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Receptor-Binding Domain of Severe Acute Respiratory Syndrome Coronavirus Spike Protein Contains Multiple Conformation-Dependent Epitopes that Induce Highly Potent Neutralizing Antibodies

Yuxian He,* Hong Lu,* Pamela Siddiqui,* Yusen Zhou,[†] and Shibo Jiang^{1*}

The spike (S) protein of severe acute respiratory syndrome associated coronavirus (SARS-CoV) is a major antigenic determinant capable of inducing protective immunity. Recently, a small fragment on the SARS-CoV S protein (residues 318–510) was characterized as a minimal receptor-binding domain (RBD), which mediates virus binding to angiotensin-converting enzyme 2, the functional receptor on susceptible cells. In this study, we demonstrated that a fusion protein containing RBD linked to human IgG1 Fc fragment (designated RBD-Fc) induced high titer of RBD-specific Abs in the immunized mice. The mouse antisera effectively neutralized infection by both SARS-CoV and SARS pseudovirus with mean 50% neutralization titers of 1/15,360 and 1/24,737, respectively. The neutralization determinants on the RBD of S protein were characterized by a panel of 27 mAbs isolated from the immunized mice. Six groups of conformation-dependent epitopes, designated as Conf I–VI, and two adjacent linear epitopes were identified by ELISA and binding competition assays. The Conf IV and Conf V mAbs significantly blocked RBD-Fc binding to angiotensin-converting enzyme 2, suggesting that their epitopes overlap with the receptor-binding sites in the S protein. Most of the mAbs (23 of 25) that recognized the conformational epitopes possessed potent neutralizing activities against SARS pseudovirus with 50% neutralizing dose ranging from 0.005 to 6.569 $\mu\text{g/ml}$. Therefore, the RBD of SARS S protein contains multiple conformational epitopes capable of inducing potent neutralizing Ab responses, and is an important target site for developing vaccines and immunotherapeutics. *The Journal of Immunology*, 2005, 174: 4908–4915.

Severe acute respiratory syndrome (SARS)² is a recently recognized febrile severe lower respiratory illness that is caused by infection with a novel coronavirus (SARS-CoV) (1–4). The global outbreak of SARS was contained, but concerns remain over the possibility of future recurrences, especially with recent reports of laboratory-acquired infections (5). However, no effective treatment or prophylaxis is currently available to fight against this deadly virus (5, 6). Similar to other coronaviruses, SARS-CoV is an enveloped virus containing a large, positive-stranded RNA genome that encodes viral replicase proteins and structural proteins including spike (S), membrane, envelope, nucleocapsid, and several uncharacterized proteins (4, 7, 8). Phylogenetic analyses indicate that SARS-CoV is distinct from the three known antigenic groups of coronaviruses. Therefore, postgenomic characterization of SARS-CoV is important for developing anti-SARS therapeutics and vaccines (9, 10).

Coronavirus infection is initiated by attachment of the S protein to the specific host receptor, which triggers a conformational change in the S protein. The S protein of SARS-CoV is a type I transmembrane glycoprotein with a predicted length of 1255 aa that contains a leader (residues 1–14), an ectodomain (residues 15–1190), a transmembrane domain (residues 1191–1227), and a short intracellular tail (residues 1227–1255) (4). Unlike many other coronaviruses, such as mouse hepatitis virus (MHV) (11, 12), in which the S protein is posttranslationally cleaved into S1 and S2 subunits, no typical cleavage motif has been identified in the SARS-CoV S protein (4). Nonetheless, its S1 and S2 domains were predicted by sequence alignment with other coronavirus S proteins (4, 13). The S2 domain (residues 681–1255) of SARS-CoV S protein containing a putative fusion peptide and two heptad repeat (HR1 and HR2) regions is responsible for fusion between viral and target cell membranes. We and others found that the HR1 and HR2 regions can associate to form a six-helix bundle structure (14–17) resembling the fusion-active core of the HIV gp41 (18) and the MHV S protein (19, 20). The S1 domain of SARS-CoV S protein mediates virus binding with angiotensin-converting enzyme 2 (ACE2), the functional receptor for SARS-CoV on susceptible cells (21–24). Recently, a 193-aa small fragment within S1 domain (residues 318–510) was identified as a minimal receptor-binding domain (RBD), which is sufficient to associate with ACE2 (25–27).

The S proteins of coronaviruses are also major antigenic determinants that induce neutralizing Abs (28, 29). Thus it is a rationale to use S protein as an Ag for vaccine development (29). Recently, it has been shown that the S protein of SARS-CoV is a major inducer of protective immunity among structural proteins (30). Yang et al. (31) reported that a DNA vaccine candidate encoding the S protein induced SARS-CoV-neutralizing Abs and protective

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² Abbreviations used in this paper: SARS, severe acute respiratory syndrome; SARS-CoV, SARS coronavirus; S, spike; RBD, receptor-binding domain; RBD-Fc, RBD linked to human IgG-Fc; ACE2, angiotensin-converting enzyme 2; MHV, mouse hepatitis virus; HR, heptad repeat; TMB, 3,3',5,5'-tetramethylbenzidine; CPE, cytopathic effect.

immunity in mice. They also demonstrated that the protection was mediated by neutralizing Abs but not a T cell-dependent mechanism. Bisht et al. (32) demonstrated that the S protein of SARS-CoV expressed by attenuated vaccinia virus (modified vaccinia Ankara) elicited protective immunity against SARS-CoV infection as shown by reduced titers of SARS-CoV in the respiratory tracts of mice after challenge. Bukreyev et al. (33) reported that mucosal immunization of African green monkeys with an attenuated parainfluenza virus (BHPV3) expressing the SARS-CoV S protein induced neutralizing Abs and protected animals from the challenge infection. These data indicate that the S protein of SARS-CoV is a protective Ag capable of inducing neutralizing Abs, although its antigenic determinants remain to be defined.

Most recently, we have demonstrated that a fusion protein containing the RBD of SARS-CoV S protein linked to a human IgG1 Fc fragment (RBD-Fc) can induce highly potent neutralizing Abs against SARS-CoV in the immunized rabbits, suggesting that the RBD has a potential to be developed as an effective and safe subunit anti-SARS vaccine (34). To identify the neutralization determinants on the SARS-CoV S protein, we used the RBD-Fc as an immunogen for immunization of mice to generate polyclonal and mAbs. We demonstrated that the RBD-Fc induced high titers of RBD-specific Abs in the immunized mice and the mouse antisera possessed highly potent neutralizing activity against SARS-CoV and SARS pseudovirus. We found that the RBD contains multiple conformation-dependent neutralization epitopes as shown by a panel of 27 mAbs isolated from mice immunized with RBD-Fc. These data indicate that the RBD of SARS-CoV S protein is a critical target for developing vaccines and therapeutics.

Materials and Methods

Expression of recombinant RBD-Fc and S1-C9 proteins

Plasmids encoding the fusion proteins RBD-Fc (RBD linked to the Fc domain of human IgG1) and S1-C9 (S1 protein tagged with C9 at the C terminus) were kindly provided by Dr. M. Farzan at the Harvard Medical School (Boston, MA) (22, 25). RBD-Fc and S1-C9 proteins were expressed in 293T cells transfected with the plasmids using Fugene 6 reagents (Boehringer Mannheim) according to the manufacturer's protocol. Supernatants were harvested 72 h posttransfection. RBD-Fc was purified by protein A-Sepharose 4 Fast Flow (Amersham Biosciences), and S1-C9 was purified by affinity chromatography with anti-C9 mAb 1D4 (National Cell Culture Center).

Immunization of mice and generation of mAbs

Five BALB/c mice (4 wk old) were s.c. immunized with 20 μ g of purified RBD-Fc resuspended in PBS (pH 7.2) in the presence of MLP+TDM Adjuvant System (Sigma-Aldrich) and boosted with 10 μ g of the same Ag plus the MLP+TDM adjuvant at 3-wk intervals. Preimmune sera were collected before starting the immunization and antisera were collected 4 days after each boost. Sera were kept at 4°C before use.

Hybridomas for producing anti-RBD mAbs were generated using standard protocol. Briefly, the splenocytes from the immunized mice were harvested and fused with SP2/0 myeloma cells. Cell culture supernatants from the wells containing hybridoma colonies were screened by ELISA using S1-C9 as a coating Ag. Cells from positive wells were expanded and retested. Cultures that remained positive were subcloned to generate stable hybridoma cell lines. All mAbs were purified from culture supernatants by protein A-Sepharose 4 Fast Flow (Amersham Biosciences). The isotypes of mAbs were determined with Mouse MonoAb-ID Kit (Zymed Laboratories).

ELISA and binding competition

Reactivity of mouse sera or mAbs with various Ags was determined by ELISA. Briefly, 1 μ g/ml recombinant proteins (RBD-Fc or S1-C9) or purified human IgG (Zymed Laboratories) were used, respectively, to coat 96-well microtiter plates (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 2% nonfat milk, serially diluted mouse sera or mAbs were added and incubated at 37°C for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Bound Abs were detected with HRP-conjugated goat anti-mouse IgG (Zymed Laboratories) at 37°C for 1 h, followed by washes. The reaction was visualized by ad-

dition of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and absorbance at 450 nm was measured by an ELISA plate reader (Tecan US).

To determine the effect of disulfide bond reduction on the binding of RBD-specific mAbs, an ELISA plate was coated with recombinant RBD-Fc or S1-C9 at a concentration of 1 μ g/ml and then treated for 1 h at 37°C with DTT at a concentration of 10 mM, followed by washes. Then the wells were treated with 50 mM iodoacetamide for 1 h at 37°C. After washes, a standard ELISA was performed as described above.

A competitive ELISA was performed to determine the inhibitory activity of the RBD-specific mAbs on binding of the biotinylated mAbs to RBD-Fc. Briefly, the wells of ELISA plates were coated with RBD-Fc at 1 μ g/ml as described above. A mixture containing 50 μ g/ml of an unlabeled mAb and 1 μ g/ml of a biotinylated mAb was added, followed by incubation at 37°C for 1 h. Binding of the biotinylated mAbs was detected after addition of HRP-conjugated streptavidin (Zymed Laboratories) and TMB sequentially. Biotinylation of mAbs was performed using the EZ-link NHS-PEO Solid Phase Biotinylation Kit (Pierce) according to the manufacturer's protocol.

Neutralization of SARS-CoV infection

Neutralization of SARS-CoV infection was assessed as previously described (16, 34). Briefly, Vero E6 cells were plated at 5×10^4 cells/well in 96-well tissue culture plates and grown overnight. One hundred 50% tissue-culture infectious dose of SARS-CoV BJ01 strain (GenBank accession no. AY278488) was mixed with an equal volume of diluted mouse sera and incubated at 37°C for 1 h. The mixture was added to monolayers of Vero E6 cells. Cytopathic effect (CPE) was recorded on day 3 postinfection. The neutralizing titers represented the dilutions of mouse antisera that completely prevented CPE in 50% of the wells as calculated by Reed-Muench method (35).

Neutralization of pseudovirus infection

The conventional neutralization assay using live SARS-CoV is cumbersome and has to be performed in biosafety level-3 facilities. Therefore, we adapted a SARS-CoV pseudovirus system (25, 31, 34, 36, 37) in our laboratory. This assay is sensitive and quantitative, and can be conducted in biosafety level-2 facilities. SARS pseudovirus bearing SARS-CoV S protein and a defective HIV-1 genome that expresses luciferase as reporter was prepared as previously described (25, 36, 37). In brief, 293T cells were cotransfected with a plasmid encoding codon-optimized SARS-CoV S protein and a plasmid encoding Env-defective, luciferase-expressing, HIV-1 genome (pNL4-3.luc.RE) using Fugene 6 reagents (Boehringer Mannheim). Supernatants containing SARS pseudovirus were harvested 48 h posttransfection and used for single-cycle infection of ACE2-transfected 293T (293T/ACE2) cells. Briefly, 293T/ACE2 cells were plated at 10^4 cells/well in 96-well tissue-culture plates and grown overnight. The supernatants containing pseudovirus were preincubated with 2-fold serially diluted mouse sera or mAbs at 37°C for 1 h before addition to cells. The culture was refed with fresh medium 24 h later and incubated for an additional 48 h. Cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar), followed by addition of luciferase substrate (Promega). Relative light units were determined immediately in the Ultra 384 luminometer (Tecan US).

Binding inhibition of RBD-Fc with receptor by mAbs

Inhibition of mAbs on RBD-Fc binding to ACE2-expressing cells was measured by flow cytometry. Briefly, 10^6 293T/ACE2 cells were detached, collected, and washed with HBSS (Sigma-Aldrich). RBD-Fc was added to the cells to a final concentration of 1 μ g/ml in the presence or absence of 50 μ g/ml mAbs, followed by incubation at room temperature for 30 min. Cells were washed with HBSS and incubated with anti-human IgG-FITC conjugate (Zymed Laboratories) at 1/50 dilution at room temperature for an additional 30 min. After washing, cells were fixed with 1% formaldehyde in PBS and analyzed in a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

Inhibition of RBD-Fc binding to soluble ACE2 by mAbs was measured by ELISA. Briefly, recombinant soluble ACE2 (R&D Systems) at 2 μ g/ml was coated onto 96-well ELISA plates (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 2% nonfat milk, 1 μ g/ml RBD-Fc was added to the wells in the presence or absence of 50 μ g/ml mouse mAbs and incubated at 37°C for 1 h. After washing, the HRP-conjugated goat anti-human IgG (Zymed Laboratories) was added and incubated an additional 1 h. After washing, the substrate TMB was used for detection.

Results

RBD-Fc effectively induced high titers of RBD-specific Abs in mice

RBD-Fc fusion protein was transiently expressed in 293T cells and purified to homogeneity by protein A. Five mice (A to E) were immunized four times with RBD-Fc in the presence of Ribi adjuvant. All animals developed appreciable Ab responses against RBD-Fc after the first boost, and their Ab titers increased with subsequent immunizations (Fig. 1A). The antisera collected 4 days after the third boost showed highest RBD-Fc binding activity and was used to measure Ab titers. As shown in Fig. 1B, the mouse antisera bound to RBD-Fc in a dose-dependent manner with a mean end-point titer of 1/1,262,500. Because RBD-Fc also contains a human IgG-Fc fragment, the Abs in the mouse sera would also bind to Fc, in addition to RBD. Therefore, we tested the binding activity of mouse antisera against S1-C9, which contains RBD but not Fc. As shown in Fig. 1C, mouse antisera bound to S1-C9

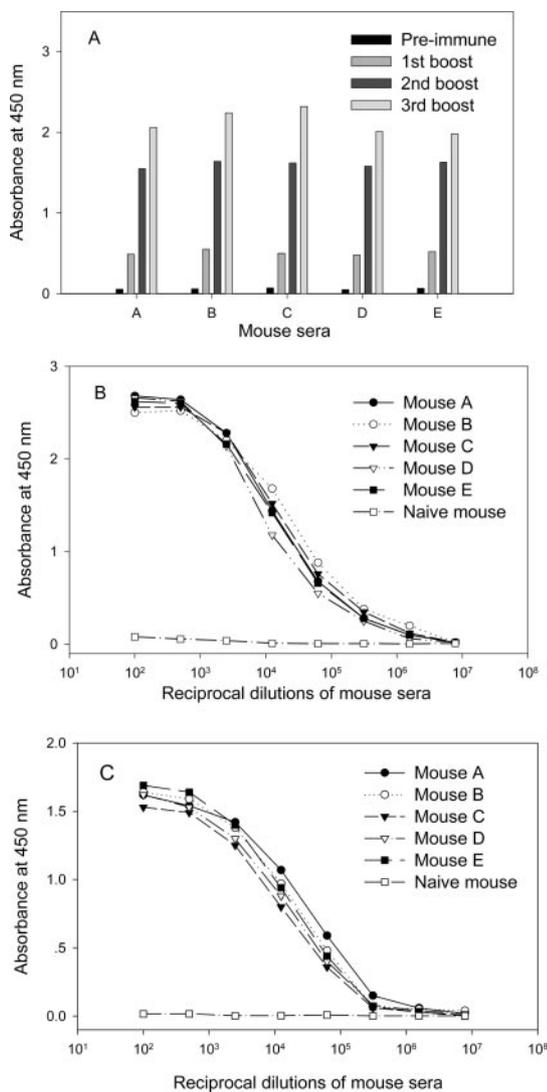


FIGURE 1. Ab responses of mice immunized with RBD-Fc. *A*, Reactivity of mouse sera with RBD-Fc. Sera were collected from mice before immunization and 4 days after each boost and tested by ELISA at 1/1000 dilution. *B*, Binding of mouse antisera to RBD-Fc. Antisera collected from the immunized mice 4 days after the third boost were tested at a series of 5-fold dilutions. *C*, Binding of mouse antisera to S1-C9. Antisera collected from the immunized mice 4 days after the third boost were tested at a series of 5-fold dilutions.

in a pattern similar to that shown in the experiments using RBD-Fc as an Ag, although the mean end-point titer (1/625,000) of the Abs against S1-C9 was ~2-fold lower than those to RBD-Fc. This suggests that RBD-Fc is capable of inducing high titers of RBD-specific Abs in the immunized mice.

Mouse antisera efficiently neutralized infection by SARS-CoV and SARS pseudovirus

A conventional neutralization assay (16) was used to measure the neutralizing activity of the mouse antisera that contain high titer of RBD-specific Abs against infection of SARS-CoV in Vero E6 cells. Strikingly, each of the mouse antisera at 1/10,240 dilutions fully protected Vero E6 cells from SARS-CoV infection (i.e., no CPE was seen and the cell monolayer remained intact). At higher serum dilutions, the cell number decreased due to the CPE induced by SARS-CoV replication in cells. All the antisera had 50% neutralizing Ab titer calculated based on Reed-Muench method (35) at 1/15,360. The preimmune mouse sera at a 1/40 dilution had no inhibitory activity on SARS-CoV infection. We also tested the neutralizing activity of the mouse antisera against SARS pseudovirus. As shown in Fig. 2, all of mouse antisera efficiently inhibited infections of 293T/ACE2 cells by SARS pseudovirus with 50% neutralizing Ab titers ranging from 1/18,505 to 1/34,574 (with a mean titer of 1/24,737), consistent with the results obtained from the conventional neutralizing assay using wild-type SARS-CoV.

Isolation and initial characterization of mAbs specific for RBD

To characterize neutralization determinants on the RBD of S protein, we generated a panel of 27 RBD-specific mAbs by fusing splenocytes from the RBD-Fc-immunized mice with Sp2/0 myeloma cells and then screening hybridomas using S1-C9 as an Ag. Epitope specificities of these mAbs were initially determined by ELISAs using RBD-Fc, DTT-reduced RBD-Fc, S1-C9, DTT-reduced S1-C9, and a purified human IgG as coating Ags (Table I). A majority of the mAbs (25 of 27) were reactive with native RBD-Fc and S1-C9, but not DTT-reduced RBD-Fc and S1-C9. This indicated that they were directed against disulfide bond-dependent conformational epitopes expressed on the RBD of S protein. Two other mAbs (4D5 and 17H9) recognized both native and reduced RBD-Fc and S1-C9, indicating that they were directed against linear epitopes presented on the RBD. None of the mAbs screened by S1-C9 reacted with human IgG, whereas control antiserum from a mouse immunized with RBD-Fc was reactive with human IgG (Table I).

Because the mAbs 4D5 and 17H9 could react with the reduced RBD-Fc and S1-C9, their epitopes might be mapped with synthetic peptides. A set of 27 overlapping peptides that cover the RBD of

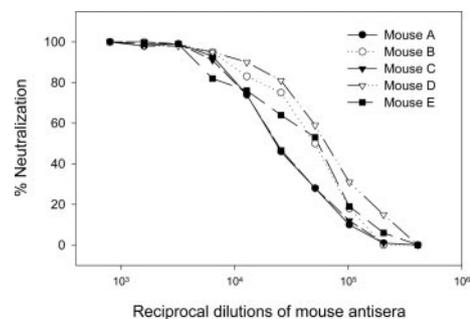


FIGURE 2. Neutralization of SARS pseudovirus infection by mouse antisera. Infection of 293T/ACE2 cells by SARS pseudovirus was determined in the presence of mouse antisera at a series of 2-fold dilutions, and percent neutralization was calculated for each sample.

Table I. Reactivities of RBD-specific mAbs against various Ags^a

mAbs	Isotype	Ag				
		RBD-Fc	Reduced RBD-Fc	S1-C9	Reduced S1-C9	Human IgG
4D5	IgG1/K	0.88	1.38	0.65	0.94	0.02
9F7	IgG1/K	1.60	0.00	1.45	0.08	0.04
10E7	IgG1/K	1.77	0.02	1.72	0.16	0.05
11E12	IgG2a/K	1.50	0.01	0.72	0.09	0.02
12B11	IgG1/K	1.37	0.04	0.78	0.00	-0.01
13B6	IgG1/K	1.58	-0.01	0.93	0.00	0.02
17H9	IgG1/K	1.72	1.71	1.21	1.15	0.07
18C2	IgG1/K	1.28	-0.01	0.80	0.01	-0.20
18D9	IgG1/K	1.47	-0.01	0.90	0.01	0.03
19B2	IgG1/K	1.63	0.00	1.55	0.12	0.01
20E7	IgG2a/K	1.50	0.00	0.98	0.01	0.02
24F4	IgG1/K	1.69	-0.01	1.08	0.08	0.04
24H8	IgG1/K	1.54	-0.01	0.94	0.12	0.01
26A4	IgG1/K	1.60	0.00	0.89	0.09	0.01
26E1	IgG1/K	1.91	0.07	1.85	0.06	0.01
27C1	IgG1/K	1.46	0.00	1.57	0.07	0.01
28D6	IgG1/K	2.06	0.01	1.60	0.16	0.00
29G2	IgG2a/K	1.69	0.00	0.96	0.17	0.04
30F9	IgG1/K	1.66	0.04	1.21	0.12	0.01
31H12	IgG1/K	1.72	0.08	1.91	0.22	0.03
32H5	IgG1/K	1.54	0.06	1.55	0.51	0.00
33G4	IgG2a/K	1.79	0.02	1.76	0.20	-0.01
34E10	IgG1/K	1.62	0.10	1.82	0.18	0.04
35B5	IgG1/K	1.74	0.06	1.72	0.25	0.02
38D4	IgG1/K	1.63	-0.01	1.20	0.07	0.00
44B5	IgG1/K	1.57	0.09	1.64	0.16	0.00
45F6	IgG1/K	1.61	0.11	1.43	0.15	-0.01
Antiserum		2.22	1.78	2.32	1.68	2.07
Naive serum		0.01	0.02	0.02	0.01	0.04

^a Ags were used at 1 μ g/ml; mAbs were tested at 10 μ g/ml and sera were tested at 1/100 dilution. Positive reactivities are highlighted in boldface.

S protein was used to localize 4D5 and 17H9 epitopes by ELISA. As shown in Fig. 3, 4D5 reacted with the peptide 435–451 (NYNYKYRYLRHGKLRPF), and 17H9 reacted with two overlapped peptides 442–458 (YLRHGKLRPFERDISNV) and 449–465 (RPFERDISNVPFSPDGK). Although the epitope of 17H9 was clearly mapped to the overlapped sequence (RPFERDISNV) of the peptides 442–458 and 449–465, the epitope for 4D5 requires most sequence of the peptide 435–451 which overlaps partial sequences of the peptides 442–458 and 449–465. Therefore, these two mAbs recognize neighboring linear epitopes that reside

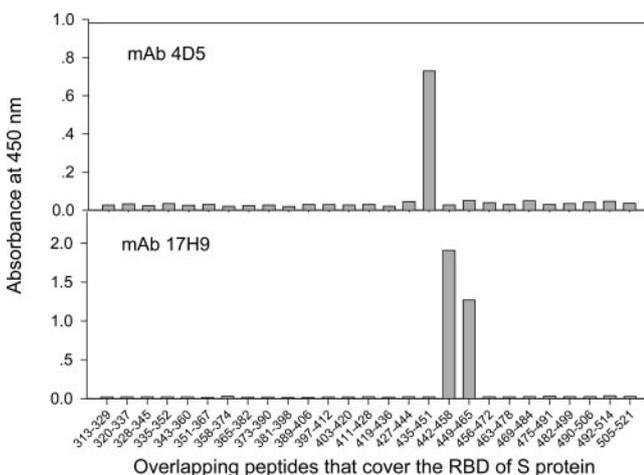


FIGURE 3. Epitope mapping of mAbs 4D5 and 17H9 by overlapping peptides that cover the RBD of S protein. Each of the peptides was coated at 5 μ g/ml, and mAbs were tested at 10 μ g/ml.

within the RBD. None of the conformation-dependent mAbs reacted with any of the tested peptides (data not shown).

Epitope specificity of the RBD-specific mAbs determined by binding competition assays

To characterize the conformation-dependent epitopes, the RBD-specific mAbs were grouped by binding competition assays (Table II). One of the mAbs (10E7) was first biotinylated and the inhibitory activity of the 27 mAbs on 10E7 binding to RBD-Fc was measured. The mAbs 4D5 and 17H9 recognizing linear epitopes mapped by peptides above were included in the competition assays as a control. About half of the conformation-dependent mAbs (13 of 25) competed with biotinylated 10E7, while other mAbs did not block 10E7 binding to RBD-Fc. Another four of the noncompeting mAbs (11E12, 33G4, 45B5, and 17H9) were subsequently biotinylated and similarly tested with the binding competition assay. Five of the 13 mAbs that compete with the biotinylated 10E7 also blocked the biotinylated 45B5 binding to RBD-Fc and were designated as a separate group. Thus, the 25 conformation-specific mAbs were divided into six distinct competition groups (designated as Conf I–VI). Two linear epitope-specific mAbs (4D5 and 17H9) did not compete with any of conformation-specific mAbs. These results suggest that the RBD of S protein contains multiple antigenic structures that induce specific Ab responses in the mice. However, the immunodominant epitopes in the RBD are conformation dependent.

Characterization of the mAbs that block receptor binding

RBD-Fc could efficiently bind to ACE2 expressed on 293T/ACE2 cells and to soluble ACE2 as measured by flow-cytometry and ELISA, respectively (data not shown). We tested whether the

Table II. Percent inhibition of RBD-specific mAbs on binding of biotinylated mAbs to RBD-Fc^a

Group	Competing mAb	Biotinylated mAb				
		10 E7	11 E12	33G4	45B5	17H9
Conf I	9F7	84.5	11.7	-13.3	22.3	16.4
	10E7	91.0	5.6	-12.9	21.0	9.9
	12B11	85.8	19.3	-0.2	19.8	21.0
	18C2	84.9	19.3	4.9	18.1	19.4
	24H8	93.7	24.0	7.0	25.6	22.1
	26E1	95.1	10.5	37.4	30.4	25.0
	29G2	96.6	20.4	1.6	11.4	23.5
	32H5	98.9	18.5	4.4	9.1	20.3
	20E7	97.2	38.5	5.9	73.0	24.6
Conf II	26A4	96.3	33.1	-0.5	60.0	19.0
	27C1	97.2	36.7	14.6	73.7	20.9
	31H12	97.5	18.7	7.1	58.4	19.7
	30E10	98.3	19.3	12.9	68.9	24.6
Conf III	11E12	12.6	92.0	0.3	-3.7	20.2
	18D9	-16.2	98.3	8.3	23.6	17.1
Conf IV	28D6	39.7	99.6	13.8	67.4	26.6
	30F9	28.7	100.0	8.7	64.0	32.4
	35B5	34.9	99.9	10.0	64.7	33.6
Conf V	24F4	11.5	-1.0	95.5	2.5	24.9
	33G4	9.5	-3.7	99.5	26.4	29.1
	38D4	8.1	-14.4	82.0	-5.1	15.8
Conf VI	13B6	23.3	10.7	-4.9	72.5	12.6
	19B2	2.9	-26.4	18.0	50.0	16.1
	44B5	25.3	-20.6	10.0	95.6	19.4
	45F6	25.7	-10.4	10.8	94.8	23.5
Linear	4D5	13.0	10.6	-11.1	1.0	-10.5
	17H9	17.8	33.3	-5.8	25.0	97.8

^a Competing mAbs were tested at 100 $\mu\text{g/ml}$ for the ability to block binding of the biotinylated mAbs to the RBD-Fc in ELISA. Greater than 40% inhibition was considered positive competition (values in bold). Negative numbers indicate increased binding of the biotinylated reagent.

RBD-specific mAbs inhibit binding of RBD-Fc to cell-associated or soluble ACE2. As shown in Fig. 4, all of the mAbs from Conf IV (28D6, 30F9, and 35B5) and Conf V (24F4, 33G4, and 38D4) completely blocked RBD-Fc binding to both cell-associated and

soluble ACE2 in a highly consistent manner. All the two Conf III mAbs (11E12 and 18D9) and two of the four Conf VI mAbs (19B2 and 45F6) partially inhibited RBD-Fc binding to ACE2 expressed on 293T/ACE2 cells and soluble ACE2. All of other mAbs, including two mAbs against linear sequences, had no significant inhibitory effects on receptor binding. These results indicate that the Conf IV and Conf V mAbs recognize epitopes that may overlap with the conformational receptor-binding sites in the S protein, although these mAbs did not compete against each other in the binding competition assays. Conf III mAbs and two Conf VI mAbs (19B2 and 45F6) may also bind to the conformational epitopes involved in the receptor-binding. All the Conf I and Conf II mAbs did not block the receptor binding, suggesting that they recognize the conformational epitopes that do not overlap the receptor-binding sites in RBD. These results highlight the epitopic heterogeneity of the RBD-specific mAbs and further indicate that the RBD of S protein contains multiple antigenic conformations.

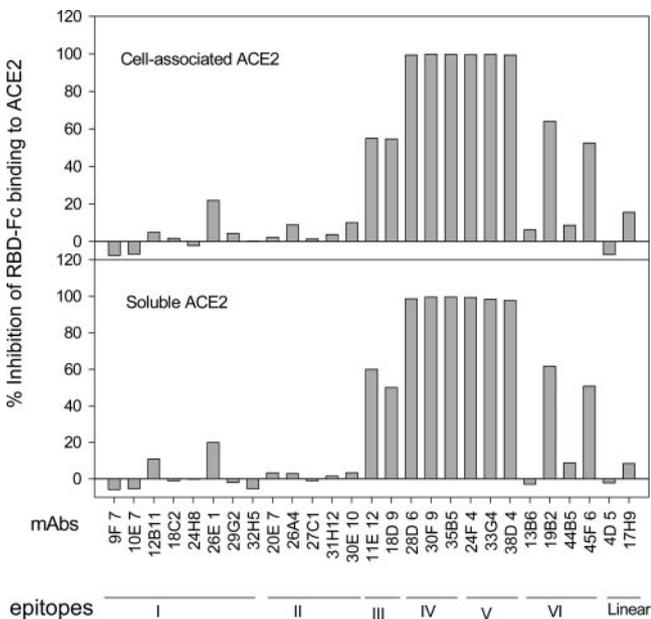


FIGURE 4. Inhibition of RBD-Fc binding to ACE2 by mAbs. *Upper panel*, Inhibition of RBD-Fc binding to cell-associated ACE2 expressed on 293T/ACE2 cells measured by flow cytometry. *Lower panel*, Inhibition of RBD-Fc binding to soluble ACE2 measure by ELISA. RBD-Fc was used at 1 $\mu\text{g/ml}$, and mAbs were used at 50 $\mu\text{g/ml}$. Percentage of inhibition was calculated for each mAb.

RBD-specific mAbs have potent neutralizing activity

Each of the RBD-specific mAbs was tested for neutralizing activity against SARS pseudovirus. Strikingly, the majority of the conformation-dependent mAbs (23 of 25) had potent neutralizing activity with 50% neutralization dose ranging from 0.005 to 6.569 $\mu\text{g/ml}$ (Table III), whereas the two mAbs directed against linear epitopes (4D5 and 17H9) and one mAb from Conf VI (44B5) at concentrations as high as 100 $\mu\text{g/ml}$ did not neutralize SARS pseudovirus infection. The mAbs 33G4 from Conf V and 30F9 from Conf IV that blocked the receptor binding had highest neutralizing activities against the pseudovirus. Interestingly, 45F6 from Conf VI had a relatively lower pseudovirus neutralizing activity, even though it partially blocked the binding of RBD-Fc with

Table III. Neutralization activity of RBD-specific mAbs against SARS pseudovirus

Group	mAb	Inhibition of ACE2 Binding ^a	ND ₅₀ (μg/ml)
Conf I	9F7	—	6.569
	10E7	—	1.673
	12B11	—	4.918
	18C2	—	5.031
	24H8	—	3.955
	26E1	—	0.354
	29G2	—	3.02
	32H5	—	0.275
Conf II	20E7	—	5.959
	26A4	—	2.815
	27C1	—	1.607
	31H12	—	0.139
Conf III	30E10	—	0.399
	11E12	+	1.39
Conf IV	18D9	+	0.02
	28D6	++	0.298
	30F9	++	0.009
Conf V	35B5	++	0.131
	24F4	++	0.052
	33G4	++	0.005
Conf VI	38D4	++	0.332
	13B6	—	1.436
	19B2	+	0.936
Linear	44B5	—	>100
	45F6	+	43.894
	4D5	—	>100
	17H9	—	>100

^a —, +, and ++ indicate no, partial, and complete inhibition, respectively.

ACE2. The dose-dependent neutralizing activity of several representative mAbs from each of groups was presented in Fig. 5. These results suggest that the RBD of S protein predominantly induces neutralizing Abs that direct against conformational epitopes.

Discussion

The S protein of coronavirus is not only responsible for receptor binding, membrane fusion and virus entry, but also a major antigenic determinant capable of inducing protective immunity. Therefore, it has been used as a major immunogen for development of coronavirus vaccines (28, 29). Recent studies have shown that the S protein of SARS-CoV is one of the major Ags eliciting immune responses during infection (38–40). Several candidate vaccines, such as DNA vaccine and vaccinia virus-based vaccines that express the full-length S protein induced SARS-CoV-specific neutralizing Abs and protective immunity against SARS-CoV challenge (31–33). These suggest that the S protein may serve as an immunogen for developing anti-SARS vaccines. The RBD of SARS-CoV is a key functional domain in the S protein responsible for virus binding to receptor on the target cells (25–27) and may contain neutralizing epitopes (41, 42). We have recently shown that the RBD of SARS-CoV S protein is a potent inducer of neutralizing Abs in the immunized rabbits (34). In the present study, the RBD-Fc was used as an immunogen to immunize mice. We found that the RBD-Fc could induce highly potent RBD-specific Ab responses in the immunized mice and the mouse antisera contain high titers (1/>15,000) of neutralizing Abs against both SARS-CoV and SARS pseudovirus. These data further confirm that the RBD of SARS-CoV S protein is an ideal immunogen to induce neutralizing Abs, thus having a potential to be developed as an effective subunit vaccine.

It seems difficult to understand why independently folded RBD, a 193-aa fragment, is actually able to elicit much higher titers of

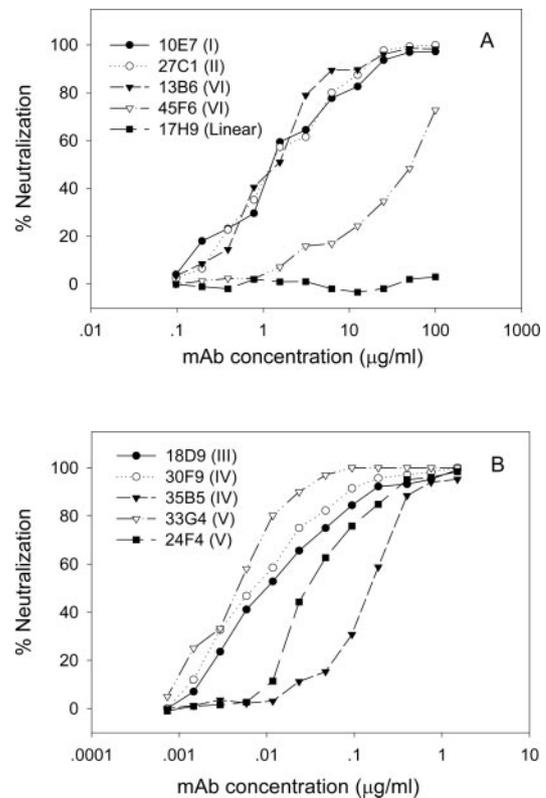


FIGURE 5. Neutralization of SARS pseudovirus by mAbs. Inhibition of SARS pseudovirus infection in 293T/ACE2 cells by representative mAbs from each group was shown. Each of the mAbs was tested at a series of 2-fold dilutions, and percentage of neutralization was calculated.

neutralizing Abs than the full-length S protein, because the latter contains more antigenic determinants and is expected to induce higher levels of neutralizing Abs than RBD alone. One of the possibilities is that the full-length S protein also contains a number of antigenic sites that induce Abs which do not neutralize, but even enhance virus infection, like those induced by antigenic sites on the envelope glycoproteins of HIV and Ebola virus (43–46). It was reported that a vaccinia virus-based vaccine encoding the full-length S protein of feline infectious peritonitis virus did not protect the immunized felines from feline infectious peritonitis virus challenge, but rather enhanced viral infection (47, 48). Recently, it has been shown that SARS-CoV infection of ferrets caused mild liver inflammation and the liver damage became much more serious if the ferrets were first given a vaccinia virus-based candidate vaccine before virus challenge (49). It is possible that the aggravation of liver impairment might be mediated by the enhancing Abs against the S protein. We have recently identified several nonneutralizing immunodominant epitopes located outside of the RBD on the SARS-CoV S protein (40). Another explanation is that the full-length S protein expressed by the DNA vaccines or recombinant viruses may be retained in the endoplasmic reticulum or Golgi after synthesis, thus limiting their ability to induce Ab responses. Therefore, a functional domain of the S protein that predominantly induces neutralizing Abs may serve as an ideal candidate for developing an effective and safe subunit vaccine. The RBD on the S protein of other coronaviruses, such as MHV, transmissible gastroenteritis virus (TGEV), and human coronavirus (HCoV-229E), has been shown to contain major antigenic determinants capable of inducing neutralizing Abs (42, 50, 51).

The neutralization determinants in the RBD were characterized with a panel of 27 mAbs isolated from the mice immunized with

RBD-Fc. Interestingly, most of the conformation-dependent mAbs had potent neutralizing activity and could be divided into six different groups (i.e., Conf I–VI) based on a binding competition experiment, suggesting that there are several distinct conformational epitopes on the RBD that can elicit neutralizing Abs. It is expected that all the neutralizing mAbs directed against the RBD can block the interaction between RBD and ACE2, the functional receptor for SARS-CoV. However, we found that only the mAbs recognizing the Conf IV and V could efficiently block RBD binding to ACE2. Some mAbs reacting with the Conf III and VI partially inhibited interaction between the RBD and ACE2. This suggests that their epitopes may overlap the receptor-binding sites on the RBD or binding of these mAbs to RBD may cause conformational change of the receptor binding sites, resulting in inhibition of RBD binding to ACE2. The mAbs that recognize the Conf I and II did not significantly affect the RBD binding with ACE2, but also possessed potent neutralizing activities, suggesting that these mAbs inhibit SARS-CoV infection without interfering in RBD-ACE2 interaction. The mechanism of action of these mAbs needs to be further investigated. These data indicate that the RBD induces neutralizing Abs specific not only for the receptor-binding sites, but also for other unique structural conformations, highlighting its antigenic heterogeneity, and suggest that the RBD of SARS-CoV S protein contains multiple conformational epitopes responsible for induction of potent neutralizing Ab responses.

The conformational sensitivity of the SARS-CoV-neutralizing mAbs described here is consistent with properties of neutralizing mAbs raised against other enveloped viruses, which generally require more native conformation for binding (52, 53). Although the RBD of SARS S protein is a 193-aa small fragment, it contains seven cysteines and five of which are essential for protein expression and ACE2 association (25). The disulfide bonds between these cysteines may form complex tertiary structures to constitute the multiple antigenic conformations. However, a neutralizing human mAb selected from a nonimmune human Ab library could react with the DTT-reduced S protein and block receptor association (41). Therefore, further characterization is needed to define the neutralization determinants on the RBD of SARS-CoV S protein, and this may provide critical information for developing anti-SARS therapeutics and vaccines. We are currently in process to generate a panel of mutant proteins for mapping the conformation-sensitive mAb epitopes and to crystallize the RBD in the presence or absence of neutralizing mAbs for visualizing the antigenic structures.

It was reported that passive transfer of mouse immune sera reduced pulmonary viral replication in the mice challenged with SARS-CoV (32, 54), and prophylactic administration of neutralizing mAbs conferred *in vivo* protection in the mice or in the ferrets (55, 56), suggesting that passive immunization with anti-SARS Abs is a viable strategy to control SARS. Thus, mAbs with high levels of SARS-CoV-neutralizing activity may be used for early treatment of SARS-CoV infection. However, application of murine mAbs in human will be limited due to human-anti-mouse Ab responses (57–59). If only a few doses of murine mAbs are used in a short period of time (1–2 wk) at the early stage of SARS-CoV infection may not cause serious human-anti-mouse Ab, but this urgent treatment may save lives of SARS patients. We have used similar strategies for early treatment of Hantaan virus infection using murine anti-Hantaan virus mAbs (60). Furthermore, the murine neutralizing mAbs can be humanized as therapeutics or immunoprophylaxis for providing immediate protection against SARS-CoV infection to those at-risk populations.

The significance of the present study is 3-fold. First, it indicates that the RBD in the SARS-CoV S protein S1 region can induce

high titers of neutralizing Abs and may be developed as a potent and safe subunit SARS vaccine for SARS prevention. Second, a number of highly potent RBD-specific neutralizing mAbs have been generated, which may be developed as immunotherapeutics for SARS treatment and as probes for studying the immunogenicity and antigenicity of SARS-CoV S protein. Third, we have demonstrated that the RBD of S protein contains multiple conformation-dependent neutralization epitopes, providing important information for understanding the structure and function of the SARS-CoV S protein.

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Disclosures

The authors have no financial conflict of interest.

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