in Vero E6 cells infected with SARS-CoV (Berry et al., 2004; Che et al., 2003; He et al., 2003; Hua et al., 2004; Li et al., 2003; Sui et al., 2004; ter Meulen et al., 2004; Traggiai et al., 2004; Wen et al., 2004; Wong et al., 2004; Zhou et al., 2004).

In the current study, a panel of monoclonal antibodies was produced from mice immunized with the inactivated SARS-CoV isolated from an infected patient (Qin et al., 2003; Tang et al., 2004) and the spectrum of antibody responses that can be elicited with an inactivated SARS-CoV was examined. Our results suggested that most mAbs generated by this approach recognized quite diverse epitopes on S protein but not against the other three structural proteins (N, E, and M). S-specific mAbs showed different biological activities. Because virion-associated antigens may resemble the true topology of SARS-CoV antigens more closely than recombinant SARS-CoV proteins, the current study produced a panel of mAbs against epitopes which may not be otherwise recognized, thus providing useful tools for a wide range of potential applications related to SARS research.

Results

Screening of antigen specificity on monoclonal antibodies produced by immunization with an inactivated SARS-CoV

Positive antibody responses against SARS-CoV were identified in five Balb/C mice after being immunized three times with inactivated SARS-CoV. After fusion of the spleen cells with $SP_2/0$ myeloma with several rounds of screening, twelve individual hybridomas were finally obtained showing antibody responses against SARS-CoV as verified by IFA staining of SARS-CoV-infected Vero E6 cells (data not shown). The antigen specificity was further mapped by ELISA against recombinant SARS-CoV structural proteins. Six mAbs (2A3, 1B4, 2B1, 1A5, 2C5, and 3A3) showed high-level reactivity against the full-length recombinant Spike (S) protein produced by the mammalian expression system (Fig. 1). The remaining mAbs showed very poor reactivity against S protein (Fig. 1) and also failed to recognize recombinant E, M, and N in the subsequent ELISA tests (data not shown).

Mapping of mAbs epitope on specific domains of S

The S protein of SARS-CoV is a large type-I transmembrane glycoprotein composed of 1255 amino acids including an N-terminal S1 domain and a C-terminal S2 domain (Marra et al., 2003; Rota et al., 2003; Spiga et al., 2003). We used individual segments of S (Fig. 2) to further map the epitopes for six mAbs that showed high reactivity against the full-length S protein. The designation of S1 (aa 12–798) and S2 (aa 797–1255) domains in the current study was based on the knowledge of S protein structure from coronaviruses in general (Lai and Holmes, 2001) and the alignment of SARS-CoV S protein sequence against S proteins from other coronaviruses with known cleavage sites between their S1 and S2 domains (Bosch et al., 2003; Krokhin et al., 2003; Marra et al., 2003; Rota et al., 2003; Spiga et al., 2003). However, there has been no direct evidence in the literature to suggest S protein of SARS-CoV was cleaved into S1 and S2. In our study, the S1 segment

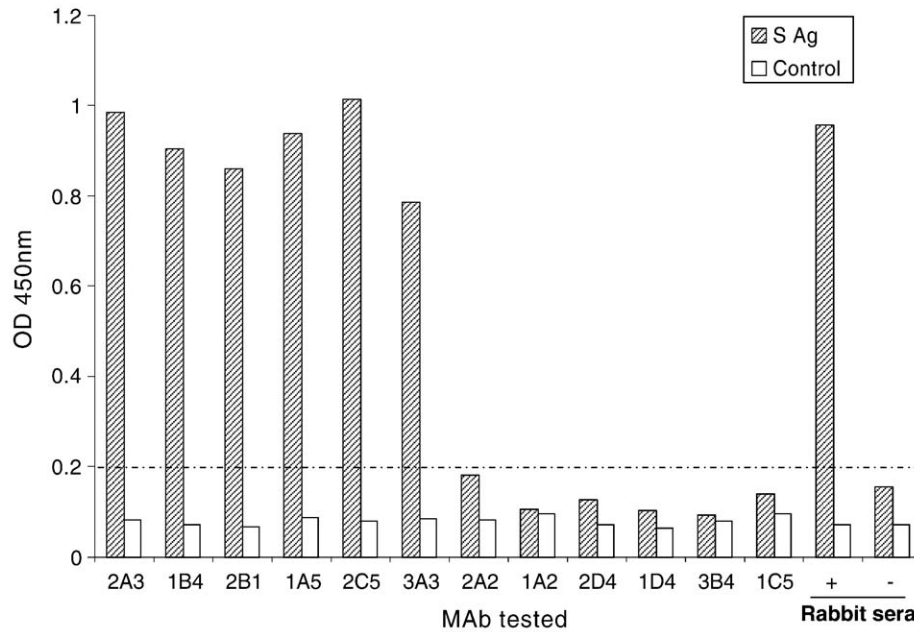

Fig. 1. Epitope mapping of mAbs to the S protein by ELISA. MAbs were added at 1:100,000 dilution to each well against either the recombinant full-length S protein (bars in shade) expressed from transiently transfected 293T cells or the vector DNA-transfected 293T cells (bars in blank). "+" and "−" were control sera from New Zealand rabbits immunized with either the full-length S DNA vaccine or the empty vector DNA, respectively. The OD_{450nm} values less than 0.2 are considered non-specific binding to the full-length S antigen in this ELISA assay.

was further divided into two sub-segments, S1.1 (aa 12–535) and S1.2 (aa 534–798), based on the distribution of the predicted N-glycan sites. The S1.1 segment was then further divided into S1.1a (aa 12–311) and S1.1b (aa 310–535), and the latter was reported to be the site for binding of SARS-CoV S protein to its receptor ACE 2 (Li et al., 2003).

These recombinant S segment proteins were produced by transiently transfected 293T cells and used as antigens in the ELISA and Western blot analyses. Three mAbs (2A3, 1B4, and 2B1) were mapped to S1.1a, two mAbs (1A5 and 2C5) to S1.1b, and one (3A3) to the S2 domain based on the result of ELISA (Table 1). The specificity of these mAbs was further confirmed by Western blot analysis recognizing specific recombinant S protein fragments. Fig. 3 shows the example of one mAb, 2A3, whose specificity was mapped to the S1.1a domain by ELISA (Fig. 3A) and confirmed by

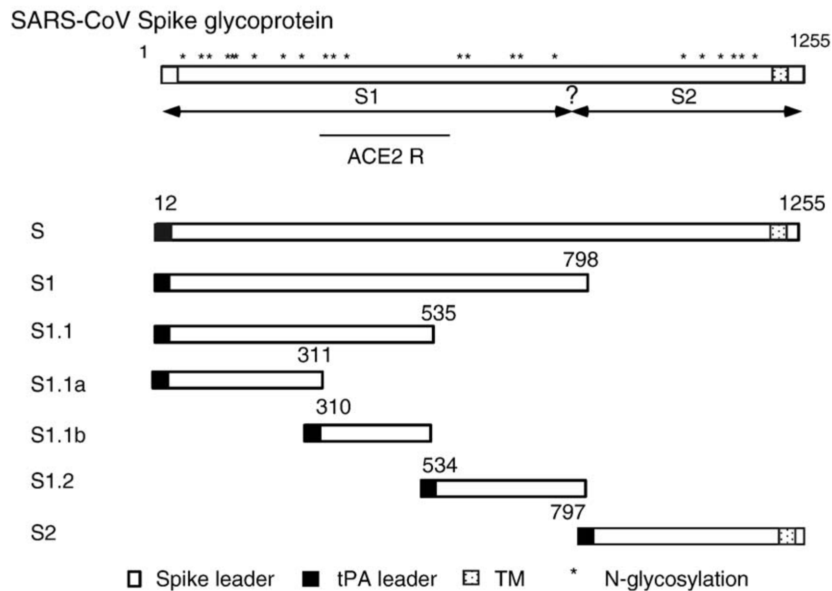

Fig. 2. Designs of recombinant S segments used in this study. Schematic representation of the entire Spike protein is shown on top, including its natural leader and its transmembrane domain close to the C terminal tail. Predicated N-glycosylation sites are marked by asterisks and the ACE2 receptor binding domain is also noted. DNA plasmids expressing different segments of the S protein were shown in the lower part of the figure with their amino acid residue numbers marked.

Table 1
Mapping of S-specific mAbs by ELISA and Western blot analyses

mAbs	Recombinant S antigens recognized in ELISA								Western blot	
	S	S1	S1.1	S1.1a	S1.1b	S1.2	S2	Vector		
2A3	+	+	+	+	–	–	–	–	S	S1
1B4	+	+	+	+	–	–	–	–	–	–
2B1	+	+	+	+	–	–	–	–	S	S1
1A5	+	+	+	–	+	–	–	–	–	–
2C5	+	+	+	–	+	–	–	–	S	S1
3A3	+	–	–	–	–	–	+	–	S	S2
2D4	–	ND	+	+	–	–	–	–	–	S1

ND: not done.

the Western blot of its recognition to recombinant S, S1, S1.1 proteins, and the SARS-CoV virion associated S antigen, but not to recombinant S1.2 or S2 protein (Fig. 3B). Similarly, the epitopes of mAbs 2B1 and 2C5 were mapped to S1.1a and S1.1b, respectively (Table 1). Another mAb, 3A3, which mapped to the S2 domain by ELISA (Table 1), recognized not only the virus-associated full-length S antigen and its oligomer but also a smaller fragment about 70 kDa which is likely to be the cleaved S2 (Fig. 4). Interestingly, the other two S-specific mAbs (1B4 and 1A5) as identified by the ELISA screening did not show any recognitions to the recombinant S proteins by the Western blot (Table 1), suggesting these mAbs may mainly recognize non-denatured epitopes.

Unexpectedly, another mAb 2D4 which failed to show reactivity against the full-length S antigen by either ELISA (Fig. 1) or Western blot recognized the recombinant S1 protein (Fig. 5A). In consistent with this finding, mAb 2D4 recognized a lower molecular band, but not the full-length S associated with the SARS-CoV virion (Fig. 5A). Additional ELISA analysis further mapped its epitope to the first 311 aa of the S protein because this mAb showed low but clearly positive reactivity against S1.1 and S1.1a antigens (Fig. 5B).

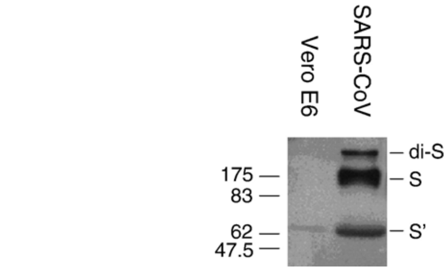

Fig. 4. Detection of SARS-CoV-associated S protein with mAb 3A3 by Western blot analysis. Samples loaded included uninfected Vero E6 lysate and SARS-CoV-infected Vero E6 lysate. Full length S protein (S), the dimmer form of S (di-S), and processed S protein (S′) are indicated.

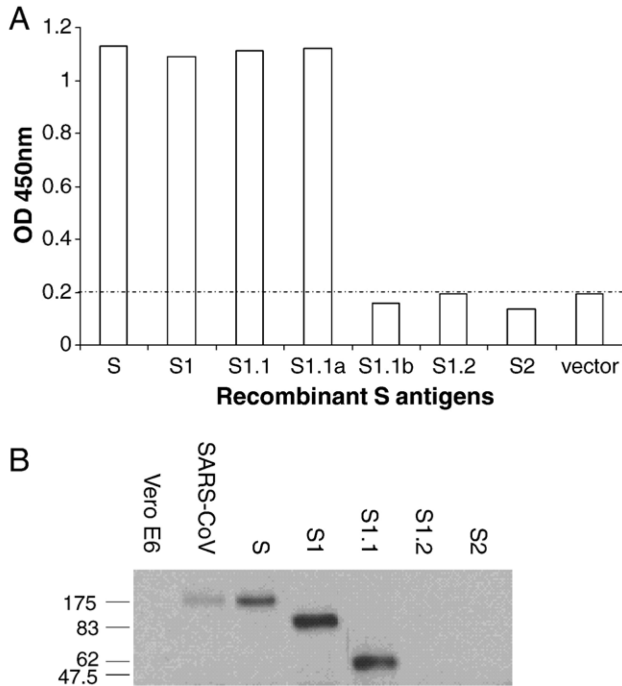

Fig. 3. Epitope mapping of mAb 2A3 using specific recombinant S antigens expressed in 293T transfected cells. (A) Detection of various recombinant S proteins as the coating antigens by ELISA. The OD_{450nm} values in ELISA less than 0.2 are considered non-specific binding to S antigens. (B) Detection of S and its subdomains by Western blot analysis. Samples included uninfected Vero E6 cell lysate (Vero E6), SARS-CoV-infected Vero E6 cell lysate (SARS-CoV), and recombinant S proteins (S, S1, S1.1, S1.2, and S2) as labeled.

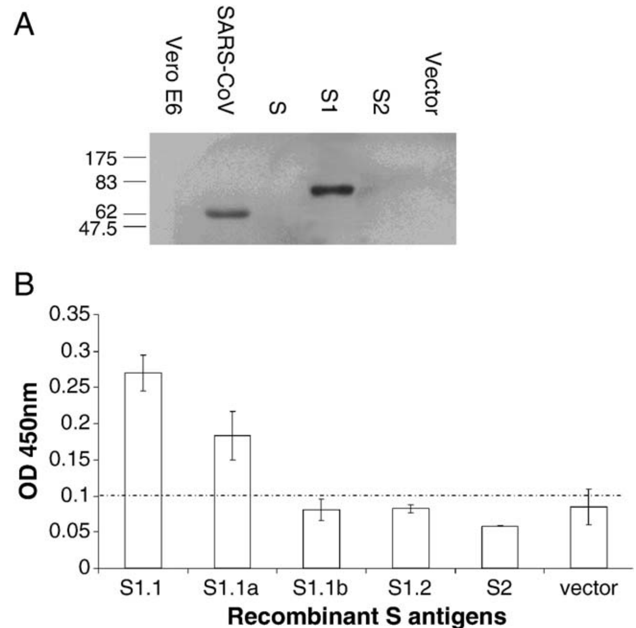

Fig. 5. Epitope mapping of mAb 2D4. (A) Western blot analysis of mAb 2D4 with the following samples: uninfected Vero E6 cells, SARS-CoV-infected Vero E6 lysate, recombinant S proteins (S, S1 and S2) expressed from transiently transfected 293T cells, and the control 293T cells transfected with vector DNA. (B) ELISA with mAb 2D4 at 1:500 dilution against different recombinant S antigens (S1.1, S1.1a, S1.1b, S1.2, and S2) expressed from transiently transfected 293T cells and control 293T cells transfected with vector DNA. OD_{450nm} values less than 0.1 are considered non-specific binding in this ELISA assay.

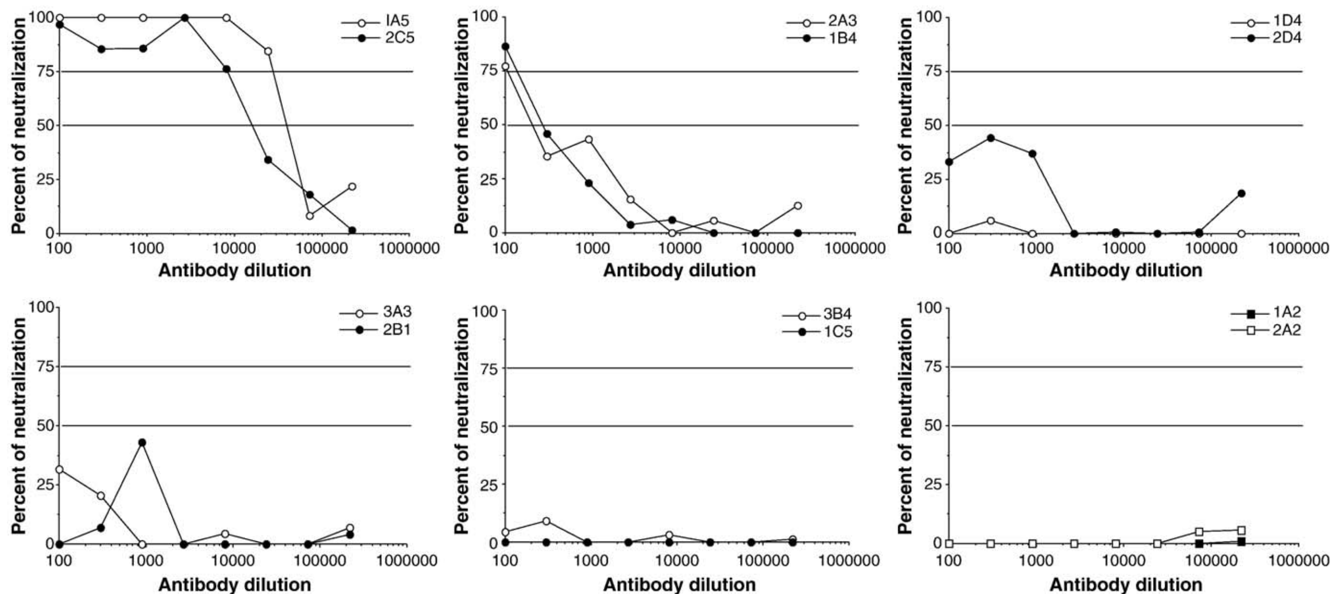

Fig. 6. Neutralizing activities of mAbs against the Urbani strain of SARS-CoV as measured by neutral red staining in infected Vero E6 cells. Neutralizing activities are plotted as the percent inhibition of viral infection against a particular rabbit serum dilution based on the geometric means from triplet wells. The 50% inhibition (IC50) or 75% inhibition (IC75) levels are marked.

Neutralization of SARS-CoV infection to Vero E6 cells

Out of twelve mAbs tested in this study, two S1.1b specific mAbs, 1A5 and 2C5, showed significant levels of neutralizing activities against SARS-CoV (Fig. 6). Two other S-specific mAbs, 2A3 and 1B4, which had their epitopes on S1.1a domain, showed only borderline neutralizing activities. The remaining three S-specific mAbs (3A3, 2B1, and 2D4) did not demonstrate any neutralizing activities (Fig. 6). The neutralizing titers of these mAbs as measured by 50% inhibition of SARS-CoV infection were summarized in Table 2 along with the original concentrations of S-specific IgG in these mAbs. This panel of mAbs was further characterized with respect to their immunoglobulin isotypes. Analyses on the constant regions of their heavy chains showed that there were five IgG1, five IgG2a, one IgG2b, and one IgM (Table 3). All of them used κ light chains instead of λ chains. Two mAbs, 1A5 and 2C5, that showed strong neutralizing activities against SARS-CoV were IgG2a and IgG1 isotypes, respectively.

Table 2
Summary of mAb neutralizing activities against SARS-CoV Urbani strain by neutral red assay

mAbs	Specificity	Concentration (μg/μl)	Neutralization titers (IC50)*
2A3	S1.1a	60	200
1B4	S1.1a	124	300
2B1	S1.1a	11	<100
2D4	S1.1a	ND	<100
1A5	S1.1b	32	45,000
2C5	S1.1b	14	11,000
3A3	S2	0.25	<100
2A2	?	ND	<100
1C5	?	ND	<100
1D4	?	ND	<100
1A2	?	ND	<100
3B4	?	ND	<100

Neutralizing titers are expressed as the highest sera dilutions that achieved 50% inhibition of SARS-CoV infection to Vero E6 cells. The values are the geometric means from 3 triplet wells.
ND: not done.

Discussion

The current study confirmed that the inactivated SARS-CoV was immunogenic in eliciting antibody responses against SARS-CoV antigens. While it is less likely that inactivated SARS-CoV can completely reflect the antigen conformation of SARS-CoV, our data revealed that the S protein is the dominant antigen among the virion-associated proteins because majority of the mAbs produced in this study were specific for S protein including two with

Table 3
Ig subtype of monoclonal antibodies against SARS-CoV

mAbs	Heavy chain						Light chain	
	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM	κ	λ
2A3	+	–	–	–	–	–	+	–
1B4	–	+	–	–	–	–	+	–
2B1	–	+	–	–	–	–	+	–
1A5	–	+	–	–	–	–	+	–
2C5	+	–	–	–	–	–	+	–
3A3	–	–	+	–	–	–	+	–
1C5	–	+	–	–	–	–	+	–
2A2	–	+	–	–	–	–	+	–
2D4	+	–	–	–	–	–	+	–
1D4	–	–	–	–	–	+	+	–
1A2	+	–	–	–	–	–	+	–
3B4	+	–	–	–	–	–	+	–

excellent neutralizing activities against SARS-CoV infection (Fig. 6). It is interesting that none of the mAb could recognize N, M, and E antigens even though anti-N antibody has been reported as one of the major antibody components in SARS-CoV-infected patient sera (Ying et al., 2004). This finding is different from the recent in vitro study using subunit SARS-CoV antigens in which M protein interacts with the N protein through its C-terminal domain to facilitate formation of nucleocapsids, and M was also reported to be able to interact with S protein to form pseudoparticles (Huang et al., 2004). In the same study, DNA vaccine expressing N antigen elicited high titer anti-N antibody responses thus ruling out the possibility that N is a poor immunogen.

No epitopes were identified for five mAbs produced by immunization with inactivated SARS-CoV. It is likely that these mAbs may have recognized some of the antigen determinants that were unique to the inactivated virion. The possibility of these mAbs to recognize non-structure proteins exists but is less likely since the mAbs were initially selected based on their reactivity against inactivated virion by ELISA. Knowledge related to the specificity of antibody responses generated by inactivated SARS-CoV vaccines is important for the evaluation of efficacy and safety profile for such vaccination approach against SARS. Viral enhancing antibody (or autoimmune responses) was reported for inactivated vaccine approach against animal coronaviruses (Marshall and Enserink, 2004; Scott, 1987). Our data will be useful for the identification of the contributing components for such autoimmune responses if the candidate inactivated SARS-CoV vaccines eventually demonstrate any similar adverse events in more advanced human testing.

In the current study, twelve mAbs against human SARS-CoV were produced by immunizing the Balb/C mice with an inactivated patient isolate of SARS-CoV. This is different from other approaches that produced anti-S mAbs. MAbs have been isolated from SARS-CoV-infected patients' IgG memory B lymphocytes and immortalized with EBV (Traggiai et al., 2004). Mice receiving the high dose of one such mAb, S3.1, were protected from viral challenge (Traggiai et al., 2004). Another study showed that a mAb specific for the nucleoprotein and five mAbs reacted with the Spike protein out of the seventeen IgG mAbs screened by ELISA were able to neutralize SARS-CoV in vitro (Berry et al., 2004). A human mAb CR3014, generated by antibody phage display technology screening a large naive antibody library, could reduce replication of SARS-CoV if prophylactic administrated to ferret (ter Meulen et al., 2004). Several mAbs were raised by using recombinant protein fragments of the SARS CoV S protein (residues 249–667 or 485–625) or N proteins expressed from *Escherichia coli* (Berry et al., 2004; Che et al., 2003; Wen et al., 2004). Synthetic gene fragments from predicted S epitopes have also been used to raise mAbs to study the S domain structure (Hua et al., 2004). There were recombinant antibodies against E and N protein isolated from the mouse synthetic $V_H + V_L$ scFv phage display library with high binding affinity to the SARS proteins E and N purified from *E. coli* (Liu et al., 2004). Our study used the inactivated vaccine approach to immunize the mice and the resulting mAbs showed diverse specificities and various biological functions.

Our data confirmed that the dominant neutralizing domain on S protein is at the S1.1b region which coincides with the ACE-2 receptor binding region of S protein as reported previously (Sui et al., 2004). Specificities and strength of two high titer neutralizing mAbs (1A5 and 2C5) were indistinguishable in our experiment, thus they probably recognized the same or a closely related epitope. The relative ease with which these mAbs were generated further supports the idea that subunit vaccination with S1- or S1.1b-like constructs may be sufficient to protect animals and perhaps humans. Two of the three S1.1a-specific mAbs (2A3 and 1B4) also showed slightly positive neutralizing activities. However, their low neutralizing titers would not support the role of S1.1a region as another neutralizing domain. These two mAbs may recognize a region on S protein which can interfere with the binding of virion to the viral receptor but may not be in a direct manner.

Seven mAbs in our panel were specific for S protein with six showing strong binding to the full-length S protein. The seventh mAb 2D4 could recognize a processed S antigen from SARS-CoV with the apparent molecular weight of ~70 kDa (Fig. 5A) and its epitope was mapped to S1.1a region (Fig. 5B). It is quite unique that 2D4 could not recognize either the full-length recombinant S protein or the virion-associated full-length S protein. The direct interpretation of this finding is that the 2D4 epitope in the S1.1a region may not be accessible on the full-length S protein given the large size and heavy glycosylation of SARS-CoV S protein. Additional analyses are needed to confirm whether this epitope may become more exposed upon the cleavage of S into smaller fragments.

As we recently demonstrated, the S protein of SARS-CoV may go through a step-wise cleavage process in which the highly specific polyclonal antibodies recognized a group of low molecular weight S fragments rather than the explicit S1 or S2 domains (Wang et al., 2005). Most of the reports suggested that the S protein of SARS-CoV was not normally processed into S1 and S2 subunits; however, it could be cleaved by exogenous trypsin (Simmons et al., 2004; Yao et al., 2004). In the current study, a cleaved S2 fragment at about 70 kDa was identified with S2-specific mAb 3A3 by Western blot analysis. This finding is consistent with the observation in a recent publication that an S2 product was detected by mAb generated by purified recombinant S2 protein (Wu et al., 2004). These data revealed important evidence that S protein can be cleaved in the lysate of SARS-CoV-infected Vero E6 cells, indicating the presence of proteolytic processing of S protein. This

process may be cell dependent because no such cleavage was observed with recombinant S protein in transfected 293T cells. The availability of specific cellular enzyme may be critical for such cleavage. The discovery of mAbs 2D4 (specific for processed S1 protein) and 3A3 (specific for S2) can aid further study on the proteocleavage of S protein.

It is known that the IgG subclasses may show differences in effector functions like antigen recognition, complement activation, and cell surface Fc receptor binding (Whitton and Oldstone, 1996). For example, human antibodies against viral capsid proteins were found to belong predominantly to the IgG1 and IgG3 subclasses, whereas IgG2 is the predominant subclass in immune responses against polysaccharides. Therefore, in this study, the immunoglobulin isotypes were also analyzed although it is known that the classification of mouse IgG subclasses does not match with that of human's. Among 11 IgG mAbs produced from this study, there are five IgG1, five IgG2a, and one IgG2b. Two strong neutralizing mAbs 1A5 and 2C5 were IgG2a and IgG1, respectively. Previously reported neutralizing mAbs against SARS-CoV included IgG1 (Sui et al., 2004), IgG2a, or IgG2b subtypes (Gubbins et al., 2005). So, there is no evidence to suggest that antibody isotypes may play any roles in determining the neutralizing activities against SARS-CoV.

In conclusion, we have characterized twelve mAbs generated against inactivated SARS-CoV. Majority of these mAbs recognized either the full-length or the processed S protein. The wide spectrum of S protein epitopes recognized by this panel of mAbs suggested the overall high immunogenicity of S protein associated with SARS-CoV particles. The antibodies to SARS-CoV S protein included both linear and conformational epitopes, thus these mAbs are useful tools for specific applications in SARS research.

Materials and methods

Viruses and cells

The SARS-CoV BJ01 strain (Qin et al., 2003) was isolated from a SARS patient in Beijing, China, by the Institute of Microbiology and Epidemiology, Beijing, China (Fang et al., 2003). Vero E6 cells (2×10^6 cells) were infected with a multiplicity of infection (MOI) of 0.01 and cultured at 37 °C with 5% CO_2 for 36 h. Cells were lysed by freeze–thaw cycles followed by centrifugation at 6000 rpm (Beckman 25R, Beckman Coulter Inc., Fullerton, CA) for 20 min. Preparation of inactivated SARS-CoV was previously reported (Tang et al., 2004). Briefly, the virus was inactivated by β-propiolactone (1:2000 dilution) from the commercial stock solution (1.146 g/mL, Sigma-Aldrich, St. Louis, MO) and incubated at 4 °C for 24–72 h, then kept at 37 °C for 2 h. The inactivated virus suspension was centrifuged at 6000 rpm (Beckman 25R, Beckman Coulter Inc.) at 4 °C for 30 min. The supernatant was harvested and concentrated with PEG20000 (Sigma-Aldrich, Inc.), then concentrated by centrifugation (30,000 × g, 20 min) using Cetriplus YM-100 (Millipore Corp., Bedford, MA), followed with purification by Sepharose 4FF column chromatography (Tang et al., 2004).

The SARS-CoV Urbani strain used for neutralization assay was obtained from U.S. Center for Disease Control and Prevention (Atlanta, GA). To inactivate this virus for ELISA and Western blot analysis, the virus stocks were first filtered through a 0.45-μm membrane to remove cell debris. Then the virus was inactivated with a buffer containing 0.2% SDS and 1% Triton-X 100 in Tris-buffered saline (pH 7.6) for 1 h at 4 °C. The inactivation of SARS-CoV was confirmed by using a Standard Operational Procedure (SOP) approved by the Institutional Biosafety Committee at the University of Massachusetts Medical School.

Production of monoclonal antibodies (mAbs) by inactivated SARS-CoV

Six-week-old Balb/C mice were injected with 2 μg of inactivated SARS-CoV BJ01 strain mixed with Complete Freund's Adjuvant (CFA, H37 Ra; Difco) on day 1. On day 10, the mice received 2 μg of inactivated SARS-CoV in incomplete Freund's Adjuvant (IFA). Mice were boosted 3 weeks later with 0.8 μg of inactivated SARS-CoV without any adjuvants. Animals were euthanized 3 days later and the mouse splenocytes were fused with SP_2/0 myeloma cells. Positive hybridomas were cloned out and supernatants were screened via ELISA using inactivated virus as coating antigen. The fused cell lines positive in ELISA were grown briefly in cell culture and then injected into mouse peritoneum. The ascites fluid which contains high titer monoclonal antibodies against SARS-CoV was collected for further analysis. Immunoglobulin isotyping was performed using a commercial dipstick test (Roche) according to the manufacturer's instruction.

Production of recombinant S, N, M, and E proteins from codon-optimized DNA expression vector

The codon usage of published SARS-CoV S, N, M, and E gene sequences (Marra et al., 2003; Rota et al., 2003) was analyzed by the MacVector software (V. 7.2, Accelrys, San Diego, CA) against that of the *Homo sapiens* genome. The less optimal codons in these genes were changed to codons more preferred in mammalian systems to promote higher expressions of these structure proteins. These codon-optimized genes were chemically synthesized by Geneart (Regensburg, Germany) and individually subcloned into the DNA vaccine vector pSW3891 (Wang et al., 2004). The human tissue plasminogen activator (tPA) leader sequence has replaced the S natural leader in these constructs as reported (Wang et al., 2005).

Each SARS-CoV DNA plasmid transformed in *E. coli* (HB101 strain) was confirmed by DNA sequencing before

large amounts of DNA plasmids were prepared with a Mega purification kit (Qiagen, Valencia, CA). The recombinant proteins used in this study were produced from transiently transfected 293T cells. DNA constructs were first transfected into 293T cells using the calcium phosphate precipitation method as reported (Wang et al., 2005). Briefly, 2×10^6 293T cell of 50% confluence in a 60-mm dish were transfected with 10 μg of plasmid DNA and were harvested 72 h later for ELISA or Western blot use.

ELISA (enzyme-linked immunosorbent assay)

High-binding 96-well flat-bottom plates (Costar) were coated overnight at 4 °C with 100 μl of SARS-CoV antigens at 1 μg/ml. To enhance S antigen binding, the plates were first incubated with 100 μl of ConA (50 μg/ml) for 1 h at room temperature, and washed 5 times with PBS containing 0.1% Triton X-100. The plates were then blocked with 200 μl/well of blocking buffer (5% non-fat dry milk, 4% whey, 0.5% Tween 20 in PBS at pH 7.2) for 1 h. After five washings, 100 μl of serially diluted mAbs was added in duplicate wells and incubated for 1 h. After another set of washings, the plates were incubated for 1 h at room temperature with 100 μl of anti-mouse IgG conjugated with horseradish peroxidase (SouthernBiotech, Birmingham, AL) diluted at 1:1000 in Whey dilution buffer (4% Whey, 0.5% Tween 20 in PBS). After the final washing, the plates were developed with 3,3′,5,5′ Tetramethybenzidine solution at 100 μl/well (Sigma, St. Louis, MO) for 3.5 min. The reactions were stopped by adding 25 μl of 2 M H_2SO_4, and the plates were read at OD 450 nm. The quantities of mouse-purified mAbs were determined using standard of IgG and IgM (Southern Biotech) in ELISA assay.

Western blot analysis

Purified SARS-CoV virions and transiently expressed S antigens were first run by SDS–PAGE electrophoresis. The samples were heated at 90 °C for 5 min in sample buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and equal amounts of transiently expressed antigens were loaded per lane of the gel. The gels were electroblotted to PVDF membranes (Bio-Rad) using 80 mA for 2 h, then blocked overnight at 4 °C in blocking buffer (0.2% I-block, 0.1% Tween 20 in 1× PBS). Membranes were incubated with a 1:200 dilution of mAbs. After being washed, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:5000 dilution, and signals were detected using a chemiluminescence Western-Light Kit (Tropix, Bedford, MA).

In vitro neutralization assays

The SARS-CoV neutralization assays using neutral red staining were performed with triplicate testing wells in 96-well flat bottom plates in the BL-3 laboratory. Initially, 400 $TCID_{50}$ of virus in 50 μl/well was incubated with 50 μl of serially diluted rabbit sera or tissue culture medium for 1 h at 37 °C. After incubation, 100 μl of Vero E6 cells (20,000 cells) was added to each well at MOI of 0.02. On day 5 of infection when more than 70% of the cells formed CPE in viral control wells, the culture medium was removed from the testing wells and 100 μl of 10% neutral red in DMEM medium was added to each well. After incubation for 1 h at 37 °C, the neutral red medium was removed, the plates were washed twice with PBS (pH 7.2), and 100 μl of acid alcohol (1% acetic acid in 50% ethanol) was added to each well. After incubation for 30 min at room temperature, the absorbance was read at A_{540}. Percent of neutralization at a given serum dilution was determined by calculating the difference in absorption (A_{540}) between test wells (cells, serum sample, and virus) and virus control wells (cells and virus), and dividing this result by the difference in absorption between cell control wells (cells only) and virus control wells (Montefiori et al., 1988). In our assay system, sera were considered positive for neutralizing antibody activities when the titers are above 50% inhibition as compared with the virus controls.

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