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Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine

Yuxian He^a, Yusen Zhou^b, Shuwen Liu^a, Zhihua Kou^b, Wenhui Li^c,
Michael Farzan^c, Shibo Jiang^{a,*}

^a *Viral Immunology Laboratory, Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10021, United States*

^b *Department of Molecular Biology, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China*

^c *Department of Medicine, Partners AIDS Research Center, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, United States*

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Abstract

The spike (S) protein of severe acute respiratory syndrome (SARS) coronavirus (CoV), a type I transmembrane envelope glycoprotein, consists of S1 and S2 domains responsible for virus binding and fusion, respectively. The S1 contains a receptor-binding domain (RBD) that can specifically bind to angiotensin-converting enzyme 2 (ACE2), the receptor on target cells. Here we show that a recombinant fusion protein (designated RBD-Fc) containing 193-amino acid RBD (residues 318–510) and a human IgG1 Fc fragment can induce highly potent antibody responses in the immunized rabbits. The antibodies recognized RBD on S1 domain and completely inhibited SARS-CoV infection at a serum dilution of 1:10,240. Rabbit antisera effectively blocked binding of S1, which contains RBD, to ACE2. This suggests that RBD can induce highly potent neutralizing antibody responses and has potential to be developed as an effective and safe subunit vaccine for prevention of SARS.

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Severe acute respiratory syndrome (SARS), a newly emerging infectious disease, is caused by a SARS-associated coronavirus (SARS-CoV) [1–7], which may originate from some wild animals [8]. A global outbreak of SARS in 2002/2003 resulted in thousands of cases and hundreds of deaths, seriously threatening public health worldwide. In early 2004, new infections caused by SARS-CoV strains different from those predominant in last year's outbreak were reported in China [9]. The latest SARS outbreak was reported in Beijing and Anhui Province, China, in April 2004. Incomplete inactiva-

tion of the virus used in a laboratory in the Center of Disease Control of China may be the major cause of this outbreak (<http://www.who.int/csr/sars/en>). These indicate that SARS epidemics may recur at any time in the future. Therefore, development of effective and safe vaccines is urgently needed for protection of at-risk populations.

Currently, one candidate vaccine using inactivated SARS-CoV is in a phase I clinical trial in China [9,10]. Although the inactivated SARS-CoV may be effective in protecting animals from challenge by SARS-CoV, its efficacy in humans is unclear. There has been a serious concern about its safety since some antigens in the virions may elicit antibodies that do not neutralize,

* Corresponding author. Fax: +1 212 570 3099.

E-mail address: sjiang@nybloodcenter.org (S. Jiang).

but rather enhance, virus infection [10]. Some viral proteins may induce harmful immune and inflammatory responses, a potential cause of SARS pathogenesis [11,12]. Most recently, it was reported that SARS-CoV infection of ferrets caused mild liver inflammation and the liver damage became much more serious if the ferrets were first immunized with vaccinia virus-based SARS vaccines before virus challenge [13].

The S proteins of coronaviruses are responsible for virus binding, fusion and entry, and are major inducers of neutralizing antibodies [14–16]. Besides, they play critical roles in viral pathogenesis and virulence [17]. The S protein of SARS-CoV is also important for viral functions and antigenicity [18,19]. It is a type I transmembrane glycoprotein consisting of two domains, S1 and S2 [18] (Fig. 1). S1 is responsible for virus binding to the receptor on the target cells. It has been demonstrated that angiotensin-converting enzyme 2 (ACE2) is a functional receptor for SARS-CoV [20–23]. A fragment located in the middle region of S1 is the receptor-binding domain (RBD) [24–26]. S2 domain, which contains a putative fusion peptide and two heptad repeat (HR1 and HR2) regions (Fig. 1), is responsible for fusion between viral and target cell membranes. Like the anti-HIV peptides derived from the HIV-1 gp41 HR2 region [27,28], a peptide derived from the HR2 region of SARS-CoV S protein had inhibitory activity on SARS-CoV infection [29]. HR1 and HR2 regions can associate to form a six-helix bundle structure [29,30], resembling the fusion-active core structure of gp41 in HIV [31] and those of the S proteins in other coronaviruses, such as mouse hepatitis coronavirus (MHV) [32,33]. These suggest that upon binding of RBD on the viral S protein to ACE2 on target cells, S2 changes conformation by interaction between the HR1 and HR2 regions to form fusogenic core and bring viral and target cell membrane into close proximity, resulting in virus fusion and entry [29]. This indicates that the fragments containing the functional domains on the S

protein may be used as antigens for inducing antibodies to block virus binding or fusion.

Several live attenuated, genetically engineered or vector vaccines encoding SARS-CoV S protein have been in preclinical studies. Nabel and co-workers [34] reported that a DNA vaccine candidate encoding the S protein induced T-cell and neutralizing-antibody responses (neutralizing antibody titers range from 1:50 to 1:150), and protected mice from SARS-CoV challenge as shown by reduced titers of SARS-CoV in the respiratory tracts. They proved that the protection was mediated by neutralizing antibodies but not a T-cell-dependent mechanism. Moss and co-workers [35] demonstrated that the intranasal or intramuscular inoculations of mice with highly attenuated modified vaccinia virus vectors virus Ankara (MVA) containing the gene encoding full-length SARS-CoV S protein (MVA/S) produced S-specific antibodies with SARS-CoV-neutralizing activity (mean neutralizing titer is 1:284), and protected mice from SARS-CoV infection after transfer of serum from immunized mice. Most recently, Collins and co-workers [36] reported that mucosal immunization of African green monkeys with an attenuated parainfluenza virus expressing SARS-CoV S protein resulted in production of neutralizing antibodies (mean neutralizing titer of about 1:16) and protected animals against challenge infection. These data suggest that the S protein can induce protective neutralizing antibodies, although the neutralizing antibody titers are relatively low [34–36].

Here we report that a recombinant fusion protein containing the RBD of SARS-CoV S protein (residues 318–510) linked to a human IgG1 Fc fragment (designated RBD-Fc, see Fig. 1) can induce high titer of RBD-specific antibodies in the immunized rabbits, and rabbit antisera can effectively inhibit binding of S1 protein to ACE2 and potently neutralize SARS-CoV and SARS pseudovirus, suggesting that the RBD of S protein may be developed as a subunit vaccine for prevention of SARS.

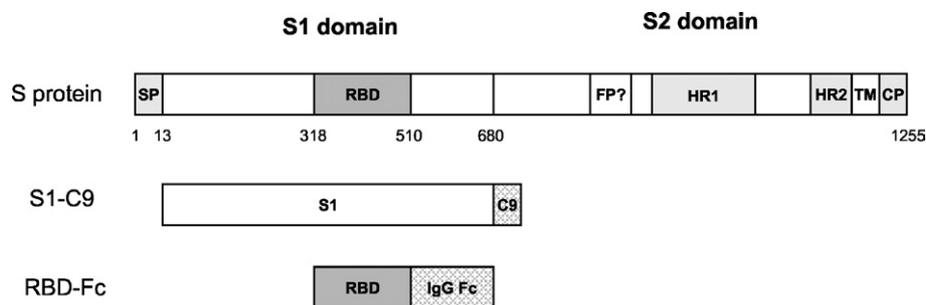


Fig. 1. Schematic diagram of SAR-CoV S protein and the recombinant fusion protein RBD-Fc. The S protein consists of S1 and S2 domains. There is a signal peptide (SP) located at the N-terminus of the S protein. The S1 domain contains a receptor-binding domain (RBD). The S2 domain contains a cytoplasm domain (CP), a transmembrane domain (TM), and an ectodomain composed of a putative internal fusion peptide (FP) and heptad repeat 1 and 2 (HR1 and HR2) regions. RBD-Fc consists of RBD and a human IgG-Fc fragment. S1-C9 contains S protein S1 domain and a C9 fragment.

Materials and methods

Expression of recombinant RBD-Fc and S1-C9 proteins. Plasmid encoding a 193-amino-acid fragment of SARS-CoV S protein (residues 318–510), corresponding to the receptor-binding domain, fused with the Fc domain of human IgG1 (RBD-Fc) and plasmid encoding S1 protein (residues 12–672) tagged with C9 at the C-terminus (S1-C9) have been described previously [20,24]. The RBD-Fc and S1-C9 proteins were expressed by transfecting 293T cells with the plasmids using Fugene 6 reagents (Boehringer–Mannheim, Indianapolis, IN) according to the manufacturer's protocol. Supernatants were harvested 72 h post-transfection. Recombinant RBD-Fc fusion proteins were purified by Protein A–Sephacryl 4 Fast Flow (Amersham Biosciences, Piscataway, NJ), and S1-C9 proteins were purified by affinity chromatography with anti-C9 mouse monoclonal antibody (mAb) 1D4 (National Cell Culture Center, Minneapolis, MN).

Immunization of rabbits. Two NZW rabbits were immunized intradermally with 150 µg purified RBD-Fc resuspended in phosphate-buffered solution (PBS, pH 7.2) in the presence of Freund's complete adjuvant (FCA), and boosted three times with freshly prepared emulsion of 150 µg immunogen and Freund's incomplete adjuvant (FIA) at 3-week intervals. Pre-immune sera were collected before starting the immunization and antisera were collected 10 days after each boost. Sera were kept at 4 °C before use.

Enzyme-linked immunosorbent assay. The reactivity of rabbit sera with various antigens was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 1 µg/ml recombinant proteins (RBD-Fc or S1-C9) or purified human IgG (Zymed, South San Francisco, CA) were used, respectively, to coat 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, serially diluted rabbit sera were added and incubated at 37 °C for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Bound antibodies were detected with HRP-conjugated 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 (TMB) and absorbance at 450 nm was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC).

Immunoabsorption assay. The anti-human IgG-Fc in the rabbit antisera were depleted by immunoabsorption. Briefly, the immunoaffinity resins were prepared by coupling the human IgG (Zymed) to cyanogens bromide-activated Sepharose beads (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Immunoabsorption was performed by diluting rabbit antisera 10-fold with PBS and incubating with the human IgG resin overnight at 4 °C with constant rotation. Resins were then packed into columns and flow-throughs (anti-Fc depleted antisera) were collected.

Neutralization of SARS-CoV infection. Neutralization of SARS-CoV infection was assessed as previously described [29]. Briefly, Vero E6 cells were plated at 5×10^4 cells/well in 96-well tissue culture plates and grown overnight. One hundred TCID₅₀ (50% tissue-culture infectious dose) of SARS-CoV BJ01 strain (GenBank Accession No. AY278488) was mixed with an equal volume of diluted rabbit 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 days 3 post-infection as previously described [29]. The neutralizing titers represented the dilutions of rabbit antisera that completely prevented CPE in 50% of the wells [34] as calculated by Reed–Muench method [40].

Neutralization of pseudovirus infection. A sensitive, quantitative, and safe neutralization assay based on reported SARS-CoV pseudovirus system [34,41–43] was developed. HIV pseudotyped with SARS-CoV S protein was prepared as previously described [23,24,43]. In brief, 293T cells were co-transfected 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 HIV/SARS-CoV S

protein were harvested 48 h post-transfection and used for single-cycle infection of ACE2-transfected 293T cells. Briefly, ACE2-expressed 293T cells were plated at 10^4 cells/well in 96-well tissue-culture plates and grown overnight. The pseudovirus was preincubated with serially diluted rabbit sera at 37 °C for 1 h before addition to cells. The culture was re-fed 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-bottomed luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). Relative light units were determined immediately on the Ultra 384 luminometer (Tecan US).

Inhibition of S1-protein binding to soluble ACE2. Recombinant soluble ACE2 (R&D systems, Minneapolis, MN) at 2 µg/ml was coated to 96-well ELISA plates (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, 2 µg/ml S1-C9 was added to the wells in the presence or absence of serially diluted rabbit sera. After incubation at 37 °C for 1 h, the anti-C9 mAb 1D4 was added and incubated at 37 °C for an additional 1 h. After washing, the HRP-conjugated goat anti-mouse IgG (Zymed) and the substrate TMB were used for detection.

Measurement of inhibition of S1 binding to cell-expressed ACE2 by flow cytometry. 10^6 stable 293T/ACE2 cells were detached and washed with Hanks' balanced salt solution (HBSS) (Sigma, St. Louis, MO). S1-C9 was added to the cells to a final concentration of 1 µg/ml in the presence or absence of rabbit sera at indicated dilutions, followed by incubation at room temperature for 30 min. After thorough washes, the mAb 1D4 was added to the cells to a final concentration of 10 µg/ml and incubated at room temperature for 30 min. Cells were washed with HBSS and incubated with anti-mouse IgG-FITC conjugate (Sigma) 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 Becton FACSCalibur flow cytometer (Mountain View, CA) using CellQuest software.

Results

Rabbit antisera directed against RBD-Fc contained high levels of anti-RBD antibodies

Rabbit antisera were collected before immunization (pre-immune) and 10 days after each boost at intervals of 3 weeks. The serum samples at 1:10,000 dilution were tested for binding to the recombinant fusion protein RBD-Fc by ELISA. As shown in Fig. 2A, the antisera collected 10 days after the 1st boost had the maximum reactivity with RBD-Fc and retained the high levels after the 2nd and 3rd boosts. The mean end-point titers of the antisera collected after the 1st boost were 1:7,812,500 (Fig. 2B). Therefore, we used these antisera samples for subsequent experiments. Since RBD-Fc also contains a human IgG-Fc fragment, the antibodies in the rabbit sera may also bind to Fc, in addition to RBD. Therefore, we tested the binding activity of rabbit antisera against the recombinant protein S1-C9, which contains RBD but not Fc. As shown in Figs. 2C and D, rabbit antisera bound to S1-C9 in a pattern similar to that shown in the experiments using RBD-Fc as antigen shown in Figs. 2A and B, although the reactivity and titers (1:312,500) of the antibodies against S1-C9

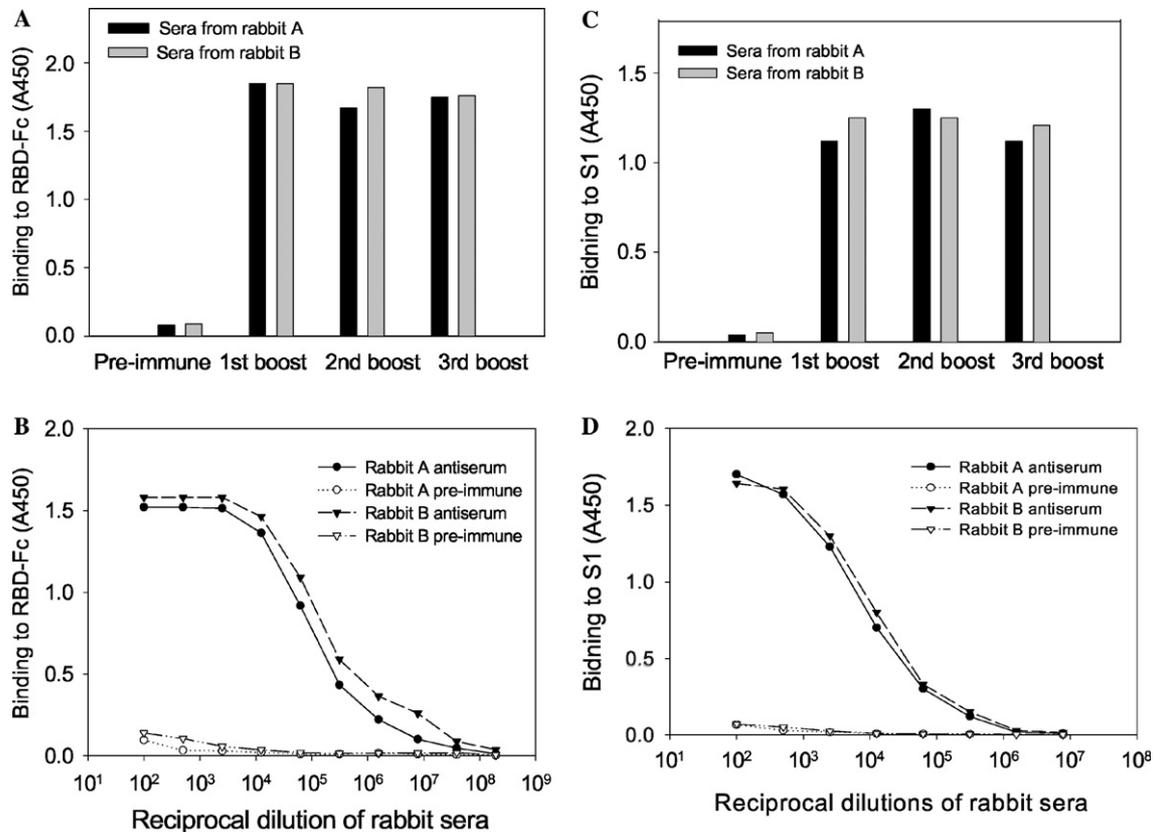


Fig. 2. Rabbit antisera contained high titers of antibodies binding to RBD. (A) Binding to RBD-Fc by antisera (1:10,000) collected from rabbits before immunization (pre-immune) and 10 days after each boost; (B) binding to RBD-Fc by rabbit antisera collected 10 days after the first boost at a series of 5-fold dilutions; (C) binding to S1-C9 by antisera (1:10,000) collected from rabbits before immunization (pre-immune) and 10 days after each boost; and (D) binding to S1-C9 protein by rabbit antisera collected 10 days after the first boost at a series of 5-fold dilutions. All samples were tested in duplicate and data presented are mean values of two tests (same for the following figures).

were lower than those to RBD-Fc. This suggests that anti-RBD antibody is one of the major antibody populations in rabbit antisera.

Rabbit antisera against RBD-Fc contained high titers of SARS-CoV-neutralizing antibodies

The rabbit antisera were tested for their neutralizing activity using two different assay systems, i.e., infection of SARS-CoV in Vero E6 and of SARS pseudovirus in 293T cells expressing ACE2. As shown in Fig. 3, both of rabbit antisera from the 1st boost at 1:10,240 or lower 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 mediated by SARS-CoV replication in cells. Mean neutralizing antibody titer calculated based on Reed–Meunch method [40] was 1:15,360. The pre-immune rabbit sera at a 1:40 dilution had no inhibitory activity on SARS-CoV infection. The anti-RBD antisera from rabbits A and B were also highly effective in inhibiting infection by SARS pseudovirus with 50% neutralizing titers of 1:73,627 and 1:131,956, respectively (Fig. 4), suggesting that the

anti-RBD antibodies can inhibit infection by both SARS-CoV and SARS pseudovirus. The rabbit antisera after 2nd and 3rd boost possessed comparable neutralizing activities against SARS virus (data not shown).

Depletion of anti-Fc antibodies from the antisera directed against RBD-Fc did not affect the RBD-binding and neutralizing activity

Since the recombinant fusion protein RBD-Fc also contains a human IgG-Fc fragment, it was expected that this antigen also induced anti-Fc antibodies. Indeed, the rabbit antisera reacted with human IgG-Fc coated in the wells of plates (Fig. 5A). However, the anti-Fc antibodies could be depleted from the antisera by immunoabsorption since the anti-Fc-depleted antisera had no reaction with human IgG in ELISA (Fig. 5A). Anti-Fc-depleted antisera retained the RBD-binding activity (Fig. 5B) and neutralizing activity (Fig. 5C), comparable with untreated rabbit antisera. These results suggest that the anti-Fc antibodies in the antisera induced by human IgG-Fc had no contribution to the RBD-binding and virus-neutralizing activity of the rabbit antisera.

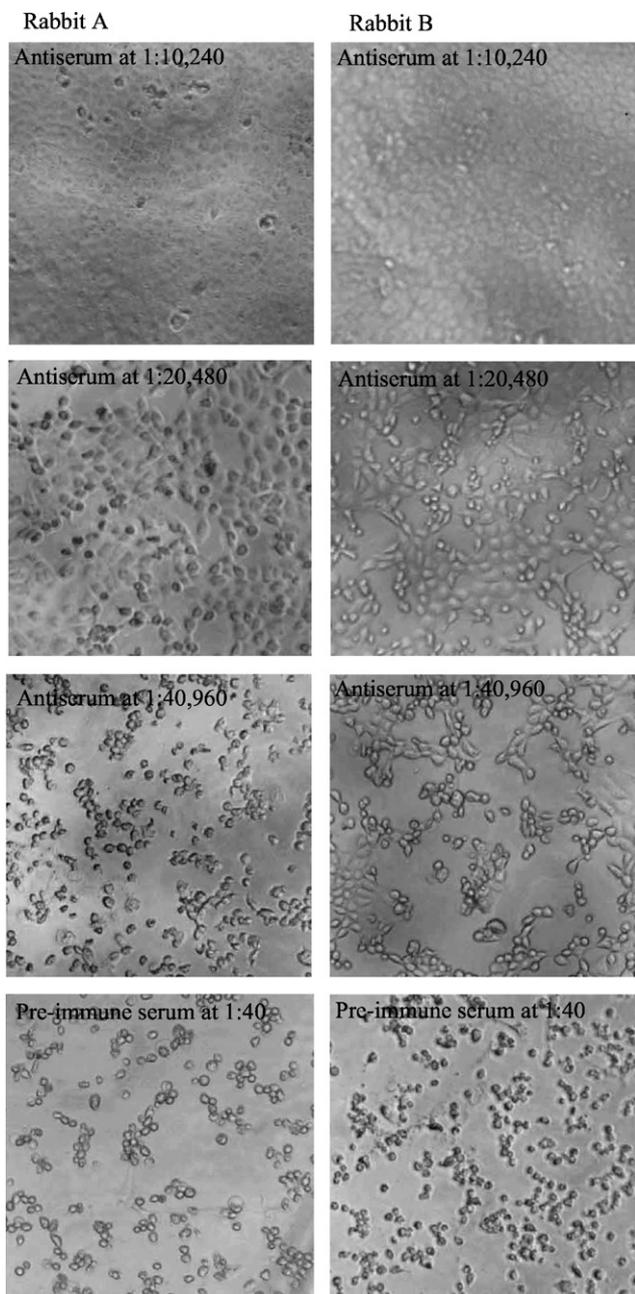


Fig. 3. Neutralization of SARS-CoV by rabbit antisera directed against RBD-Fc. SARS-CoV was incubated with Vero E6 monolayer in the presence of rabbit antisera in a series of 2-fold dilutions. The CPE caused by SARS-CoV infection was recorded under microscope and the virus-neutralizing titers were calculated.

Rabbit anti-RBD-Fc effectively blocked RBD binding to ACE2

We tested whether the anti-RBD antibodies block RBD binding to soluble and cell-associated ACE2 using ELISA and flow cytometry, respectively. Since RBD-Fc can also react with the anti-Fc antibodies in the antisera directed against RBD-Fc, we used S1-C9 which contains only RBD, but not Fc in all the experiments for deter-

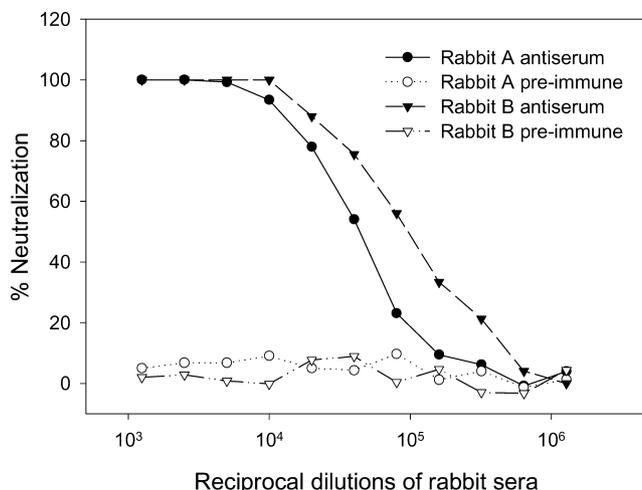


Fig. 4. Neutralization of HIV/SARS-CoV S pseudovirus infection by rabbit antisera. Inhibition of a single-cycle infection of 293T cells expressing ACE2 by the pseudovirus was determined in a luciferase assay.

mining the binding of RBD. In ELISA, soluble ACE2 was coated on the wells of ELISA plates and S1-C9 was able to bind to ACE2 (data not shown). Rabbit anti-RBD antisera effectively blocked S1 binding to ACE2 in a dose-dependent manner while the pre-immune sera had no inhibitory activity (Fig. 6A). Soluble ACE2 coated on plastics may lose the native conformation, so we also used cell expressed ACE2, which is expected to retain the native conformation, for detecting the RBD-binding activity in a flow-cytometric assay. As shown in Fig. 6B, S1-C9 significantly bound to ACE2-expressed cells as measured using anti-C9 mAb 1D4 (positive control). If no S1-C9 was added (negative control), only background signals were detected. Rabbit antisera at 1:100 effectively blocked S1 binding to ACE2-expressed cells while pre-immune rabbit sera at the same dilution had no inhibitory activity (Fig. 6B). The inhibitory activity of the rabbit antisera on S1 binding to ACE2-expressed cells was dose-dependent. Depletion of anti-Fc antibodies from the rabbit antisera did not affect the inhibitory activity of the rabbit antisera on S1-ACE2 interaction (Fig. 6C), confirming that the anti-RBD activity is not mediated by anti-Fc antibodies.

Discussion

During the SARS pandemic of 2002/2003, despite the lack of effective and specific therapy, most SARS patients recovered from the acute illness and few were re-infected by SARS-CoV (<http://www.who.int/csr/sars/en>). Neutralizing antibodies were detectable in the convalescent sera of SARS patients [43,44]. Inoculation of hyperimmune sera from mice infected by SARS-CoV [45] or immunized with MVA/S [35] in mice, or

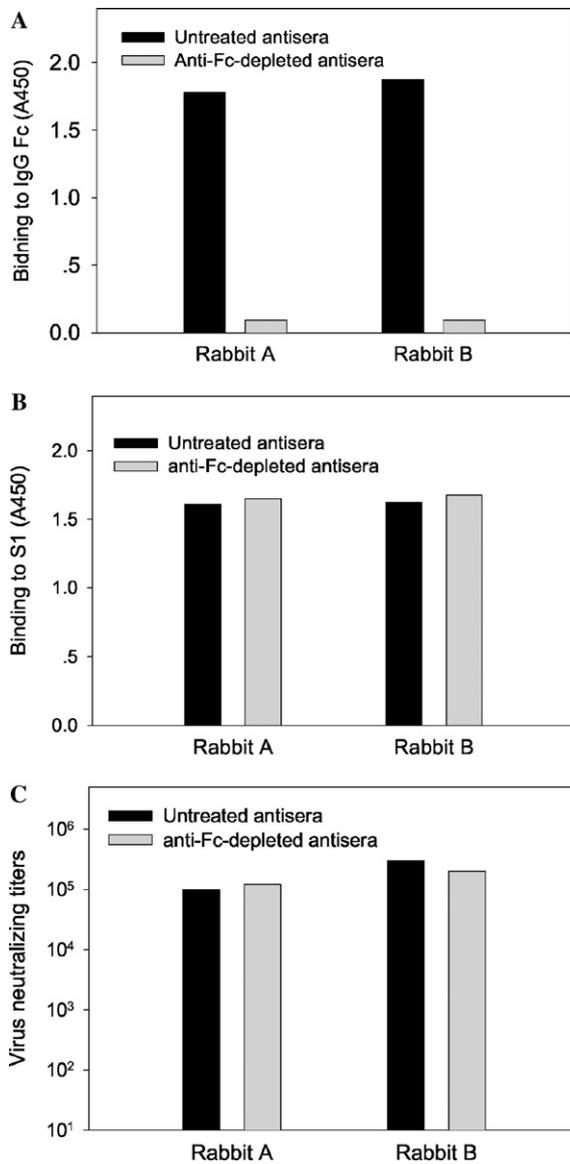


Fig. 5. Effect of depletion of human IgG-Fc specific antibodies from the rabbit antisera on binding to S1 and virus-neutralizing activity. The binding activity of anti-Fc-depleted and untreated rabbit antisera to human IgG (A) and S1 (B) was tested at 1:50 dilution by ELISA. The neutralizing activity of the anti-Fc-depleted rabbit antisera against HIV/SARS-CoV S was compared with that of untreated rabbit antisera (C).

administration of neutralizing monoclonal antibodies into ferrets [46] reduced the titers of SARS-CoV in the respiratory tracts of these animals after challenge. These data suggest that protective humoral immunity is achievable and vaccines can be developed for prevention of SARS.

A number of vaccine candidates are of preclinical study, including inactivated vaccines, DNA vaccines, and attenuated viruses encoding SARS-CoV S protein [9,10,34,35]. These agents are effective in inducing a protective neutralizing-antibody response in animals [34–36]. In the present study, we used a recombinant fusion

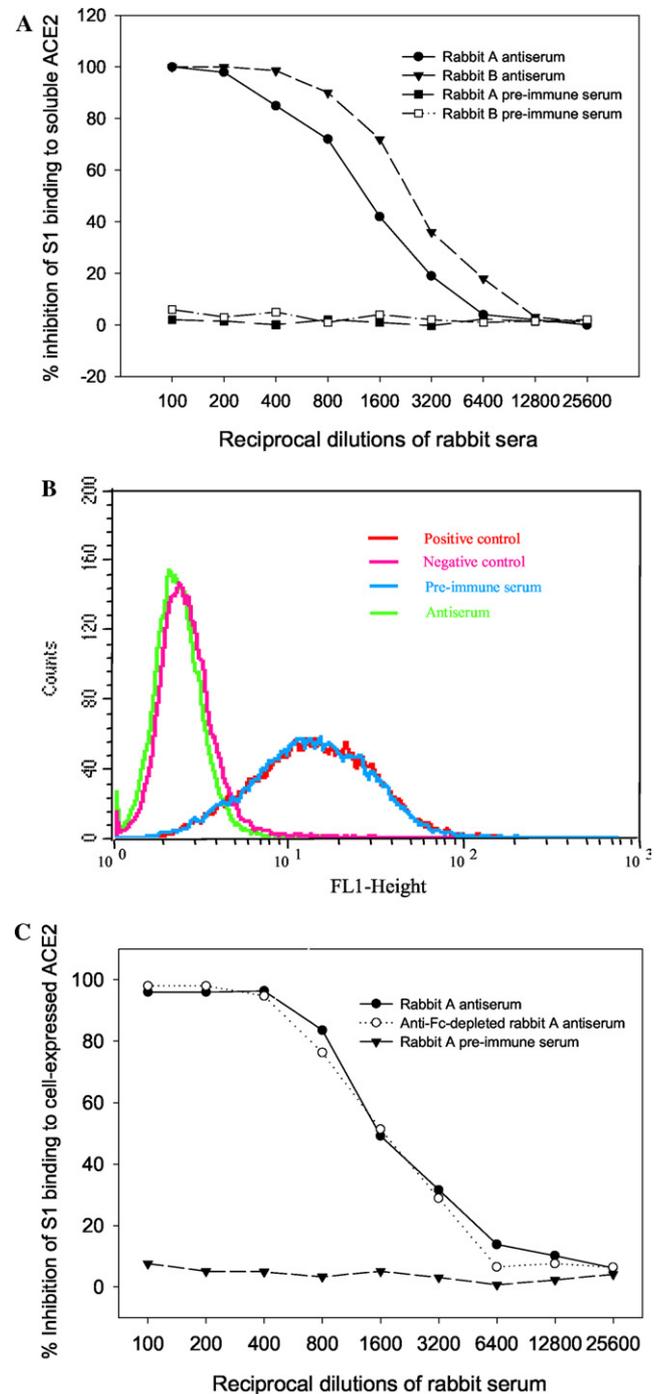


Fig. 6. Rabbit antisera inhibited S1 binding to ACE2. (A) inhibition of S1 binding to soluble ACE2 by rabbit antisera was measured by ELISA; (B) inhibition of S1 binding to cell-expressed ACE2 by rabbit antisera was measured by flow cytometry. In the positive control, no rabbit serum was added while in the negative control, neither rabbit serum nor S1-C9 was added; (C) rabbit antisera inhibited S1 binding to ACE2-expressing cells in a dose-dependent manner.

protein (RBD-Fc) as an immunogen to immunize rabbits since RBD is a key functional domain in the S protein responsible for viral binding to receptor on the target cell [24–26] and contains neutralizing epitopes [47]. Antibodies against RBD on the S proteins of other

coronaviruses, such as MHV, transmissible gastroenteritis virus (TGEV), and human coronavirus (HCoV-229E) [48–50], and those against receptors for the coronaviruses [51,52] are highly effective in blocking RBD-receptor interaction and neutralizing infection by the corresponding coronaviruses. Here we found that the RBD-Fc fusion protein elicited robust antibody responses in the immunized rabbits and the rabbit antisera could completely block SARS-CoV infection at the serum dilution of 1:10,240 (Fig. 3).

Since full-length S protein contains RBD and other viral functional domains and multiple neutralizing epitopes, it is expected to induce more potent neutralizing antibodies than RBD alone. One possible reason why RBD actually elicited much higher titers of neutralizing antibodies than full-length S protein is that the latter contains non-neutralizing epitopes that may elicit enhancing antibodies, like those induced by antigenic sites on the envelope glycoproteins of HIV and Ebola virus [39,53–55]. The S proteins from some coronaviruses could also induce enhancing antibodies. For example, immunization of felines with a vaccinia virus vector encoding the S protein of feline infectious peritonitis virus (FIPV) resulted in enhancement of virus replication after virus challenge [37,38] and the epitopes that elicit enhancing antibodies were localized in the S protein [56]. Although no enhanced virus replication may be observed [34,35], this may not exclude the possibility that the enhancing antibody titers are lower than neutralizing antibody titers. In such a case, enhancing antibodies may suppress or “neutralize” the neutralizing-antibody activity, resulting in reduced neutralizing-antibody titers.

The rabbit antisera directed against RBD are effective in binding to RBD on the S1 domain of SARS-CoV S protein (Fig. 2) and blocking RBD binding to soluble and cell-expressed ACE2 (Fig. 6). These confirm that the rabbit antisera contain antibodies specifically targeted to RBD. Although we have not tested the protective activity of the rabbit anti-RBD antibodies in animal models against SARS-CoV challenge, the high neutralizing titers of these antisera tested *in vitro* suggest that RBD-Fc may induce strong protective immunity in animals and humans, considering that the effective protection against SARS-CoV infection can be achieved by the convalescent sera from SARS patients with neutralizing antibody titers ranging from 1:20 to 1:1280 [44] and by antisera from mice and monkeys immunized by DNA vaccines and attenuated viruses encoding S protein, respectively, with low neutralizing antibody titers (1:16–1:284) [34,35].

The sequences of S proteins, especially the S1 domains, of most coronaviruses are highly variable [14], which is a major concern in developing effective vaccines against virus strains with distinct genotypes and phenotypes. However, recent studies have shown that SARS-CoV strains are quite stable and do not change

as much as that was originally predicted [10]. At the early phase of SARS endemic in 2002/2003, 5 out of the 193 amino acid residues in the RBD of SARS-CoV S protein are variable due to the positive selection pressure in the process of transition from animal (e.g., palm civet) SARS-like-CoV to human SARS-CoV. However, at the middle and late phases (most virus strains were isolated from SARS patients during these two phases), there is no mutation in the RBD sequence [6]. Furthermore, the conformation of RBD is relatively conserved to ensure the binding of virus with different subtypes to a specific receptor on the target cells, even though the linear sequence of RBD may be variable. One example is B12 mAb which recognizes the neutralizing epitopes on the CD4-binding domain on HIV-1 gp120 and neutralizes a broad range of HIV-1 primary isolates, although the linear sequences of CD4-binding regions in gp120 from the corresponding strains are highly variable [57,58]. These suggest that anti-antibodies against conserved RBD of the S protein may have neutralizing activity against a broad spectrum of SARS-CoV strains.

In summary, the RBD of S protein has potential to be developed as a subunit vaccine since it induces highly potent antibodies to block S protein binding with receptor and to neutralize SARS-CoV infection and has low level of risk compared with inactivated viruses or live attenuated virus vectors.

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