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Cross-Neutralization of Human and Palm Civet Severe Acute Respiratory Syndrome Coronaviruses by Antibodies Targeting the Receptor-Binding Domain of Spike Protein

Yuxian He,[1]* Jingjing Li,* Wenhui Li,† Sara Lustigman,* Michael Farzan,† and Shibo Jiang*

The spike (S) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) is considered as a protective Ag for vaccine design. We previously demonstrated that the receptor-binding domain (RBD) of S protein contains multiple conformational epitopes (Conf I-VI) that confer the major target of neutralizing Abs. Here we show that the recombinant RBDs derived from the S protein sequences of Tor2, GD03, and SZ3, the representative strains of human 2002–2003 and 2003–2004 SARS-CoV and palm civet SARS-CoV, respectively, induce in the immunized mice and rabbits high titers of cross-neutralizing Abs against pseudoviruses expressing S proteins of Tor2, GD03, and SZ3. We also demonstrate that the Tor2-RBD induced-Conf I-VI mAbs can potently neutralize both human SARS-CoV strains, Tor2 and GD03. However, only the Conf IV-VI, but not Conf I-III mAbs, neutralize civet SARS-CoV strain SZ3. All these mAbs reacted significantly with each of the three RBD variants (Tor2-RBD, GD03-RBD, and SZ3-RBD) that differ at several amino acids. Regardless, the Conf I-IV and VI epitopes were completely disrupted by single-point mutation of the conserved residues in the RBD (e.g., D429A, R441A, or D454A) and the Conf III epitope was significantly affected by E452A or D463A substitution. Interestingly, the Conf V epitope, which may overlap the receptor-binding motif and induce most potent neutralizing Abs, was conserved in these mutants. These data suggest that the major neutralizing epitopes of SARS-CoV have been apparently maintained during cross-species transmission, and that RBD-based vaccines may induce broad protection against both human and animal SARS-CoV variants. ***The Journal of Immunology,*** **2006, 176: 6085–6092.**

Severe acute respiratory syndrome (SARS)[2] is an emerging infectious disease caused by a novel coronavirus (SARS-CoV) (1–5). Its global outbreak in 2002–2003 resulted in >8000 cases with a fatality rate of ~10%. Several isolated infections occurred later due to either accidental releases of the SARS-CoV from laboratories or new acquisition from animal reservoirs (6–9). Palm civets (*Paguma larvata*) were considered as a source most often associated with interspecies transmission since the earliest SARS cases occurred in wild-game civet traders, and genetically similar viruses (SZ3, SZ16) were isolated from these animals (10, 11). In late 2003 and early 2004, a total of four patients with much less severe symptoms were independently hospitalized in Guangzhou, China, and no secondary transmission was observed (6, 8, 9). The viruses isolated from these patients, e.g., GD03T13 (GD03), GZ03–02 and GZ03–03, are genetically closer to civet SARS-CoV than those of 2002–2003 human SARS-CoV strains. However, recent studies suggest that the bats are the natural reservoir for the origin of SARS epidemic (12, 13). The human and civet isolates of SARS-CoV nestle phylogenetically within the spectrum of SARS-CoV-like (SL-CoV) strains isolated from the species of bats. The civets may have served as intermediate amplification hosts that enable SARS-CoV to cross species for animal-to-human transmission. Therefore, SARS-CoV may reemerge from the animal reservoirs and cause new SARS epidemics in future. Development of a safe and effective vaccine against both human and animal SARS-CoV strains is highly important to prevent future SARS outbreaks.

Coronavirus spike (S) protein mediates infection of targeted cells that bear the specific receptor (14–16). The S protein of SARS-CoV, a type I transmembrane glycoprotein containing putative S1 and S2 domains (Fig. 1), binds to angiotensin-converting enzyme 2 (ACE2), a functional cell receptor, with high affinity (14, 17–20). A fragment located in the middle region of the S1 domain (residues 318–510) has been defined as a discrete receptor-binding domain (RBD) (21–23). The S2 domain contains two heptad repeat (HR1 and HR2) regions that mediate membrane fusion between viruses and cells (24–27). The SARS-CoV S protein, which is responsible for receptor-binding and viral entry, is a major Ag inducing neutralizing Abs and thus protective immunity. Several live attenuated, genetically engineered, or vector-based vaccines encoding the S protein have been tested in preclinical studies (28–32). These vaccine candidates are effective in terms of eliciting protective immunity against homologous SARS-CoV strains in vaccinated animals. However, there are some concerns over the safety of full-length S protein-based immunogens because they may induce harmful immune or inflammatory responses (33, 34). For example, the Abs induced by a DNA vaccine encoding the full-length S protein (Urbani) efficiently neutralized infection by 2002–2003 human SARS-CoV isolates, but were not effective in neutralizing infection by the 2003–2004 human SARS-CoV (e.g., GD03), and even enhanced infection by civet SARS-CoV (e.g., SZ3 and SZ16) (35). Unexpectedly, Abs induced by the S proteins of GD03 and SZ3 could not neutralize either homologous or heterologous SARS-CoV strains (35). Weingartl et al. (36) reported that vaccination of ferrets with vaccinia virus-based SARS vaccine

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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; RBM, receptor-binding motif.

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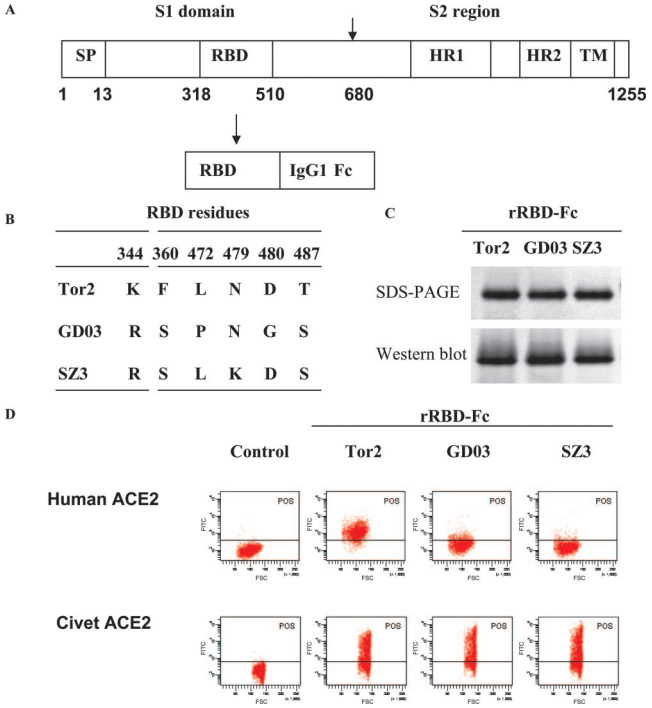

FIGURE 1. Design, expression and characterization of recombinant RBD variants. *A*, Schematic diagram of SARS-CoV S protein and the fusion protein RBD-Fc. The RBD of S protein contains residues 318–510 in the S1 domain. RBD-Fc consists of RBD and a human IgG1-Fc fragment. *B*, Amino acid differences among the RBDs of the S proteins of the indicated isolates (Tor2, GD03T0013, and SZ3). *C*, Characterization of purified RBD-Fc fusion proteins by SDS-PAGE with 10% gel and Western blot with anti-RBD mAb 17H9. *D*, Binding of RBD variants to human or palm civet ACE2 analyzed by flow cytometry.

expressing a full-length S protein caused liver damage after animals were challenged with SARS-CoV. We found that S protein contains five linear immunodominant domains that induce high titers of non-neutralizing Abs (37), which may have potential to mediate harmful immune responses. Therefore, further characterization on the antigenic properties of the S protein is critical for the rational design of SARS vaccines.

We and others have recently demonstrated that the RBD of SARS-CoV S protein is a major target of neutralizing Abs induced in patients infected with SARS-CoV and in animals immunized with inactivated viruses or S proteins (31, 38–41). The removal of RBD-specific Abs from the immune sera by immunoadsorption could reduce 60–100% sera-mediated neutralizing activities (31, 41). Single-point mutations of the conserved amino acid residues in the RBD completely abolished the capacity of full-length S protein to induce neutralizing Abs (42). We found that the RBD contains multiple conformation-dependent epitopes (Conf I-VI) capable of inducing highly potent neutralizing Abs (40), suggesting its potential application as a subunit vaccine. However, the RBDs among the human 2002–2003 and 2003–2004 and civet SARS-CoV isolates differ in several amino acids (Fig. 1*B*), some of which determine the receptor adaptation of SARS-CoV from palm civet to human (43, 44). It is important to know whether these sequence variations can determine the antigenic epitopes in the RBD and whether the recombinant RBDs can induce Abs with broad cross-neutralizing activity. Here we have demonstrated that each of three RBD variants (Tor2-RBD, GD03-RBD, and SZ3-RBD) can induce high titers of neutralizing Abs against human and civet SARS-CoV variants and that a panel of anti-RBD mAbs possess potent cross-neutralizing activities, suggesting that the RBD of S protein might maintain its conformation-dependent neutralizing epitopes during cross-species transmission and the conserved residues are critical to determine the antigenic structure of the RBD.

Materials and Methods

Expression of recombinant RBD-Fc and S1-C9 proteins

Plasmids encoding the corresponding S proteins of the representative strains of human 2002–2003 and 2003–2004 SARS-CoV isolate Tor2 (GenBank accession no. AY274119) and GD03T0013 (GenBank accession no. AY525636, denoted GD03 herein); and palm civet SARS-CoV isolate SZ3 (GenBank accession no. AY304486) were constructed as previously described (43). The sequences encoding Tor2-RBD, GD03-RBD, and SZ3-RBD were amplified by PCR with specific primers from the corresponding plasmids and cloned into vectors to express RBD-Fc fusion proteins (RBD linked to the Fc domain of human IgG1) as previously described (38, 40). RBD-Fc mutants were generated by mutagenesis using the QuikChange XL kit (Stratagene) and verified by DNA sequencing. Plasmid encoding the S1 domain (residues 12–672 tagged with C9 at the C terminus) was previously described (38, 40). The recombinant 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 proteins were 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 rabbits

Three RBD-Fc fusion proteins (Tor2-RBD, GD03-RBD, and SZ3-RBD) were, respectively, used to immunize mice and rabbits. Four female BALB/c mice (6 wk old) per group were subcutaneously immunized with 20 μg of purified proteins resuspended in PBS plus MPL + TDM (monophosphoryl-lipid A + trehalose dicorynomycolate) adjuvant (Sigma-Aldrich) and boosted with 10 μg of the same Ag plus the MPL + TDM adjuvant at 3-wk intervals. Two New Zealand White rabbits (12 wk old) per group were immunized intradermally with 150 μg of purified proteins resuspended in PBS (pH 7.2) in the presence of Freund's complete adjuvant, and boosted three times with freshly prepared emulsion of 150 μg of immunogen and incomplete Freund's adjuvant at 3-wk intervals. Preimmune sera were collected before starting the immunization, and antisera were collected 10 days after each boost. Sera were kept at 4°C before use.

Binding assays by flow cytometry

Association of RBD-Fc with ACE2 was determined by flow cytometry as previously described (38, 40). Briefly, HEK293T cells were transfected with a plasmid encoding human ACE2 or civet ACE2. At 2 days posttransfection, 10^6 stable 293T/ACE2 cells were detached and washed with HBSS (Sigma-Aldrich). The RBD-Fc was added to the cells to a final concentration of 1 μg/ml, and the mixture was incubated at room temperature for 30 min. Cells were washed three times with HBSS and then incubated with anti-human IgG-FITC conjugate (Sigma-Aldrich) at room temperature for an additional 30 min. After washing, cells were fixed with 1% formaldehyde in PBS and analyzed in a BD Biosciences FACSCalibur flow cytometer using CellQuest software.

ELISA

The reactivity of rabbit and mouse antisera with S proteins were determined by ELISA. Briefly, 1 μg/ml recombinant protein (RBD-Fc or S1-C9) was used 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 or rabbit sera 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 or goat anti-rabbit IgG (Zymed) 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).

Neutralization of SARS pseudoviruses

SARS-CoV pseudovirus system was developed in our laboratory as previously described (38, 45). In brief, HEK293T cells were cotransfected with a plasmid encoding the S protein corresponding to SARS-CoV Tor2, GD03, or SZ3 isolate and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4–3.luc.RE) by using Fugene 6 reagents (Boehringer Mannheim). Supernatants containing SARS pseudovirus were harvested 48 h posttransfection and used for single-cycle infection of human or civet ACE2-transfected 293T (293T/ACE2) cells. Briefly, 293T/

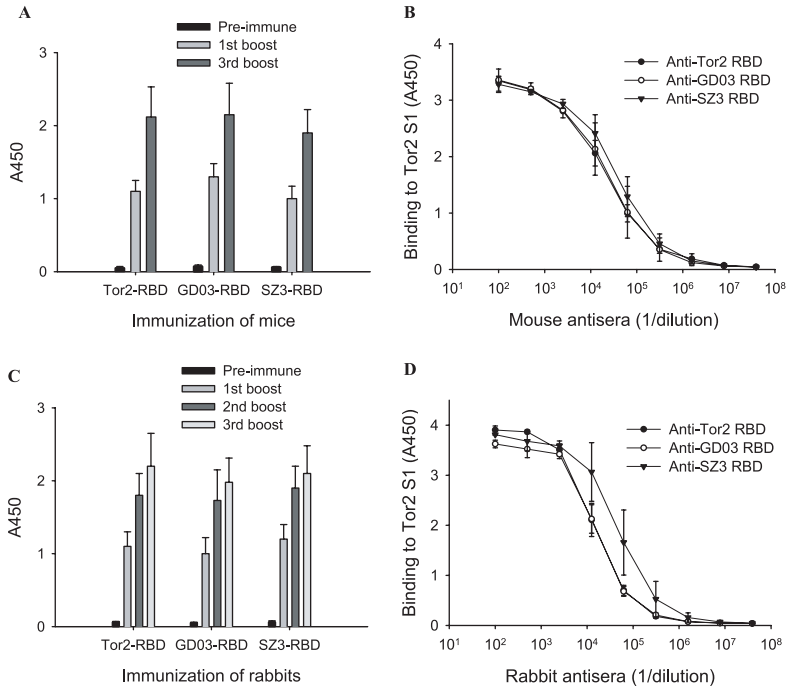

FIGURE 2. Ab responses in mice and rabbits immunized with RBD-Fc fusion proteins. *A*, Reactivity of mouse sera with RBD-Fc variants in ELISA. *B*, Binding of serially diluted mouse antisera collected after the third boost to S1-C9. *C*, Reactivity of rabbit sera with RBD-Fc variants in ELISA. *D*, Binding of serially diluted rabbit antisera collected after the third boost to S1-C9.

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). 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).

Results

RBD variants induced strong cross-reactive Abs in mice and rabbits

Three RBD-Fc fusion proteins containing the RBDs of representative SARS-CoV isolates (Tor2-RBD, GD03-RBD, and SZ3-RBD) were expressed in 293T cells and purified to homogeneity by protein A. The purified proteins were then analyzed by SDS-PAGE and Western blotting with anti-RBD mAb 17G9 (Fig. 1*C*). Association of each of the RBD-Fc variants with cell-associated human or civet ACE2 was determined by flow cytometry. As shown in Fig. 1*D*, Tor2-RBD, derived from 2002–2003 human SARS-CoV, bound efficiently to human ACE2, while GD03-RBD and SZ3-RBD, derived from 2003–2004 human SARS-CoV and civet SARS-CoV, respectively, bound marginally to human ACE2. However, all three proteins could efficiently interact with civet ACE2, in agreement with the previous results obtained by immunoprecipitation assays (43). This result indicates that these independently folded RBD variants maintain their functional conformation and bind with their specific receptor.

These three fusion proteins were, respectively, used to immunize four mice and two rabbits. As shown in Fig. 2, *A* and *C*, both mice and rabbits developed appreciable Ab responses against the corresponding immunogen after the first boost, and their Ab titers increased with subsequent immunizations. We used S1-C9, which contains S1 subunit of Tor2 S protein, to measure Ab titers specific for the RBD in the antisera collected after the third boost (Fig. 2, *B* and *D*). Similar to our previous results, Tor2-RBD could induce high titers of anti-S1 Abs in mice (mean end point titer was 1/1,562,500) and rabbits (mean end point titer was 1/312,500). Promisingly, both GD03-RBD and SZ3-RBD also induced comparable levels of Abs cross-reactive with Tor2 S1 protein in mice (mean end point titers were 1/937,500 and 1/1,562,500, respectively) and rabbits (mean end point titers were 1/312,500 and 1/937,500, respectively). In contrast, all mouse and rabbit preimmune sera did not react with S1-C9 (data not shown).

Cross-neutralization of human and civet SARS-CoV variants by polyclonal anti-RBD Abs

It is critical to know whether the RBD of S protein, a major target of neutralizing Abs, can elicit Abs that have broad neutralizing activity, considering that the full-length S protein of SARS-CoV could not induce Abs capable of neutralizing heterologous isolates (35). We previously developed a SARS pseudovirus with the S sequence of 2002–2003 human SARS-CoV strain Tor2 (38, 41), which effectively infects 293T cells expressing human and civet ACE2 (Fig. 3). Using a similar approach, we also prepared the pseudoviruses expressing S proteins of 2003–2004 human

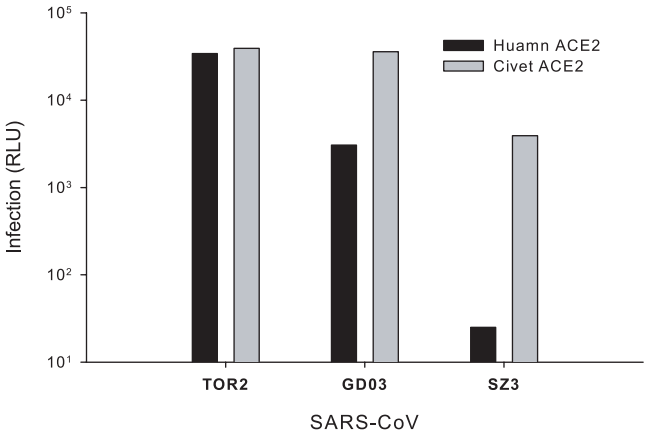

FIGURE 3. Infection of SARS pseudoviruses expressing S protein of the Tor2, GD03, or SZ3 isolate in HEK293 cells expressing human and palm civet ACE2, respectively. Infection, measured as luciferase activity of cell lysates, was assayed 2 days postinfection.

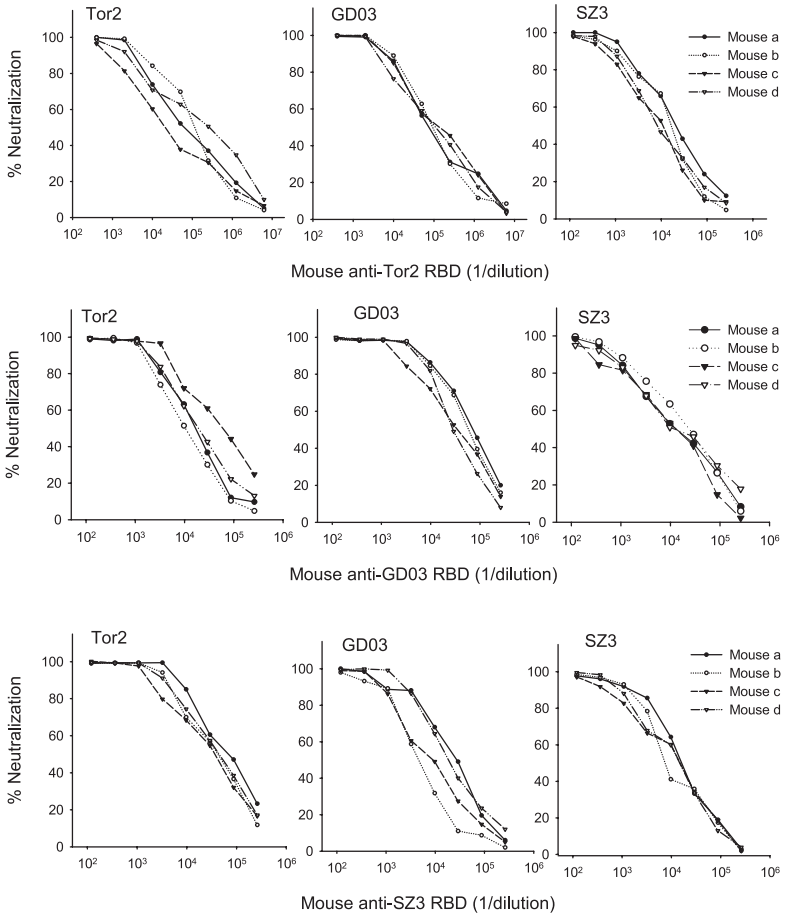

FIGURE 4. Potent neutralization of SARS pseudovirus infection by mouse anti-RBD Abs. Infection of HEK293 cells expressing human ACE2 or palm civet ACE2 by SARS pseudoviruses (Tor2, GD03, or SZ3) was determined in the presence of mouse antisera at a series of 3-fold dilutions. Percentage of neutralization was calculated for each sample, and the average values were plotted.

SARS-CoV isolate GD03 and civet SARS-CoV isolate SZ3. Like Tor2 pseudovirus, both GD03 and SZ3 pseudoviruses could infect 293T cells expressing civet ACE2, but unlike Tor2 and GD03 pseudoviruses, SZ3 pseudovirus was unable to infect cells expressing human ACE2. The efficiency of viral entry was consistent with the ability of each RBD variant to bind to the corresponding ACE2, in agreement with our previous report (43). Each of these pseudoviruses was incubated with serially diluted mouse anti-Tor2 RBD antisera to evaluate their sensitivity to Ab-mediated neutralization. Strikingly, the mouse anti-Tor2 RBD antisera potently neutralized both homologous and heterologous SARS-CoV strains - Tor2, GD03 and SZ3 with mean 50% neutralizing titers (NT_{50}) at 1/39,291, 1/62,229 and 1/16,112, respectively (Fig. 4). We then tested whether GD03-RBD and SZ3-RBD also induced cross-reactive neutralizing Abs. Similarly, mouse antisera induced by GD03-RBD and SZ-RBD neutralized each of the three pseudoviruses, Tor2 (mean NT_{50} = 1/28,756 and 1/53,793, respectively), GD03 (mean NT_{50} = 1/56,383 and 1/17,439, respectively), and SZ3 (mean NT_{50} = 1/14,377 and 1/12,102, respectively) (Fig. 4). Consistently, each of the three RBD fusion proteins could induce in rabbits high titers of neutralizing Abs against each of the three pseudoviruses with mean NT_{50} ranging from 1/6,407 to 1/29,079 (Fig. 5). In contrast, all mouse and rabbit preimmune sera at a 1/100 dilution had no inhibitory activity on the S protein-pseudotyped viruses (data not shown). These data indicate that the RBD of S protein can induce potent cross-reactive neutralizing Abs against human and civet SARS-CoV variants.

Neutralization of human and civet SARS-CoVs by anti-RBD mAbs

We previously isolated a panel of 27 anti-RBD mAbs from mice immunized with Tor2-RBD. Using these mAbs, we defined six groups of conformation-dependent neutralization epitopes (Conf I-VI), and found that the Conf III-VI mAbs effectively blocked the receptor binding, but the Conf I, II, and VI mAbs could not (40) (Table I). It was important to determine whether these Tor2-neutralizing mAbs also neutralize GD03 and SZ3. Accordingly, representative mAbs from each group were tested against each of the three pseudoviruses. As shown in Table I, Conf IV-VI mAbs could potently neutralize the three pseudoviruses with 50% neutralizing dose (ND_{50}) ranging from 0.02 to 0.6 μg/ml, except two Conf V mAbs (24F4 and 38D4) that had relatively lower activity on GD03 (ND_{50} = 3.61 and 35.14 μg/ml, respectively), suggesting that these mAbs have cross-neutralizing activities against human and civet SARS-CoV variants. Although all Conf I-III mAbs effectively neutralized the two human isolates (Tor2 and GD03), they had no neutralizing activities against the civet isolate (SZ3) even at a concentration as high as 50 μg/ml. Notably, the Conf I and II mAbs had relatively higher potency to neutralize heterologous isolate GD03. These results suggest that Conf IV-VI neutralizing epitopes are conserved among all three isolates, which might have conferred cross-neutralizing activities mediated by polyclonal antisera. In comparison, the Conf I-III neutralizing epitopes were only shared by two human isolates. Although the Conf I-III mAbs could not neutralize SZ3, no Ab-mediated enhancement of viral entry was observed. As expected, the two control mAbs against linear epitopes (4D5 and 17H9) had no neutralizing activities against any of these three pseudoviruses.

Characterization of the antigenic structures of the neutralization epitopes in the RBD by natural and non-natural mutations

Unlike the Abs induced by the full-length S protein, both polyclonal and monoclonal anti-RBD Abs had potent neutralizing activities

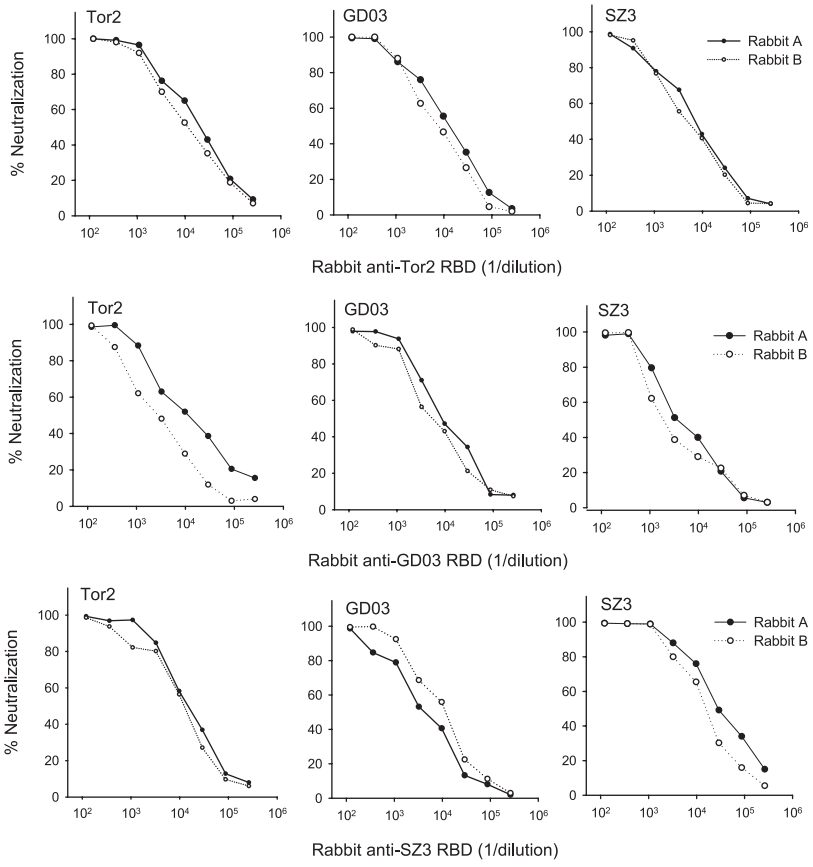

FIGURE 5. Cross-neutralization of SARS pseudoviruses (Tor2, GD03, or SZ3) by rabbit anti-RBD Abs. The neutralizing activity of the Abs against SARS pseudovirus infection in HEK293 cells expressing human or palm civet ACE2 was determined as described above.

against SARS-CoV variants. The RBD sequences are highly conserved among the isolates from 2002 to 2003 outbreak, but differ at several positions of the RBDs from isolates of the 2003–2004 outbreak and the palm civets (Fig. 1). Considering that the corresponding pseudoviruses had different sensitivity to neutralization by the mAb panel, we thereby tested the reactivity of each mAb with a panel of natural RBD mutants by ELISA to determine the epitopic specificity. First, all of the conformational and linear epitope-dependent mAbs strongly reacted with the RBDs derived from Tor2, GD03, and SZ3 (Table II). Second, the RBD bearing a point mutation from Tor2 to SZ3 (K344R, F360S, N479K, or T487S) had no effects on the binding by each group of mAbs (Table II). Although Conf I-III mAbs failed to neutralize SZ3, they bound to SZ3-RBD efficiently. These data further suggest that the RBDs derived from the S proteins of SARS-CoV variants may share common antigenic structures that induce neutralizing Abs. The mechanism by which SZ3 has evaded neutralization by anti-Conf I-III mAbs needs to be further investigated.

Table I. *Neutralization of SARS pseudoviruses by anti-RBD mAbs*

Group	mAb	Inhibition of ACE2 Binding	ND_{50} of Pseudovirus (μg/ml)		
			Tor2	GD03	SZ3
Conf I	9F7	−	6.22	0.15	>50
	12B11	−	4.56	0.7	>50
	29G2	−	3.24	0.16	>50
Conf II	26A4	−	2.85	0.49	>50
	27C1	−	1.47	0.06	>50
	31H12	−	0.21	0.02	>50
Conf III	11E 12	+	1.28	3.91	>50
	18D9	+	0.03	5.29	>50
Conf IV	28D6	+	0.21	0.16	0.64
	30F9	+	0.02	0.08	0.21
	35B5	+	0.09	0.06	0.35
Conf V	24F4	+	0.05	3.61	0.14
	33G4	+	0.01	0.02	0.01
	38D4	+	0.35	35.14	0.36
Conf VI	13B6	−	1.32	1.5	1.25
	19B2	−	0.76	0.62	0.41
Linear	4D5	−	>50	>50	>50
	17H9	−	>50	>50	>50

Subsequently, we assessed the reactivity of two truncated RBD fragments with the panel of mAbs in ELISA. As shown in Table III, a majority of neutralization epitopes (Conf I-IV and VI) were completely disrupted by the deletion of amino acids at the N terminus (residue 318–326) or the C terminus (residues 491–510) of the RBD. This result suggests that both N- and C-terminal sequences are essential for maintaining the proper conformation of these neutralizing epitopes.

To further map the critical epitopes in the RBD, a set of RBD variants with non-natural mutations of the conserved residues were expressed and used in ELISA to measure their reactivity with the panel of mAbs induced by Tor2-RBD. Interestingly, even single amino acid substitutions of some conserved residues (D429A, R441A, or D454A) abolished the recognition of the Conf I-IV and VI epitopes. Conversely, the Conf V epitope was not sensitive to these point mutations or to the N- and C-terminal deletions, suggesting that this epitope within residues 327–490 in the RBD is highly conserved. Moreover, the reactivities of two mAbs from Conf I (12B11 and 18C2) with the RBD bearing E452A mutation completely disappeared, while the reactivities of Conf III mAbs (11E12 and 18D9) and some mAbs of Conf I and II were significantly affected by the E452A mutation. Furthermore, D463A substitution disrupted the Conf III epitope and significantly affected the 12B11 and 18C2 epitopes. In addition, the 17H9 epitope, a linear epitope mapped to residues 449–458, could be knocked down by the D454A mutation. In contrast, K390R substitution did not interfere with both conformational and linear epitopes in the RBD.

Table II. *Characterization of neutralization epitopes with natural mutants by ELISA*[a]

		RBD Variant			Tor2 to SZ3 Point Mutation			
	mAb	Tor2	GD03	SZ3	K344R	F360S	N479K	T487S
Conf I	9F7	**2.72**	**2.97**	**3.13**	**2.34**	**2.07**	**2.01**	**1.91**
	10E 7	**2.83**	**3.23**	**3.29**	**3.00**	**2.08**	**2.19**	**1.93**
	12B11	**2.96**	**3.53**	**3.10**	**2.71**	**1.80**	**2.04**	**2.13**
	18C2	**2.75**	**3.35**	**3.32**	**2.75**	**1.93**	**2.13**	**1.75**
	24H8	**2.70**	**2.89**	**2.96**	**2.91**	**2.03**	**2.11**	**2.07**
	29G2	**2.56**	**2.88**	**2.63**	**2.27**	**1.84**	**1.98**	**1.96**
	32H5	**2.59**	**2.86**	**2.86**	**2.66**	**2.10**	**2.16**	**2.03**
Conf II	20E 7	**2.72**	**2.54**	**2.58**	**2.43**	**1.95**	**1.90**	**1.90**
	26A4	**2.78**	**3.60**	**3.38**	**2.63**	**2.23**	**2.09**	**1.91**
	27C1	**2.59**	**2.75**	**2.57**	**2.83**	**2.08**	**2.13**	**2.12**
	31H12	**2.69**	**3.15**	**2.86**	**2.69**	**2.17**	**2.32**	**2.08**
	34E 10	**2.65**	**3.22**	**3.06**	**3.07**	**2.31**	**2.46**	**1.97**
Conf III	11E 12	**2.51**	**1.80**	**2.66**	**1.99**	**1.39**	**1.64**	**1.57**
	18D9	**2.49**	**2.28**	**2.77**	**2.28**	**1.48**	**1.30**	**1.78**
Conf IV	28D6	**2.68**	**3.21**	**3.03**	**3.10**	**2.23**	**2.18**	**2.05**
	30F9	**2.73**	**3.15**	**3.35**	**3.28**	**2.24**	**2.26**	**2.17**
	35B5	**2.59**	**3.42**	**3.22**	**3.21**	**2.29**	**2.38**	**2.20**
Conf V	24F4	**2.75**	**2.53**	**3.31**	**3.58**	**2.41**	**2.39**	**1.95**
	33G4	**2.68**	**2.85**	**3.05**	**3.11**	**2.50**	**2.48**	**2.34**
	38D4	**2.56**	**2.02**	**2.98**	**2.85**	**2.09**	**2.40**	**1.96**
Conf VI	13B6	**2.73**	**2.75**	**2.80**	**3.14**	**1.67**	**2.06**	**2.02**
	19B2	**2.72**	**2.72**	**2.67**	**2.77**	**1.94**	**1.78**	**2.00**
	44B5	**2.61**	**3.11**	**2.76**	**2.77**	**2.07**	**2.12**	**2.02**
	45F6	**2.56**	**3.10**	**2.78**	**2.66**	**2.06**	**2.17**	**2.08**
Linear	4D5	**2.72**	**3.19**	**3.56**	**1.42**	**1.04**	**1.08**	**1.22**
	17H9	**2.75**	**3.51**	**3.38**	**3.77**	**2.32**	**2.62**	**2.16**
Control	3A3[b]	0.03	0.05	0.02	0.03	0.04	0.03	0.02

[a] Ags were coated to ELISA plates at 1 μg/ml and mAbs were tested at 10 μg/ml. OD_{450} value >0.2 was considered positive reaction and highlighted in boldface.
[b] 3A3 is a control mAb targeting the S2 domain.

Discussion

Molecular characterization of the SARS-CoV has shown genetic diversity among isolates and raised concerns about the breadth and efficacy of immune protection induced by specific vaccines (35, 46). We previously demonstrated that a major mechanism for neutralizing SARS-CoV is through blockage of the interaction between the S

Table III. *Characterization of neutralization epitopes with non-natural RBD mutants by ELISA*[a]

		Deletion of		Single-Point Mutation					
Group	MAb	aa 318–326	aa 491–510	K390A	D429A	R441A	E452A	D454A	D463A
Conf I	9F7	0.08	0.00	**2.15**	0.18	0.02	**1.36**	0.01	**1.47**
	10E 7	0.11	0.00	**2.39**	0.19	0.01	**1.44**	0.03	**1.71**
	12B11	0.03	0.00	**2.40**	0.09	0.03	0.19	0.00	**0.45**
	18C2	0.03	0.01	**2.45**	0.12	0.01	0.03	0.00	**0.34**
	24H8	0.07	−0.01	**2.32**	0.19	0.02	**0.29**	0.00	**1.35**
	29G2	0.06	0.00	**2.41**	0.17	0.01	**0.71**	0.02	**1.37**
	32H5	0.08	0.00	**2.51**	0.20	0.01	**0.82**	0.01	**1.46**
Conf II	20E 7	0.02	−0.01	**2.36**	0.12	0.02	**0.65**	0.00	**1.24**
	26A4	0.02	0.00	**2.42**	0.14	0.02	**0.65**	0.00	**1.08**
	27C1	0.07	0.03	**2.36**	0.19	0.12	**0.79**	0.01	**1.32**
	31H12	0.08	0.00	**2.48**	0.20	0.01	**1.03**	0.00	**1.87**
	34E 10	0.08	−0.01	**2.74**	0.20	0.01	**1.02**	0.01	**1.72**
Conf III	11E 12	0.01	0.00	**2.05**	0.01	0.02	**0.25**	0.01	0.05
	18D9	0.01	0.00	**2.18**	0.01	0.02	**0.39**	0.00	**0.44**
Conf IV	28D6	0.07	0.00	**2.37**	0.07	0.02	**1.69**	0.00	**1.49**
	30F9	0.06	0.00	**2.43**	0.05	0.02	**1.59**	0.00	**1.37**
	35B5	0.08	−0.01	**2.48**	0.11	0.02	**1.65**	0.01	**1.57**
Conf V	24F4	**1.73**	**1.22**	**2.75**	**1.45**	**0.73**	**2.59**	**1.95**	**0.86**
	33G4	**2.03**	**1.56**	**2.50**	**1.71**	**1.05**	**2.56**	**2.06**	**2.02**
	38D4	**1.27**	**1.06**	**2.49**	**0.77**	**0.50**	**2.41**	**1.30**	**0.77**
Conf VI	13B6	0.00	−0.01	**2.32**	0.15	0.02	**1.40**	0.00	**1.28**
	19B2	0.04	−0.01	**2.36**	0.00	0.01	**1.15**	0.00	**0.86**
	44B5	0.13	0.00	**2.41**	0.20	0.03	**1.70**	0.04	**1.99**
	45F6	0.08	0.00	**2.43**	0.20	0.02	**1.78**	0.03	**1.62**
Linear	4D5	**2.70**	**2.20**	**1.75**	**0.89**	**0.83**	**3.03**	**2.44**	**0.82**
	17H9	**2.95**	**3.62**	**2.63**	**2.78**	**3.59**	**3.23**	0.19	**4.18**
Control	3A3	0.01	0.02	0.02	0.00	0.03	0.02	0.01	0.03

[a] Ags were coated to ELISA plates at 1 μg/ml, and mAbs were tested at 10 μg/ml. OD_{450} value >0.2 was considered positive reaction and highlighted in boldface.

protein and the cellular receptor ACE2, and that an independently folded RBD-Fc fusion protein can induce highly potent neutralizing Abs against SARS-CoV variants that have predominated in the 2002–2003 outbreak (38–41). We proposed that the RBD of the S protein can be used for the development of a SARS vaccine, because it is not only a functional domain that mediates virus-receptor binding but also a critical neutralization determinant of SARS-CoV (47, 48). However, the evolving genetic heterogeneity of SARS-CoV has raised concerns that viruses may have developed immune escape mechanisms to neutralization by Abs targeting the RBD (35). In this study, three RBD fusion proteins that contain the RBDs corresponding to the S proteins of representative SARS-CoV isolates, e.g., Tor2 that predominated in the 2002–2003 outbreak and caused severe disease, GD03 isolated from the 2003–2004 outbreak that caused a mild disease, and the SZ3 variant isolated from palm civet, were generated and their immunogenicity were evaluated in mice and rabbits. All RBD fusion proteins induced high titers of Abs that cross-reacted with the S protein derived from Tor2 and potently neutralized each of the pseudoviruses constructed by three representative S proteins (Tor2, GD03, and SZ3). These data suggest that the SARS-CoV spike RBD can induce broadly neutralizing Abs against diverse isolates, a key aspect for SARS vaccine development. In contrast, a full-length S protein expressed by a DNA vaccine was recently reported to be effective in inducing neutralizing Abs and protective immunity against homologous SARS-CoV, but the Abs could not neutralize GD03 and even enhanced SZ3 infection (35). Moreover, it was unexpected that the full-length S proteins derived from GD03 and SZ3 could not elicit Abs capable of neutralizing any of the S protein-pseudotyped viruses (35). It was proposed that the evasion of Ab neutralization might be mediated by the RBD of S protein (35). The mechanism by which heterologous SARS-CoV evaded the neutralization by the Abs induced by S protein-based DNA vaccine needs to be elucidated. Our present findings, however, further imply that an RBD-based vaccine may be superior to the full-length S protein in terms of its safety and efficacy.

Although the RBD is a 193-aa small fragment in the S protein, it contains multiple conformation-dependent neutralizing epitopes (Conf I-Conf VI) (40). The Conf III-VI epitopes may overlap the receptor-binding motif (RBM), since the mAbs that recognize these epitopes can efficiently block the receptor binding. We previously showed that the Conf I-VI mAbs had potent neutralizing activities against the Tor2 pseudovirus (40). In this study, we found that these anti-RBD mAbs also potently neutralized the GD03 that was markedly resistant to the Abs induced by the vaccines expressing the full-length S protein. Interestingly, whereas the SZ3 could be efficiently neutralized by the Conf IV-VI mAbs, it was resistant to the Conf I-III mAbs. It is unclear why the Conf I-III mAbs bound to the RBD variants equally well, but they could not neutralize the palm civet virus. Further characterization and comparison of the structures of the neutralization determinants in Tor2-RBD and SZ3-RBD may provide an answer to this question. The full-length sequence of S protein shows some degree of variation (up to 17 substitutions) among the human and civet SARS-CoV isolates. Some of the substitutions outside RBD may indirectly affect the conformations of the neutralizing epitopes in RBD. For example, the V1/V2 domain of HIV-1 gp120 is a global regulator of the sensitivity of primary isolates to neutralizing Abs (49).

Even the Conf I-III mAbs could not neutralize the civet isolate, they did not mediate enhancement of SZ3 entry. It is possible that the Ab-mediated enhancement is dependent on the type of cells used for infection. We thus repeated our neutralization assays with 786-O cells, a human kidney cell line, in which the enhancement was observed (35). Unexpectedly, none of our three pseudotype viruses (Tor2, GD03, and SZ3) could infect 786-O cells (data not shown), consistent with the recent report by Yi et al. (42). The viral and cellular determinants responsible for S protein-induced harmful immune responses remain to be characterized. However, our data here indicate that Abs targeting the RBD of S protein are unlikely to mediate the enhancement of SZ3 infection.

The RBD sequence of Tor2 displays 5- and 4-aa differences with those of GD03 and SZ3, respectively. The residues at the positions 479 and 487 are critical for virus adaptation from civet ACE2 to human ACE2 (43, 44). However, it is obvious that these sequence variations do not significantly affect the neutralizing epitopes that reside within the RBD. Indeed, three RBD variants and four RBD mutants bearing Tor2 to SZ3 point mutations efficiently bound with the mAbs from each of antigenic groups, indicating that the RBD might maintain its antigenic configuration during the cross-species transmission. The cross-species antigenic conservation of RBD may provide a basis of potent cross-neutralization of SARS-CoV variants, and suggests that a vaccine based on one RBD, e.g., Tor2-RBD, may prevent infection by SARS-CoV variants with distinct genotypes and phenotypes.

The RBD of S protein is well exposed on surface of virion and responsible for attachment and binding with receptor ACE2. It contains seven cysteines, and five of them are essential for protein expression and ACE2 binding (22), highlighting its structural complexity. Crystal structure of the RBD bound with the peptidase domain of human ACE2 reveals that the disulfide bonds connect cysteines 323–348, 366–419, and 467–474 to form loops, and that residues 424–494 form the RBM to makes all contacts with ACE2 (50). The complex tertiary structures in the RBD may confer the multiple antigenic conformations. Single-point mutations of the conserved amino acid residues in the RBD could abolish the ability of the full-length S protein to induce neutralizing Abs (42), indicating the importance of RBD-based neutralizing epitopes for the immunogenicity of S protein. In this study, the antigenic structures of the RBD were also characterized by a panel of RBD mutants with substitutions or deletion of conserved amino acid residues. We found that both N- and C-terminal residues of the RBD are essential to maintain its multiple neutralizing epitopes. Outstandingly, single amino acid substitutions either abolished the Conf I-IV and VI conformational epitopes (D429A, R441A, or D454A) or significantly disrupted the Conf I and Conf III epitopes (E452A and D463A). These results indicate that these particular RBD residues, unlike those of naturally mutated residues among SARS-CoV variants, are critical to maintain its antigenic integrity. These residues were previously characterized to be essential for receptor binding or viral entry (22, 42, 51). However, K390A substitution did not impact any of the antigenic epitopes significantly even though it completely disrupted the functionality of ACE2 binding (51). Importantly, we found that Conf V epitope was not affected by the truncation or point mutation in the RBD, suggesting it is a conserved epitope. These data suggest that SARS-CoV may maintain its antigenic structures during cross-species transmission and that the RBD of S protein can be developed as a safe and effective vaccine against multiple human and animal SARS-CoV variants for preventing future SARS outbreaks.

Disclosures

The authors have no financial conflict of interest.

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