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Antigenic and Immunogenic Characterization of Recombinant Baculovirus-Expressed Severe Acute Respiratory Syndrome Coronavirus Spike Protein: Implication for Vaccine Design

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The spike (S) glycoprotein of severe acute respiratory syndrome coronavirus (SARS-CoV) mediates the receptor interaction and immune recognition and is considered a major target for vaccine design. However, its antigenic and immunogenic properties remain to be elucidated. In this study, we immunized mice with full-length S protein (FL-S) or its extracellular domain (EC-S) expressed by recombinant baculoviruses in insect cells. We found that the immunized mice developed high titers of anti-S antibodies with potent neutralizing activities against SARS pseudoviruses constructed with the S proteins of Tor2, GD03T13, and SZ3, the representative strains of 2002 to 2003 and 2003 to 2004 human SARS-CoV and palm civet SARS-CoV, respectively. These data suggest that the recombinant baculovirus-expressed S protein vaccines possess excellent immunogenicity, thereby inducing highly potent neutralizing responses against human and animal SARS-CoV variants. The antigenic structure of the S protein was characterized by a panel of 38 monoclonal antibodies (MAbs) isolated from the immunized mice. The epitopes of most anti-S MAbs (32 of 38) were localized within the S1 domain, and those of the remaining 6 MAbs were mapped to the S2 domain. Among the anti-S1 MAbs, 17 MAbs targeted the N-terminal region (amino acids [aa] 12 to 327), 9 MAbs recognized the receptor-binding domain (RBD; aa 318 to 510), and 6 MAbs reacted with the C-terminal region of S1 domain that contains the major immunodominant site (aa 528 to 635). Strikingly, all of the RBD-specific MAbs had potent neutralizing activity, 6 of which efficiently blocked the receptor binding, confirming that the RBD contains the main neutralizing epitopes and that blockage of the receptor association is the major mechanism of SARS-CoV neutralization. Five MAbs specific for the S1 N-terminal region exhibited moderate neutralizing activity, but none of the MAbs reacting with the S2 domain and the major immunodominant site in S1 showed neutralizing activity. All of the neutralizing MAbs recognize conformational epitopes. These data provide important information for understanding the antigenicity and immunogenicity of S protein and for designing SARS vaccines. This panel of anti-S MAbs can be used as tools for studying the structure and function of the SARS-CoV S protein.

The sudden appearance of highly contagious severe acute respiratory syndrome coronavirus (SARS-CoV) caused a global epidemic in 2002 and 2003 (11, 29, 37, 41, 43), which resulted in more than 8,000 cases, with a fatality rate of about 10%. After the outbreak was contained, several isolated infections were reported during 2003 to 2004 in the Guangzhou region of China, which caused much less severe symptoms (35, 40, 44). The viruses isolated during the mild 2003 to 2004 outbreak, e.g., GD03T13 (GD03) and GZ03-02, are genetically closer to palm civet SARS-CoV (e.g., SZ3 and SZ16) than those predominating in the 2002 to 2003 outbreak, e.g., Tor2 and Urbani. The civets (*Paguma larvata*) may play a role in both the major (2002 to 2003) and the minor (2003 to 2004) SARS outbreaks and were initially considered an animal reservoir for SARS-CoV (8, 14, 44). However, recent studies suggest that the bats are the natural reservoir for the origin of SARS-CoV and that the civets may have served as intermediate amplification hosts that enable SARS-CoV interspecies transmission (30, 33). Therefore, SARS-CoV may reemerge from the animal reservoirs after its adaptation to humans. In preparedness, the need to develop an effective prophylactic vaccine remains of high importance.

Similar to other coronaviruses, the spike (S) protein of SARS-CoV is a large type I transmembrane glycoprotein with multiple biological functions (13, 23, 24). The predicted S1 subunit corresponding to the region of amino acids (aa) 13 to 680 contains the minimal receptor-binding domain (RBD) and mediates binding of the S protein to angiotensin-converting enzyme 2 (ACE2), a functional receptor on susceptible cells (1, 10, 32, 42, 50). The predicted S2 subunit (aa 681 to 1255) contains two heptad repeat regions (HR1 and HR2) and is responsible for fusion between viral and cellular membranes (3, 25, 36, 47). A second major feature of coronavirus S protein is its capacity to induce neutralizing antibodies and protective immunity, and it is thereby considered a major target for vaccine development. Several live virus and DNA vaccines expressing the S protein have been tested in preclinical studies (2, 4–6, 51). However, recent reports have raised some serious concerns over the full-length S protein-based immunogen, since it may induce harmful immune or inflammatory responses (12, 38, 52). For example, a DNA vaccine that expresses the full-length S protein (Urbani), the first vaccine approved for clinical trial in the United States, induced antibodies that neutralized the 2002 to 2003 human SARS-CoV

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strains but could not effectively neutralize the 2003 to 2004 human SARS-CoV isolates (28, 51, 52). Unexpectedly, these antibodies even enhanced infection mediated by the S proteins of palm civet SARS-CoV variants (52). It was also reported that a recombinant vaccinia virus-expressed full-length S protein caused serious liver damage in challenged vaccinated ferrets (9, 49). Therefore, structural and functional characterization of the SARS-CoV S protein remains a priority.

In the present study, the antigenicity and immunogenicity of recombinant baculovirus-expressed S proteins have been finely characterized. Our findings have significant impact for designing a safe and effective SARS vaccine.

MATERIALS AND METHODS

Recombinant S proteins and synthetic peptides. The full-length S protein (FL-S) and its extracellular domain (EC-S) truncated at amino acid 1190 of SARS-CoV Urbani (GenBank accession number AY278741) were expressed in expresSF$^+$ insect cells with recombinant baculovirus D3252 by the Protein Sciences Corporation (Bridgeport, CT). The plasmids encoding truncated S fragments corresponding to the S1 subunit (aa 12 to 672), the N-terminal domain (aa 12 to 327), and the receptor-binding domain (aa 318 to 510) of the 2002 to 2003 human SARS-CoV Tor2 (accession no. AY274119), fused with the Fc portion of human immunoglobulin G1 (IgG1) (designated S1-Fc, NT-Fc, and RBD-Fc, respectively), were kindly provided by M. Farzan at the Harvard Medical School (Boston, MA) (50). Each of three recombinant S fusion proteins was expressed in 293T cells transfected with the plasmid using Fugene 6 reagents (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol and purified by protein A-Sepharose 4 fast flow (Amersham Biosciences, Piscataway, NJ).

A set of 168 peptides spanning the entire sequence of the S protein of SARS-CoV strain Tor2 (each peptide contains 17 residues, with 9 residues overlapping with the adjacent peptides) were synthesized at Gene Gateway, LLC (Hayward, CA), as previously described (20). A panel of 14 longer peptides that overlap with the S1 subunit (aa 19 to 48, aa 278 to 312, aa 378 to 419, aa 511 to 552, aa 536 to 566, and aa 603 to 634) or S2 subunit (aa 823 to 856, aa 892 to 931, aa 920 to 953, aa 975 to 1010, aa 981 to 1014, aa 1149 to 1186, aa 1152 to 1188, and aa 1162 to 1198), with lengths ranging from 30 to 40 amino acids, were synthesized at CytoMol Corp. (Mountain View, CA). A standard solid-phase 9-fluorenylmethoxy carbonyl method was used for peptide synthesis. Peptides were purified to homogeneity (purity, >90%) by high-performance liquid chromatography and identified by laser desorption mass spectrometry.

Immunization of mice and generation of anti-S monoclonal antibodies (MAbs). Four BALB/c mice (6 weeks old) per group were subcutaneously immunized with 20 μg of FL-S or EC-S proteins resuspended in phosphate-buffered saline (PBS) plus MLP+TDM adjuvant (Sigma, Saint Louis, MO) and boosted with 10 μg of the same antigen plus the MLP+TDM adjuvant at 3-week intervals. Preimmune sera were collected before starting the immunization, and antisera were collected 7 days after each boost. Sera were kept at 4°C before use.

Hybridomas producing anti-S MAbs were generated using standard protocols, as previously described (17). Briefly, the splenocytes from the FL-S-immunized mice were harvested and fused with SP2/0 myeloma cells. Cell culture supernatants from the wells containing hybridoma colonies were screened by enzyme-linked immunosorbent assay (ELISA) using the FL-S as a coating antigen. 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 a mouse monoclonal antibody isotyping kit (Amersham Biosciences).

ELISA and binding competition. The reactivity of mouse sera or MAbs with various antigens was determined by ELISA. Briefly, 0.5 μg/ml recombinant protein was used to coat 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After the plates were blocked with 2% nonfat milk, serially diluted mouse sera or MAbs were added and incubated at 37°C for 1 h, followed by three washes with PBS containing 0.1% Tween 20. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories) at 37°C for 1 h, followed by washes. The reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine, and absorbance at 450 nm was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC). To determine the effect of disulfide bond reduction on the binding of anti-S MAbs, an ELISA plate was coated with recombinant FL-S or EC-S at a concentration of 0.5 μg/ml and then treated for 1 h at 37°C with dithiothreitol 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 anti-S MAb on binding of the biotinylated MAbs to truncated S proteins. Briefly, the wells of ELISA plates were coated with NT-Fc or RBD-Fc as described above. A mixture containing 50 μg/ml of an unlabeled MAb and 1 μg/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 horseradish peroxidase-conjugated streptavidin (Zymed Laboratories) and 3,3′,5,5′-tetramethylbenzidine sequentially. Biotinylation of MAbs was performed using the EZ-link NHS-PEO solid-phase biotinylation kit (Pierce, Rockford, IL) according to the manufacturer's protocol.

Inhibition of RBD-Fc binding to the receptor ACE2 by MAbs. The inhibition of anti-S MAbs on RBD-Fc binding to ACE2-expressing cells was measured by flow cytometry as previously described (18, 21). Briefly, 10^6 293T/ACE2 cells were detached, collected, and washed with Hanks' balanced salt solution (Sigma, St. Louis, MO). RBD-Fc was added to the cells to a final concentration of 1 μg/ml in the presence or absence of 50-μg/ml MAbs, followed by incubation at room temperature for 30 min. Cells were washed with Hanks' balanced salt solution and incubated with anti-human IgG-fluorescein isothiocyanate conjugate (Zymed laboratories) at a 1:50 dilution at room temperature for an additional 30 min. After being washed, cells were fixed with 1% formaldehyde in PBS and analyzed on a FACSCanto flow cytometer (BD Biosciences, Mountain View, CA) using FACSDiva software.

Neutralization of SARS pseudoviruses. Plasmids encoding the S proteins corresponding to the SARS-CoV Tor2, GD03, or SZ3 isolate were provided by M. Farzan at Harvard Medical School (34). The SARS-CoV pseudovirus system was developed in our laboratory as previously described (18, 39). In brief, HEK293T cells were cotransfected with a plasmid encoding the S protein and a plasmid encoding the Env-defective, luciferase-expressing human immunodeficiency virus type 1 genome (pNL4-3.luc.RE) by using Fugene 6 reagents (Boehringer Mannheim). Supernatants containing SARS pseudoviruses were harvested 48 h posttransfection. The infectivity of each SARS pseudovirus was titrated and adjusted to an equivalent luciferase unit (~10,000 relative light units [RLU]) and used for single-cycle infection of human or civet 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 serially diluted antisera 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, Madison, WI). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). RLU were determined immediately in the Ultra 384 luminometer (Tecan US).

RESULTS

Recombinant S proteins induced high titers of antibodies that potently neutralized human and palm civet SARS-CoV variants. To evaluate the immunogenicity of FL-S and EC-S, which were expressed in expresSF$^+$ insect cells by recombinant baculoviruses as protein vaccines, four mice were immunized four times with the FL-S or EC-S in the presence of MLP+TDM adjuvant. As shown in Fig. 1A and C, both groups of mice developed significant antibody responses against the corresponding immunogen after the first boosting immunization, and their serum reactivity apparently increased with subsequent boosts. The reactive titers of mouse antisera collected after the third boost against the FL-S or EC-S were measured by ELISA. As shown in Fig. 1B and D, the mouse antisera bound to the FL-S or EC-S in a dose-dependent manner with a mean end point titer of 1/218,700 or 1/256,000. This result suggests that recombinant baculovirus-expressed FL-S and its EC-S are highly immunogenic in mice.

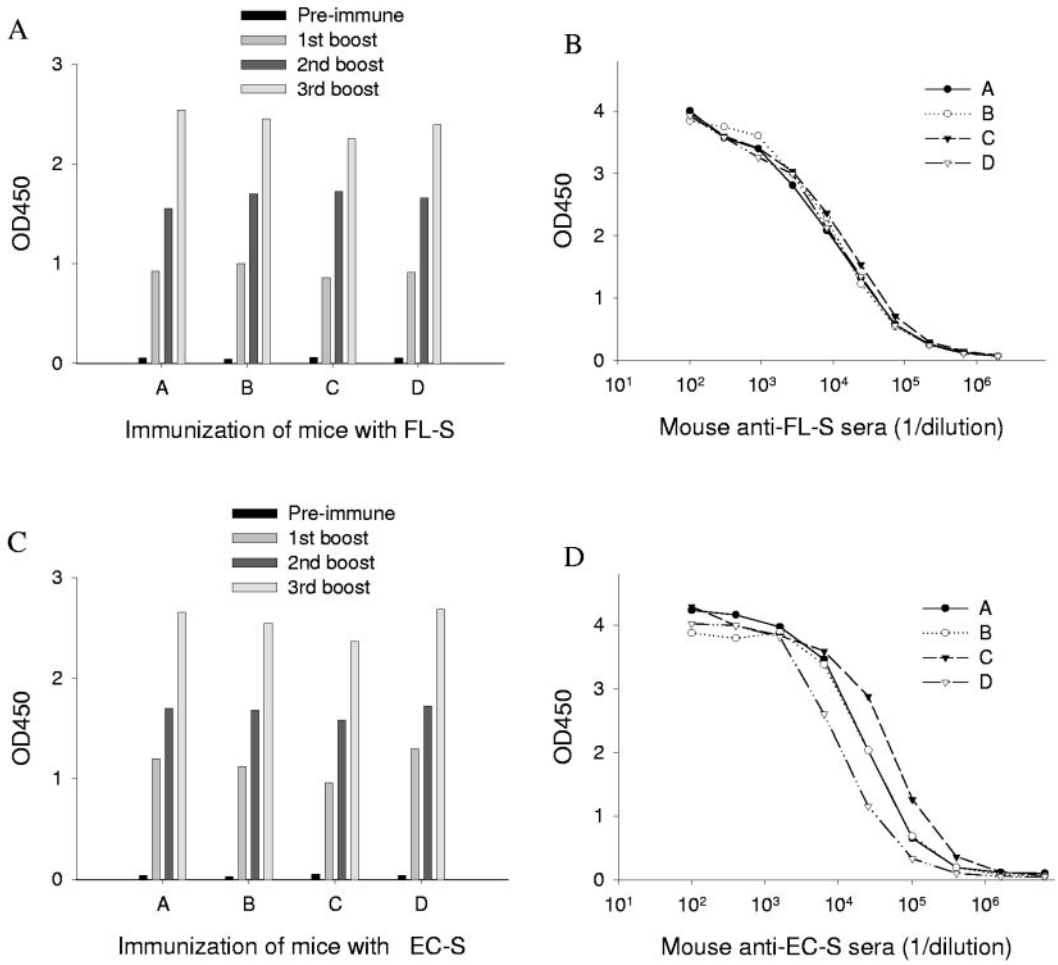

FIG. 1. Induction of anti-S antibodies in mice immunized with S proteins. (A) Antibody responses of mice immunized with FL-S. Sera were tested by ELISA at a 1/100 dilution. (B) Titers of mouse anti-FL-S sera, collected after the third boost, binding to the FL-S. (C) Antibody responses of mice immunized with EC-S. Sera were tested by ELISA at a 1/100 dilution. (D) Titers of mouse anti-EC-S sera, collected after the third boost, binding to the EC-S. OD450, optical density at 450 nm.

We then tested whether the mouse antisera induced by the FL-S and EC-S had neutralizing activities against SARS pseudoviruses, a key aspect for vaccine development. We previously developed a SARS pseudovirus system with the S sequence of 2002 to 2003 human SARS-CoV strain Tor2, which effectively infects 293T cells expressing human ACE2 (18). As shown in Fig. 2, both groups of mouse antisera potently neutralized Tor2 pseudoviruses, with mean 50% neutralizing titers (NT_{50}) at 1/59,291 and 1/44,362, respectively. It was important to know whether these two protein vaccines were able to elicit antibodies that had broad neutralizing activity, considering that the full-length S protein encoded by a DNA vaccine could not induce antibodies capable of neutralizing heterologous isolates (52). Using a similar approach, we therefore prepared the pseudoviruses expressing the S proteins of 2003 to 2004 human SARS-CoV isolate GD03 and civet SARS-CoV isolate SZ3. As previously described (34), the GD03 and SZ3 pseudoviruses infect target cells bearing human ACE2 and civet ACE2, respectively. Each of these two pseudoviruses was incubated with serially diluted mouse anti-S antisera to evaluate their sensitivity to antibody-mediated neutralization. Markedly, both mouse anti-FL-S and anti-EC-S serum samples could potently neutralize GD03 (mean NT_{50}, 1/60,931 and 1/55,992, respectively) and SZ3 (mean NT_{50}, 1/29,418 and 1/24,591, respectively) pseudoviruses (Fig. 2). In contrast, none of the preimmune sera had neutralizing activity against any of the pseudoviruses. Therefore, both FL-S and EC-S can induce potent cross-reactive neutralizing antibodies against human and civet SARS-CoV variants.

Isolation of anti-S MAbs and localization of their epitopes with truncated S proteins. To characterize the antigenic properties of S protein, we generated a panel of 38 anti-S MAbs by fusing splenocytes from the FL-S-immunized mice with Sp2/0 myeloma cells and then screening hybridomas using the FL-S as an antigen (Table 1). All of the MAbs reacted with both FL-S and EC-S, suggesting that they target the extracellular domain of S protein. Of them, 21 anti-S MAbs were reactive with native FL-S and EC-S but not with dithiothreitol-reduced FL-S and EC-S, suggesting that they were directed against putative disulfide bond-dependent conformational epitopes expressed on the S protein (designated Conf). Thirteen of the anti-S MAbs were directed against putative linear epitopes,

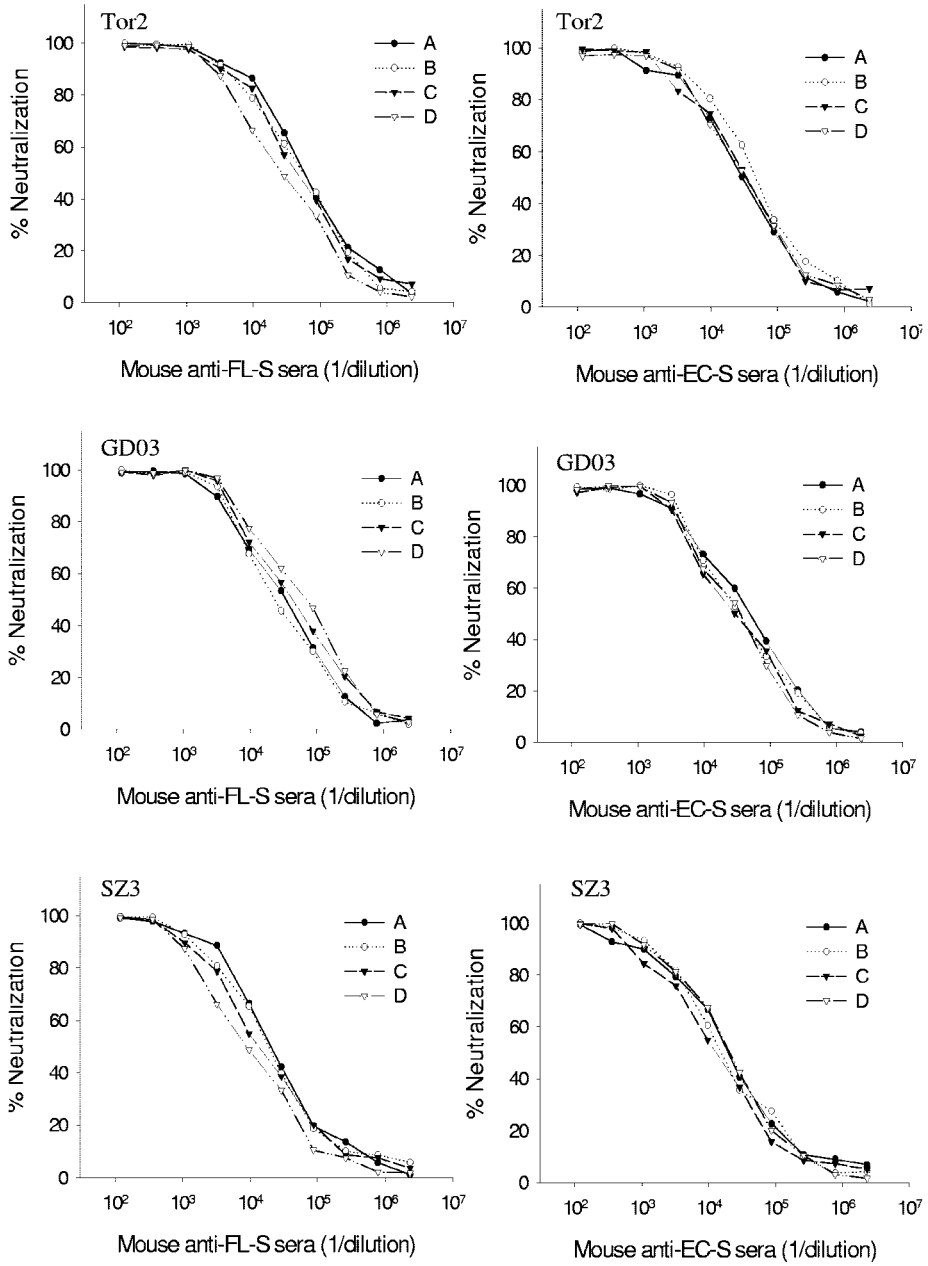

FIG. 2. Potent neutralization of SARS pseudovirus infection by mouse anti-S antibodies. The infectivity of each SARS pseudovirus was assessed by luciferase reporter gene expression (RLU) and adjusted to an equivalent titer for use. Infection of HEK293 cells expressing human ACE2 or palm civet ACE2 by SARS pseudoviruses (Tor2, GD03, or SZ3) was determined in the presence of mouse anti-FL-S or anti-EC-S sera in a series of threefold dilutions. Percent neutralization was calculated by the reduction in luciferase activity relative to values achieved in the absence of sera for each sample, and the average values were plotted.

since they recognized both native and reduced FL-S and EC-S. The remaining 4 anti-S MAbs (109D7, 120B11, 121E9, and 102B11) bound strongly to the native and weakly to the reduced S proteins, suggesting that they can react with both conformational and linear epitopes (designated Conf/linear), which may be dependent on the disulfide bonds. All of the MAbs also significantly reacted with the purified SARS-CoV lysate, as measured by ELISA, and with the S protein expressed on the transfected 293T cells, as measured by flow cytometry (data not shown), implying that these anti-S MAbs recognize the epitopes presented on the native S protein. Therefore, the recombinant baculovirus-expressed S proteins may maintain their native antigenic structures to induce antibody responses in vaccinated hosts.

Seventeen anti-S MAbs reacted with the truncated protein NT-Fc (Table 2), suggesting that their epitopes locate in the N-terminal region of the S1 subunit (S1-NT, aa 12 to 327). Of them, 11 MAbs recognized the conformation-dependent epitopes, 3 MAbs (114E3, 115H2, and 116F8) recognized linear epitopes, and 3 MAbs targeted the Conf/linear epitopes.

TABLE 1. Reactivity of anti-S MAbs with S proteins in ELISA

MAb	OD_{450} of antigen[a]				Epitope
	FL-S	Reduced FL-S	EC-S	Reduced EC-S	
101E7	**2.44**	0.08	**2.58**	0.01	Conf
101F10	**4.51**	**3.91**	**4.51**	**4.51**	Linear
102A12	**4.56**	0.04	**4.51**	0.15	Conf
102B11	**2.64**	**0.45**	**1.83**	**0.72**	Conf/linear
102D7	**2.75**	**2.38**	**2.00**	**2.06**	Linear
103D1	**3.66**	0.01	**4.13**	0.00	Conf
103D3	**4.52**	0.03	**4.52**	0.22	Conf
103F2	**4.67**	**2.72**	**5.21**	**4.51**	Linear
104D4	**3.73**	**3.66**	**4.51**	**4.52**	Linear
104F10	**2.86**	0.00	**2.92**	0.00	Conf
106B3	**2.98**	0.04	**4.19**	0.05	Conf
106C5	**3.06**	0.03	**4.52**	0.07	Conf
106C7	**2.87**	0.01	**3.66**	0.03	Conf
106H2	**3.67**	0.00	**4.51**	0.01	Conf
108G2	**3.04**	**1.92**	**2.83**	**2.08**	Linear
109B4	**2.99**	0.00	**3.35**	0.01	Conf
109D7	**3.16**	**0.66**	**3.38**	**0.56**	Conf/linear
109G7	**3.26**	0.02	**4.51**	0.02	Conf
111E3	**2.99**	0.04	**4.13**	0.09	Conf
111A7	**3.63**	**4.52**	**4.52**	**4.52**	Linear
112B7	**3.69**	0.00	**4.02**	0.00	Conf
112D5	**3.43**	0.05	**5.51**	0.05	Conf
112F9	**2.66**	0.01	**2.66**	0.01	Conf
112H6	**3.39**	0.05	**4.51**	0.05	Conf
113G3	**3.22**	0.01	**3.56**	0.01	Conf
114E3	**2.98**	**1.58**	**2.88**	**1.83**	Linear
114F7	**1.71**	**1.32**	**2.43**	**1.86**	Linear
114G5	**2.03**	**2.12**	**2.65**	**3.32**	Linear
115H2	**3.54**	**1.26**	**3.25**	**1.15**	Linear
116F8	**3.85**	**2.51**	**4.44**	**3.06**	Linear
117F7	**2.70**	0.07	**2.93**	0.01	Conf
119F6	**3.71**	**3.77**	**3.36**	**3.52**	Linear
119F8	**2.72**	0.00	**2.96**	0.00	Conf
120B11	**3.34**	**0.57**	**3.30**	**0.47**	Conf/linear
120D9	**2.94**	0.07	**2.35**	0.02	Conf
121E9	**3.19**	**0.85**	**2.68**	**0.60**	Conf/linear
121B8	**4.81**	**3.94**	**4.51**	**4.51**	Linear
121D7	**3.19**	0.01	**4.67**	0.00	Conf

[a] ELISA plates were coated with antigens at 0.5 μg/ml, and MAbs were tested at 10 μg/ml. An optical density at 450 nm (OD_{450}) of >0.2 was considered a positive reaction and is highlighted in boldface type.

TABLE 2. Localization of epitopes for anti-S MAbs with truncated S proteins by ELISA

Epitope	MAb	OD_{450} of truncated S protein[a]		
		S1-Fc	NT-Fc	RBD-Fc
S1-NT linear	114E3	**0.65**	**3.67**	0.00
	115H2	**0.55**	**3.75**	0.01
	116F8	**1.40**	**3.48**	0.01
S1-NT Conf	101E7	0.11	**2.07**	0.02
	103D1	0.01	**2.78**	0.01
	104F10	0.01	**1.11**	0.01
	106B3	0.06	**2.86**	0.05
	109B4	0.00	**2.10**	0.01
	111E3	0.17	**2.67**	0.01
	112D5	0.05	**2.95**	0.02
	112F9	0.04	**3.39**	0.04
	112H6	0.03	**2.43**	0.05
	113G3	0.01	**2.22**	0.01
	120D9	0.08	**3.44**	0.01
S1-NT Conf/linear	109D7	**0.48**	**3.51**	0.01
	120B11	**0.39**	**3.44**	0.01
	121E9	**0.28**	**3.38**	0.02
S1-RBD Conf	102A12	**3.25**	0.01	**3.23**
	103D3	**3.54**	0.00	**3.74**
	106C5	**2.84**	0.01	**3.32**
	106C7	**1.77**	0.00	**1.93**
	106H2	**1.38**	0.00	**3.16**
	109G7	**1.94**	0.03	**3.45**
	112B7	**1.22**	0.00	**2.91**
	119F8	**0.74**	0.02	**1.67**
	121D7	**1.49**	0.00	**2.89**
S1-CT linear	101F10	**1.99**	0.00	0.09
	103F2	**1.58**	0.03	0.03
	104D4	**2.06**	0.00	0.01
	111A7	**2.24**	0.00	0.01
	114G5	**0.30**	0.00	0.01
	121B8	**1.89**	0.01	0.01
S2 linear	114F7	0.00	0.00	0.02
	102D7	0.01	0.00	0.01
	119F6	0.02	0.00	0.00
	108G2	0.02	0.00	0.01
S2 Conf	117F7	0.16	0.01	0.02
S2 Conf/linear	102B11	0.00	0.01	0.01

[a] ELISA plates were coated with antigens at 0.5 μg/ml, and MAbs were tested at 10 μg/ml. An optical density at 450 nm (OD_{450}) of >0.2 was considered a positive reaction and is highlighted in boldface type.

Nine anti-S MAbs reacted with both S1-Fc and RBD-Fc, indicating that their epitopes are presented in the RBD (aa 318 to 510) in the S1 subunit. Interestingly, all of these were directed against the conformation-dependent epitopes. The epitopes of 6 anti-S MAbs that recognized the reduced S proteins might locate in the C-terminal region of the S1 subunit (S1-CT, aa 511 to 672) since they reacted with S1-Fc but not with S1-NT-Fc or S1-RBD-Fc. The other 6 anti-S MAbs could not react with any of the truncated S1 proteins (S1-Fc, S1-NT-Fc, and S1-RBD-Fc), suggesting that they might target the S2 subunit. Therefore, the epitope specificity of all 38 anti-S MAbs could be initially determined with these truncated S proteins. Noticeably, S1-Fc, a molecule previously used for the identification of the SARS-CoV receptor and receptor-binding domain (32, 50), reacted with the anti-RBD MAbs but not with the anti-S1-NT MAbs that recognize the putative conformational epitopes, implying that the N-terminal region of this protein may not be correctly folded. Further characterization of the structure of S1-Fc will help to elucidate its antigenic integrity.

Mapping of the anti-S MAbs that recognize the linear epitopes by synthetic peptides. Thirteen of the 38 anti-S MAbs reacted with the reduced S proteins, suggesting that their epitopes might be mapped by synthetic peptides. We previously synthesized a set of 168 peptides that overlap the entire S protein sequence to identify the immunodominant sites of the SARS-CoV (20). In the present study, these peptides were used as probes to localize the epitopes for the anti-S MAbs that recognized the linear sequences, and the reactive peptides in ELISA are presented in Table 3. The epitopes of three MAbs that recognized the linear sequences in S1-NT were

TABLE 3. Mapping of anti-S MAbs targeting linear epitopes by peptide-based ELISA

MAb	OD_{450} of S peptide from aa[a]:						
	236–253	528–545	544–558	549–566	807–823	814–831	1125–1141
Anti-S1-NT							
114E3	**0.43**	0.01	0.03	0.01	0.02	0.02	0.03
115H2	**1.25**	0.02	0.03	0.02	0.03	0	0
116F8	**1.26**	0.03	0.01	0.02	0.03	0.01	0.02
Anti-S1-CT							
114G5	0	**0.41**	0.01	0.02	0	−0.01	0.01
101F10	−0.01	0.01	**2.71**	**2.94**	0.02	0	−0.01
103F2	0.04	0.03	**2.65**	**2.86**	0.03	0.03	0.02
104D4	0.01	0.03	**2.71**	**2.79**	0.02	0.03	0.01
111A7	0.02	0.03	**2.89**	**3.03**	0.01	0.03	0.02
121B8	0.02	0.03	**2.51**	**2.89**	0.02	0	−0.01
Anti-S2-NT 102D7	0.02	0.03	0	0.01	**3.16**	**2.87**	0.03
Anti-S2-CT 119F6	0.02	0.03	0.01	0.02	0.03	0.01	**0.59**

[a] ELISA plates were coated with each of the peptides at 5 μg/ml, and MAbs were tested at 10 μg/ml. An optical density at 450 nm (OD_{450}) of >0.2 was considered a positive reaction and is highlighted in boldface type.

mapped to the peptide sequence from aa 236 to 253. In the control, none of the MAbs that recognized the Conf or Conf/linear epitopes reacted with the peptides. Interestingly, all six MAbs targeting S1-CT were mapped to the previously identified major immunodominant site (site IV, aa 528 to 635). MAb 114G5 reacted with the peptide sequence from aa 528 to 545, and the other five anti-S1-CT MAbs (101F10, 103F2, 104D4, 111A7, and 121B8) reacted with two overlapping peptide sequences, aa 544 to 558 and aa 549 to 566. The epitopes of this group of MAbs were further mapped by two longer peptides that overlap site IV (aa 511 to 552 and aa 536 to 566). As shown in Fig. 3, MAb 114G5 reacted strongly with the peptide sequences aa 511 to 552 and aa 536 to 566, suggesting that the overlapped sequence (GVLTPSSKRFQPFQQFG) is its epitope. In comparison, five anti-S1-CT MAbs that reacted with the peptide sequences from aa 544 to 558 and aa 549 to 566 also reacted with aa 536 to 566 but not with aa 511 to 552, indicating that the overlapped shorter sequence (QQFGRDV SDF) is a core of their epitopes. The epitopes for two anti-S2 MAbs (102D7 and 119F6) were also localized by the peptides. 102D7 reacted with two overlapping peptides from aa 807 to 823 and aa 814 to 831, which are located in the N-terminal region of the S2 subunit (S2-NT); 119F6 recognized the peptide sequence from aa 1125 to 1141, which is located in the C-terminal region of the S2 subunit (S2-CT). Although two of the other four probable anti-S2 MAbs (108G2 and 114F7) reacted with the reduced S proteins, their epitopes could not be mapped with the peptides, including those longer peptides derived from the S2 subunit (aa 823 to 856, aa 892 to 931, aa 920 to 953, aa 975 to 1010, aa 981 to 1014, aa 1149 to 1186, aa 1152 to 1188, and aa 1162 to 1198). Further localization for their epitopes is needed.

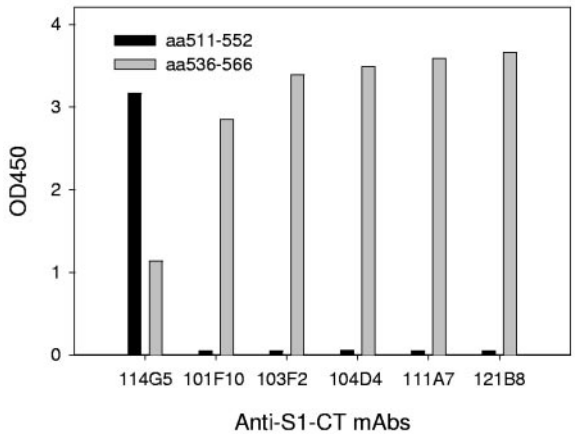

FIG. 3. Reactivity of anti-S1-CT MAbs with the peptides from aa 511 to 552 and aa 536 to 566 measured by ELISA. Coating was done with each of the peptides at 5 μg/ml, and the MAbs were tested at 10 μg/ml. OD450, optical density at 450 nm.

Mapping of the anti-S MAbs that recognize the conformational epitopes by binding competition assays. First, the epitopes for the anti-S1-NT MAbs that depend on the disulfide bonds were characterized by binding competition assays (Table 4). One of the MAbs (103D1) was initially biotinylated, and its binding activity to NT-Fc was measured in the presence or absence of each of 17 anti-S1-NT MAbs. Three MAbs recognizing the linear epitopes mapped by the peptides described above were also included in the binding competition assays as a control. As shown in Table 4, half of the anti-S1-NT MAbs (7 of 14) competed with biotinylated 103D1, suggesting that their epitopes may be identical or overlapped. Another 3 of the noncompeting MAbs (111E3, 112F9, and 120D9) were subsequently biotinylated and similarly tested with the binding competition assays. Four of the 7 MAbs that competed with the biotinylated 103D1 also blocked binding of the biotinylated 112F9 and 120D9 to the NT-Fc and were designated as a separate group. Thus, the 14 conformation-specific MAbs against S1-NT were divided into five distinct competition groups (designated NT I to V). Interestingly, three linear epitope-specific MAbs (114E3, 115H2, and 116F8) significantly competed with the biotinylated 112F9 and 120D9, suggesting that aa 236 to 253 have been complicated in the constitution of the conformational epitopes for 112F9 and 120D9.

TABLE 4. Mapping of anti-S MAbs targeting the S1-NT by binding competition assays[a]

Group	Competing MAb	% Inhibition of binding by biotinylated MAb:			
		103D1	111E3	112F9	120D9
S1-NT-I	103D1	**82.75**	25.27	−0.10	5.51
	109B4	**58.59**	24.47	−10.16	9.37
	113G3	**59.19**	24.60	−0.74	8.39
S1-NT-II	120D9	**87.34**	−12.14	**59.09**	**96.81**
	109D7	**49.59**	−5.93	**73.88**	**97.16**
	120B11	**49.42**	−17.07	**72.97**	**96.70**
	121E9	**48.83**	−40.66	**66.13**	**96.35**
S1-NT-III	106B3	−10.83	**90.58**	2.36	8.95
	111E3	3.22	**89.40**	−3.05	5.44
	112D5	−36.58	**87.75**	−2.32	−3.31
	112H6	7.48	**86.32**	4.23	7.74
S1-NT-IV	101E7	15.67	6.03	**40.22**	3.35
	112F9	2.86	7.10	**96.50**	29.56
S1-NT-V	104F10	31.58	12.85	−3.71	3.76
S1-NT linear	114E3	25.15	5.90	**54.26**	**92.98**
	115H2	27.03	−19.54	**60.03**	**98.01**
	116F8	32.49	−21.40	**72.74**	**98.21**

[a] Competing MAbs were tested at 100 μg/ml for the ability to block binding of the biotinylated MAbs to NT-Fc by ELISA. Greater than 40% inhibition was considered positive competition (values are shown in boldface type). Negative numbers indicate increased binding of the biotinylated reagent.

TABLE 5. Mapping of anti-S MAbs targeting S1-RBD by binding competition assays[a]

Group	Competing MAb	% Inhibition of binding by biotinylated MAb:		
		102A12	119F8	121D7
S1-RBD-I	102A12	**98.85**	12.37	−213.26
	106C5	**96.00**	9.63	−108.06
	112B7	**89.18**	5.30	−131.89
S1-RBD-II	103D3	−18.56	**95.07**	4.52
	119F8	−28.16	**87.67**	−3.21
S1-RBD-III	106C7	−26.06	−4.80	**91.27**
	106H2	1.62	19.34	**96.32**
	109G7	−2.23	11.93	**98.25**
	121D7	14.11	30.25	**97.97**

[a] Competing MAbs were tested at 100 μg/ml for the ability to block binding of the biotinylated MAbs to RBD-Fc by ELISA. Greater than 40% inhibition was considered positive competition (values are shown in boldface type). Negative numbers indicate increased binding of the biotinylated reagent.

Similarly, three anti-RBD MAbs (102A12, 119F8, and 121D7) were sequentially biotinylated and used in binding competition assays (Table 5). This allowed 9 anti-RBD MAbs to be divided into three distinct competition groups (designated RBD I to III). Interestingly, a group of three RBD I MAbs (102A12, 106C5, and 112B7) could significantly enhance the binding of biotinylated 121D7 to RBD-Fc, which was efficiently blocked by a group of RBD III MAbs. These data suggest that RBD I and RBD III are two independent conformational epitopes, but binding of the RBD I MAbs to the RBD I epitope may result in the increased exposure of the RBD III epitope, which becomes more accessible to the RBD III MAbs.

Potent inhibition of receptor binding and S protein-mediated virus entry by the MAbs that target the RBD of S protein. The S protein of SARS-CoV mediates receptor binding through its RBD. We previously demonstrated that some of the anti-RBD MAbs could efficiently block receptor binding (17). We tested whether newly isolated 9 RBD-specific MAbs can inhibit the binding of RBD-Fc to cell-associated ACE2 by flow cytometry. As shown in Fig. 4, RBD-Fc could bind to ACE2-expressed 293T cells, but the binding could be effectively inhibited by all three RBD I MAbs (102A12, 106C5, and 112B7) and by three MAbs from the RBD III group (106H2, 109G7, and 121D7). However, two anti-RBD MAbs corresponding to the competitive group RBD II (103D3 and 119F8) and one of the RBD III group, similarly to the control MAbs targeting the S1-NT, S1-CT, or S2 region (Table 6), had no significant inhibitory effects on receptor binding. These results indicate that the RBD I and RBD III MAbs may target the epitopes overlapping the receptor-binding motif (aa 424 to 494) (31) or that their binding can trigger conformational changes on the RBD.

Immunization of mice with the recombinant S proteins induced high titers of antibodies that potently neutralized SARS-CoV. It was important to determine the antigenic epitopes that mediate the neutralizing and nonneutralizing antibody responses. Each of the anti-S MAbs isolated from the immunized mice was tested for neutralizing activity against SARS pseudovirus (Tor2). Surprisingly, only anti-RBD MAbs had potent neutralizing activity, with a 50% neutralization dose (ND_{50}) ranging from 0.01 to 2.25 μg/ml (Table 6). Several MAbs targeting the conformational epitope in the N-terminal region of the S1 subunit (conformational S1-NT II and III) neutralized the pseudovirus with much lower activity (ND_{50} values were in a range of 24.78 to 46.82 μg/ml). None of the MAbs directed against the linear or Conf/linear epitopes in the S1-NT, S1-CT, and S2 regions and that target the conformational S1-NT I, VI, and V epitopes had neutralizing activity at concentrations up to 50 μg/ml (Fig. 5). This result provides direct evidence to support the finding that the RBD of S protein is a major target of neutralizing antibodies.

DISCUSSION

Antigenic and immunogenic characterization of the SARS-CoV is highly important for developing a safe and effective SARS vaccine. In this study, the antigenic and immunogenic properties of SARS-CoV S protein expressed by recombinant baculovirus have been finely characterized by generation of polyclonal and monoclonal antibodies and determination of their epitopes and neutralizing activities. We found that both FL-S and its EC-S were able to induce high titers of neutralizing antibodies in immunized mice. Importantly, the mouse antibodies potently neutralized not only the SARS pseudovirus constructed with the S protein of Tor2, a SARS-CoV strain isolated during the severe 2002 to 2003 SARS outbreak, but also those of GD03 and SZ3, the SARS-CoV isolates obtained during the mild 2003 to 3004 outbreak and from palm civets. These data are consistent with our recent findings that a panel

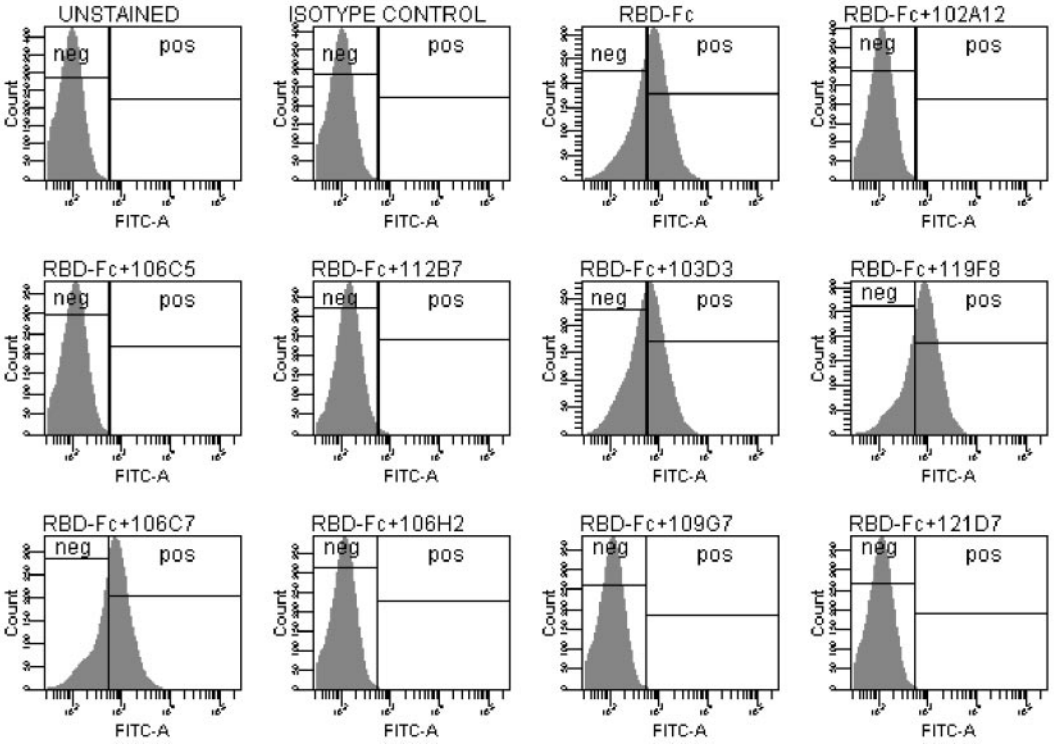

FIG. 4. Inhibition of RBD-Fc binding to cell-associated human ACE2 expressed on 293T/ACE2 cells measured by flow cytometry. RBD-Fc was used at 1 μg/ml, and MAbs were used at 50 μg/ml. The percent inhibition was calculated for each MAb. FITC, fluorescein isothiocyanate.

of RBD-induced MAbs can recognize the putative conformational epitopes in the RBDs derived from Tor2, GD03, and SZ3 and potently cross-neutralize the virions pseudotyped with S proteins of these human and civet SARS-CoV strains (22). These data suggest that the S protein can induce cross-neutralizing antibodies against human and animal SARS-CoV variants, a key aspect for SARS vaccine development.

Since the emergence of the SARS epidemic, a number of candidate vaccines comprising the S proteins expressed by attenuated viruses or DNA vectors have been tested in preclinical studies (2, 4–6, 51). These immunogens are effective in terms of their capacities to induce neutralizing antibodies and protective immunity against the 2002 to 2003 human SARS-CoV isolates. However, limited information is available about whether the antibodies induced by these S protein-based vaccines can neutralize heterologous SARS-CoV, e.g., GD03 and SZ3. An ideal SARS vaccine should elicit cross-reactive neutralizing antibodies that confer broad protection against variant isolates. Recently, it was reported that a DNA vaccine that expresses the full-length S protein (Urbani) induced antibodies that neutralized the 2002 to 2003 human SARS-CoV isolates but were not effective in neutralizing the 2003 to 2004 human SARS-CoV isolates and even enhanced infection mediated by the S proteins of palm civet SARS-CoV (52), raising some serious concerns about the breadth and efficacy of immune protection induced by S protein-based vaccines. Molecular characterization of the SARS-CoV has revealed evolving heterogeneity among isolates. The S protein, which not only mediates receptor binding but also is a major antigen to induce neutralizing antibodies, shows some degree of variation (up to 17 substitutions) in its 1,255-aa sequence from early to late outbreak phases (7). It is possible that the SARS-CoV strains with genetic diversity may escape neutralization by antibodies targeting the S protein. However, our present study indicates that recombinant baculovirus-expressed S protein vaccines can elicit potent cross-neutralizing antibodies against SARS-CoV variants, suggesting that the difference in S sequences may not significantly confer the antibody-mediated enhancement of the civet SARS-CoV. Indeed, a modified DNA vaccine encoding a secreted form of S protein, truncated at amino acid 1153, induced antibodies that failed to cause the enhancement of the civet SARS-CoV infection (52). Therefore, a SARS vaccine based on the S protein sequence of one SARS-CoV strain may provide protection against multiple SARS-CoV variants with distinct genotypes and phenotypes.

The antigenic structure of S protein has been characterized with a panel of 38 anti-S MAbs isolated from the FL-S-immunized mice. The data suggest that the S1 subunit of S protein is a major antigenic site to induce antibody responses. We previously identified five linear immunodominant epitopes in the S protein, and four of them were located in the S1 subunit (20). Similar to the S protein of other coronavirus, the S1 subunit of SARS-CoV S protein is a peripheral fragment of the global viral envelope and is well exposed to the immune system, whereas the S2 subunit is a membrane-spanning fragment that may be buried within viral envelope glycoprotein in the native state and thus results in lower immunogenicity. Therefore, the S1 subunit of S protein not only contains the receptor-binding motif (RBM) but also is a major antigen to induce

TABLE 6. Neutralization activity of anti-S MAbs against SARS pseudovirus (Tor2)

Epitope	MAb	Inhibition of ACE2 binding[a]	ND_{50} (μg/ml)
S1-NT (linear)	114E3	−	>50
	115H2	ND	>50
	116F8	ND	>50
S1-NT-I (Conf)	103D1	−	>50
	109B4	ND	>50
	113G3	ND	>50
S1-NT-II (Conf)	120D9	−	24.78
S1-NT-II (Conf/linear)	109D7	ND	>50
	120B11	ND	>50
	121E9	ND	>50
S1-NT-III (Conf)	106B3	−	25.64
	111E3	ND	33.56
	112D5	ND	46.82
	112H6	ND	27.98
S1-NT-IV (Conf)	101E7	−	>50
	112F9	ND	>50
S1-NT-V (Conf)	104F10	ND	>50
S1-RBD-I (Conf)	102A12	+	0.02
	106C5	+	0.20
	112B7	+	0.05
S1-RBD-II (Conf)	103D3	−	0.01
	119F8	−	1.56
S1-RBD-III (Conf)	106C7	−	0.20
	106H2	+	0.10
	109G7	+	2.25
	121D7	+	0.01
S1-CT (linear)	101F10	ND	>50
	103F2	−	>50
	104D4	ND	>50
	111A7	ND	>50
	114G5	ND	>50
	121B8	ND	>50
S2 (linear)	114F7	−	>50
	102D7	ND	>50
	119F6	ND	>50
	108G2	ND	>50
S2 (Conf)	117F7	ND	>50
S2 (Conf/linear)	102B11	−	>50

[a] ND, not done; + and −, inhibition and noninhibition of receptor binding, respectively.

immune responses, whereas the main function of the S2 subunit is membrane fusion between virus and cells.

Strikingly, only the MAbs that target the RBD had potent neutralizing activity against the SARS pseudovirus, indicating further that the major mechanism of SARS-CoV neutralization is through blocking the receptor association. We have recently demonstrated that the RBD of SARS-CoV S protein is a major target of neutralizing antibodies induced by viral infection and vaccination and that it contains multiple conformation-dependent neutralizing epitopes (17–19, 21). Chen et al. have recently shown that a recombinant modified vaccinia virus Ankara expressing the S protein of SARS-CoV induces protective neutralizing antibodies that primarily target the RBD (6). Depletion of the RBD-specific antibodies from the immune sera of SARS patients or vaccinated animals could remove the majority of neutralizing activity (6, 21). Outstandingly, most of the SARS-CoV-neutralizing MAbs isolated so far are directed against the RBD of S protein (21, 45, 46, 48). The epitopes of nine newly isolated anti-RBD MAbs were mapped by binding competition to three conformational groups, which may confer polyclonal antibody-mediated potent virus-neutralizing activity. The antigenic heterogeneity of the RBD may depend on its complex tertiary conformations. Crystal structure of the RBD complexed with human ACE2 reveals that the RBD contains several loops formed between cysteines and that aa 424 to 494 constitute the RBM (31). Six of the 9 novel anti-RBD neutralizing MAbs were able to efficiently block the receptor binding, suggesting that the RBM is a key site for neutralizing antibodies. The structural and functional coincidence of the major neutralizing epitopes and the receptor-binding site of the S protein validate our proposal that the RBD can be developed as an effective SARS subunit vaccine (16, 26). Furthermore, we have recently demonstrated that independently folded RBD can induce antibodies that potently neutralize human and animal SARS-CoV variants. Therefore, the RBD-based vaccine may be superior to the full-length S protein in terms of its safety and efficacy.

Linear and conformational epitopes in the N-terminal region of S protein (S1-NT) have been characterized by 17 specific MAbs. Only four of them, which were mapped to the conformational epitopes, possessed moderate neutralizing activity, suggesting that this region is not a major target for neutralizing antibodies. Six anti-S MAbs were mapped to the C-terminal region of the S1 subunit, which overlaps the previously characterized major liner immunodominant site (site IV) (20). None of them had neutralizing activity against the SARS pseudovirus. However, it was reported that two MAbs (S34 and S84) that mapped to the overlapping aa 548 to 567 could effectively neutralize SARS-CoV (54). Whether this location

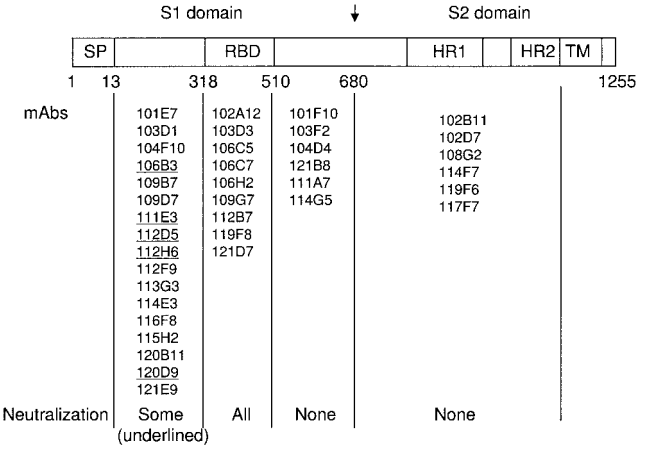

FIG. 5. Localization of the neutralizing and nonneutralizing epitopes for the anti-S MAbs.

contains a linear neutralizing epitope needs to be further investigated. We have recently demonstrated that two longer peptides (aa 511 to 552 and 536 to 566) that overlap the above immunodominant epitope can induce high titers of antibodies in immunized rabbits, but rabbit antibodies have no neutralizing activity against either SARS-CoV or pseudovirus (data not shown). In this study, six anti-S2 MAbs were also isolated and characterized, but none of them had neutralizing activity. We are in the process of expressing a panel of truncated proteins that overlap the S2 sequence to finely localize their epitopes. But it is obvious that the S2 subunit is not a major antigen to induce protective antibodies, although several lines of evidence suggest that it may contain neutralizing epitopes (27, 53). It was reported that the S2 subunit could induce high levels of total IgG in immunized mice but little neutralizing antibodies against infection by SARS-CoV (15).

In summary, the significance of the present study is threefold. First, it is first to show that recombinant S protein vaccines can induce cross-neutralizing antibodies against human and animal SARS-CoV. Second, it indicates further that the RBD of S protein not only is a functional site to mediate receptor binding and virus entry but also contains major neutralizing epitopes and thereby can serve a critical target for developing SARS vaccines. Third, this panel of anti-S MAbs will provide important tools for studying the structure and function of SARS-CoV S protein.

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