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Identification of Immunodominant Sites on the Spike Protein of Severe Acute Respiratory Syndrome (SARS) Coronavirus: Implication for Developing SARS Diagnostics and Vaccines

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The spike (S) protein of severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) is not only responsible for receptor binding and virus fusion, but also a major Ag among the SARS-CoV proteins that induces protective Ab responses. In this study, we showed that the S protein of SARS-CoV is highly immunogenic during infection and immunizations, and contains five linear immunodominant sites (sites I to V) as determined by Pepscan analysis with a set of synthetic peptides overlapping the entire S protein sequence against the convalescent sera from SARS patients and antisera from small animals immunized with inactivated SARS-CoV. Site IV located in the middle region of the S protein (residues 528–635) is a major immunodominant epitope. The synthetic peptide $S_{603-634}$, which overlaps the site IV sequence reacted with all the convalescent sera from 42 SARS patient, but none of the 30 serum samples from healthy blood donors, suggesting its potential application as an Ag for developing SARS diagnostics. This study also provides information useful for designing SARS vaccines and understanding the SARS pathogenesis. ***The Journal of Immunology*****, 2004, 173: 4050–4057.**

Severe acute respiratory syndrome (SARS)[2] is a new emerging infectious disease caused by a novel coronavirus (SARS-CoV) (1–3). During its global outbreak in 2002/2003, this catastrophic disease resulted in >8400 cases and 900 deaths according to the report by World Health Organization (WHO). Although this pandemic has been contained, there are concerns over the recurrences, especially a recent report of new SARS cases in China (www.who.int/csr/don/2004_04_23/en/). Elucidation of antigenic properties of SARS-CoV proteins is essential for developing effective diagnostics and vaccines to prevent new SARS epidemic.

Like other known coronaviruses, SARS-CoV is an enveloped positive-stranded RNA virus, featuring the large viral RNA genome (4–6). Its structural proteins contain spike (S), membrane (M), envelope (E), nucleocapsid (N), and several proteins that are uncharacterized. The S protein is a large type I transmembrane glycoprotein that is responsible for receptor binding and membrane fusion (7). Two functional domains, S1 and S2, located in the N- and C-terminal regions, respectively, of the S protein are conserved among the coronaviruses. However, no typical cleavage site was identified in the SARS-CoV S protein. By aligning the sequence of the SARS-CoV S protein with those of other coronavirus, its S1 and S2 domains were predicted to be localized at the regions of amino acid residues 17–680 and 681–1255, respectively (8). It has recently been demonstrated that the angiotensin-converting enzyme 2 on the cell surface functions as a receptor for SARS-CoV (9, 10), and a fragment of around 200 aa on the S protein S1 domain serves as a receptor-binding domain, which is responsible for binding of the viral S protein with the receptor angiotensin-converting enzyme 2 (11–15). The S protein S2 domain contains a putative fusion peptide and two heptad repeats, named HR1 and HR2. We found that one peptide, CP-1, which overlaps the HR2 sequence, inhibited SARS-CoV infection and interacted with a peptide derived from the HR1 region to form six-helix bundle (16). This is consistent with the result by Tripet et al. (17) and suggests that the S2 domain plays an important role in SARS-CoV fusion with the target cells.

The S proteins in coronaviruses are major antigenic determinants that induce immune response in the hosts (18–20). In the case of murine hepatitis virus, its immunodominant sites responsible for eliciting protective humoral responses are mainly located in the S protein S1 domain (21, 22). The S protein of transmissible gastroenteritis virus contains four major antigenic sites (A to D), and site A on the S1 subunit is the main inducer of neutralizing Abs (23–25). It has been known that significant Ab responses to SARS-CoV can be developed in SARS patients; however, its antigenic determinants remain to be elucidated (26). In this study, we identified several immunodominant sites on the S protein by Pepscan analysis with a series of peptides overlapping the entire sequence of the SARS-CoV S protein against the convalescent sera from SARS patients and antisera from small animals immunized by inactivated virus. This information may be useful for designing SARS diagnostics and vaccines.

Materials and Methods

Expression of recombinant S1 protein

A plasmid encoding SARS-CoV S protein S1 domain (residues 12–672) tagged with C9 at the S1 C terminus (S1-C9) was a generous gift from Dr. M. Farzan at the Harvard Medical School (Boston, MA) (9). 293T cells were transfected with this plasmid for transient expression using Fugene 6

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[2] Abbreviations used in this paper: SARS, severe acute respiratory syndrome; SARS-CoV, SARS-coronavirus; S, spike; HR, heptad repeat.

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reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. The medium was harvested 72 h posttransfection, and C9-tagged proteins were purified by affinity chromatography with mAb 1D4 specific for C9 (National Cell Culture Center, Minneapolis, MN).

Synthesis of peptides

A set of 168 peptides spanning the entire sequence of the S protein of SARS-CoV strain TOR2 (each peptide contains 17 amino acid residues with 9 residues overlapping with the adjacent peptides) were synthesized at Gene Gateway, LLC (Hayward, CA). Peptides S_{19-48}, $S_{278-312}$, $S_{368-419}$, $S_{511-552}$, $S_{536-566}$, and $S_{603-634}$ with lengths ranging from 30 to 42 aa were synthesized at CytoMol (Mountain View, CA). A standard solid-phase Fmoc method was used for peptide synthesis. Peptides were purified to homogeneity (purity, >90%) by HPLC and identified by laser desorption mass spectrometry.

Preparation of inactivated SARS-CoV

SARS coronavirus strain BJ01 (accession no. AY278488) was used as viral source and propagated in Vero E6 cells as described previously (5). The infected cells were harvested and completely lysed by three cycles of freeze-thaw. β-Propiolactone was then added to the lysates at 1:2000 ratio and incubated at 37°C for 2 h. The inactivated virus was centrifuged at 10,000 rpm for 20 min. After removal of cell debris, the supernatants were desalted with Sephadex G-50 (Amersham Biosciences, Piscataway, NJ), concentrated with polyethylene glycol-8000 (Sigma-Aldrich, St. Louis, MO), and filtrated with Sepharose-CL 2B (Amersham Biosciences) sequentially. The inactivated SARS-CoV in the final preparation, with >95% purity analyzed by HPLC, was confirmed by observing the coronavirus-like particles under an electron microscope and by determining the reactivity with the convalescent sera from SARS patients in Western blots.

Serum specimens from SARS patients

Serum samples were collected from 42 convalescent SARS patients 30–60 days after the onset of symptoms based on the clinical diagnosis. These patients, 22 males and 20 females ranging in age from 21 to 51 years old, were admitted to the You An Hospital, Beijing, during the 2003 SARS epidemics in Beijing. The diagnostic criteria for SARS-CoV infection followed the clinical description of SARS released by WHO (www.who.int/csr/sars/guidelines/en/). All of the sera were verified to be positive for SARS-CoV as detected by immunofluorescence assay and ELISA using commercially available diagnostic kits (Beijing Genomics Institute, Beijing, China). Sera collected from 30 healthy blood donors (15 males and 15 females ranging in age from 20 to 44 years old) at the You An Hospital, Beijing, were used as controls. Informed consent was obtained from each participant.

Immunizations

BALB/c mice and NZW rabbits were immunized intradermally with 10 and 30 μg, respectively, of purified viral particles inactivated by β-propiolactone as immunogen in the presence of CFA, and boosted with freshly prepared emulsion of the immunogen and IFA at 2-wk intervals. Antisera were collected 5 days after the third boost immunization.

Ab detection by ELISA

The reactivity of immune sera with various Ags was determined by ELISA. Briefly, the viral lysates (1 μg/ml), or recombinant S1 protein (1 μg/ml) or peptides (10 μg/ml) 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 3% nonfat milk, the plates were incubated with serially diluted antisera (human, mouse, or rabbit) at 37°C for 2 h, and then washed five times with PBS containing 0.1% Tween 20. Bound Abs were detected with alkaline phosphatase-conjugated goat anti-human (or mouse or rabbit, accordingly) IgG (Zymed, South San Francisco, CA) at 37°C for 1 h, followed by five washes. The reaction was visualized by addition of the substrate *p*-nitrophenyl phosphate (Zymed), and the absorbance at 405 nm was measured by an ELISA plate reader. Sera were considered positive when the OD values were above the cutoff value (the mean OD of negative samples plus 3 SDs).

Computational analysis of antigenic sites

Antigenic sequences of S protein were predicted using the method of Kolaskar and Tongaonkar (27) based on a table that reflects the occurrence of amino acid residues in experimentally known segmental epitopes.

Results

Identification of immunodominant domains on the S protein in the SARS patients

All of the convalescent sera from the SARS patients were positive to SARS-CoV as detected by ELISA with commercially available diagnostic kits using the mixture of proteins purified from SARS-CoV lysates as coating Ag (Fig. 1*A*). The Abs specific for S protein in the SARS convalescent sera were detected by ELISA using the rS1-C9 as an Ag. As shown in Fig. 1*B*, all of the 42 convalescent sera from SARS patients significantly reacted with the S protein, whereas none of the sera from healthy blood donors was reactive to this Ag. This result indicates that the S protein of SARS-CoV is highly immunogenic during infection.

To determine the antigenic sites on the S protein, a set of 168 peptides spanning the entire sequence of the S protein of SARS-CoV strain TOR2 was synthesized. Each of these peptides contains 17 amino acid residues with 9 residues overlapping with the adjacent peptides. Pepscan analysis of these peptides against SARS convalescent sera revealed that the S protein contained five linear immunodominant sites, designated as sites I to V corresponding to the sequences of residues 9–71, 171–224, 271–318, 528–635, and 842–913, respectively (Fig. 2). The immunodominant sites I to III, and V, reacted with >50% of the convalescent sera from SARS

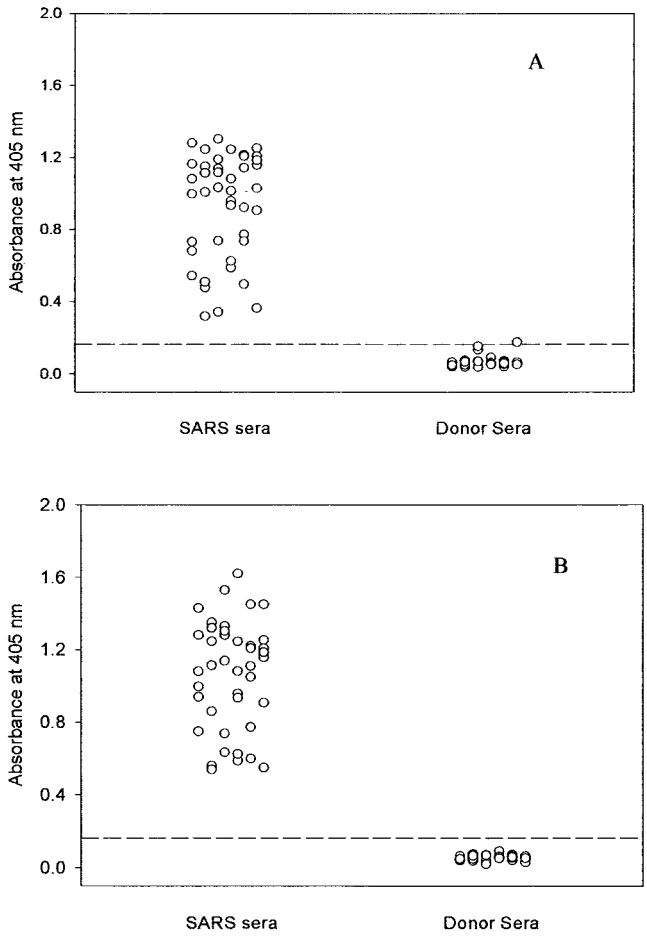

FIGURE 1. Detection of Abs specific for SARS-CoV proteins in the convalescent sera from SARS patients by ELISA. *A*, Mixture of proteins purified from SARS-CoV lysates in a commercial kit was used as Ags. *B*, The rS1-C9 protein was used as an Ag. Sera from 42 SARS patients and 30 healthy blood donors were tested at 1/50 dilution. The dashed lines represent the cutoff values (the mean absorbance at 405 nm of sera from healthy blood donors plus 3 SDs).

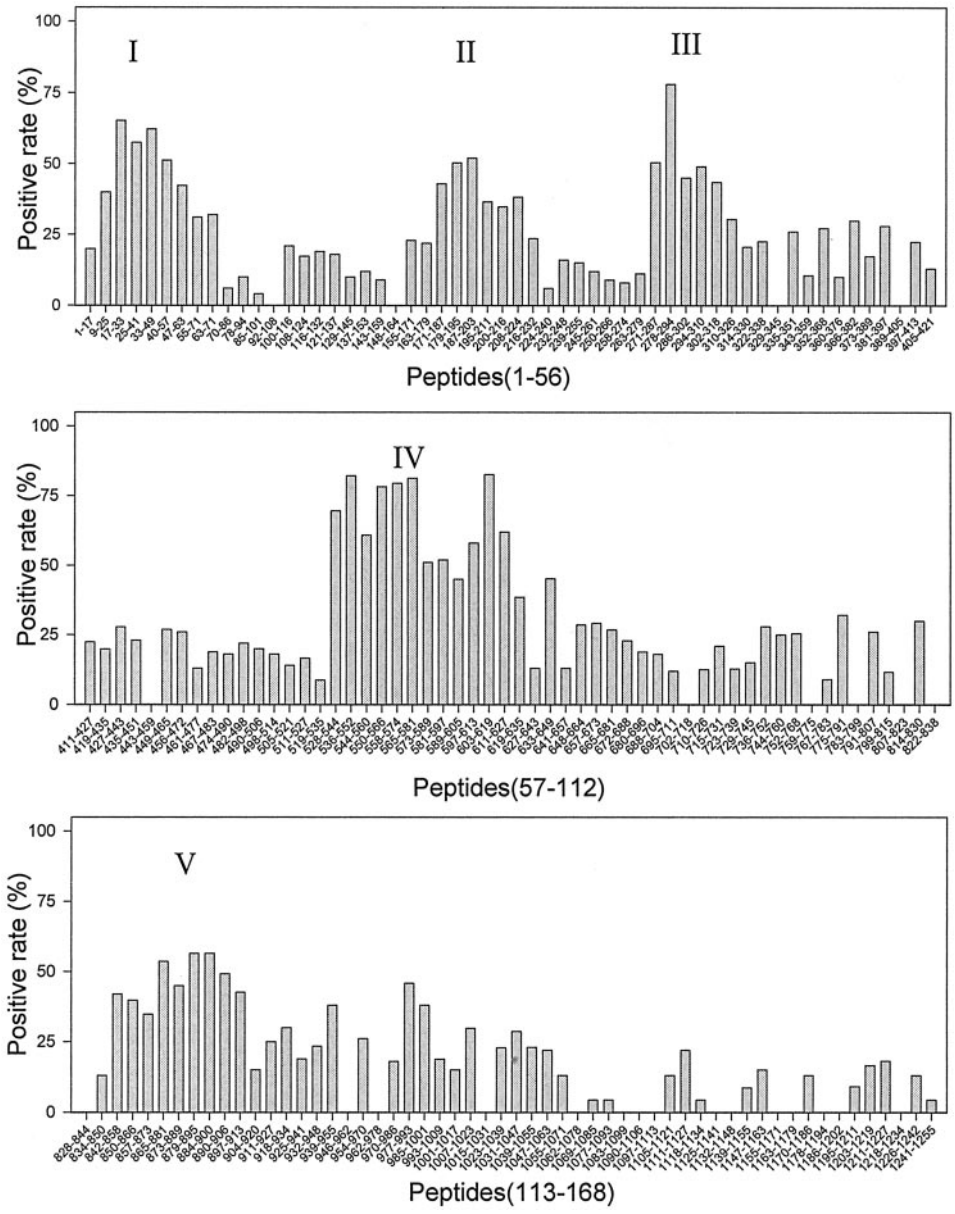

FIGURE 2. Mapping of the immunodominant epitopes on the SARS-CoV S protein by Pepscan analysis. A set of overlapping peptides that cover the entire S protein sequence were used to coat plate, and sera (1/50 dilution) from 42 SARS patients and 30 healthy blood donors were tested in ELISA. Sera were considered positive when the OD values were above the cutoff value (the mean OD of absorbance at 405 nm of sera from healthy blood donors plus 3 SDs). The positive rate of SARS sera for each peptide was calculated.

patients, and site IV was reactive with >80% of SARS sera, suggesting that site IV is the major immunodominant epitope on the S protein.

Identification of immunodominant epitopes on the S protein in mice and rabbits immunized with inactivated SARS-CoV

To determine the antigenicity and immunogenicity of the S protein in small animals, mice and rabbits were immunized with the inactivated SARS-CoV, and their sera were tested for Abs specific for SARS-CoV S protein. As shown in Fig. 3, all of the mice and rabbits, after the third boost immunization, developed substantial Ab responses against S1 protein, whereas the naive sera from control mouse and rabbit did not react with the tested Ags. This indicates that the S protein is a major Ag in the inactivated viruses that elicit humoral immune responses in the small animals.

The immunodominant epitopes on the S protein that induce Ab response in small animals were determined by Pepscan analysis of the overlapping peptides against the antisera from mice and rabbits immunized with inactivated SARS-CoV. As shown in Fig. 4, among the 168 peptides tested, only three of them (536–552, 544–560, and 603–619) reacted significantly with the three mouse antisera. All three reactive peptides reside within the immunodominant site IV that was identified by the convalescent sera from SARS patients. Similarly, peptide 536–552 corresponding to the N-terminal sequence of the site IV reacted with all of the antisera from the three rabbits immunized with inactivated SARS-CoV (Fig. 5), suggesting that the major immunodominant domain (site IV) induces Ab response not only in humans, but also in mice and rabbits. Besides reacting with site IV, the rabbit antisera also reacted with peptides 17–33 and 278–294 derived from immunodominant sites I and III, indicating that other antigenic sites on the S protein may function differently in humans, rabbits, and mice.

Detection of Abs specific for the S protein in SARS patients with synthetic peptides overlapping the sequences of the immunodominant epitopes

To approach the possibility of using synthetic peptides as Ags for developing SARS diagnostics, we designed and synthesized a set of five longer peptides that cover the immunodominant sites based

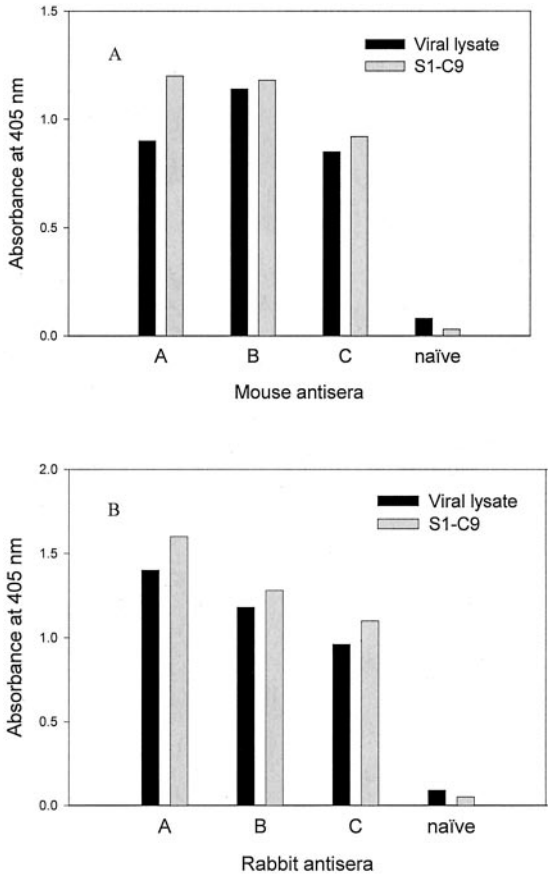

FIGURE 3. Anti-SARS-CoV Ab responses in small animals immunized with inactivated SARS-CoV. The antisera from mice (*A*) and rabbits (*B*) at 1/100 dilution were tested against the SARS-CoV lysates and the rS1-C9 protein.

on the above finding and tested their reactivity with the convalescent sera from SARS patients. Peptide S_{19-48} and $S_{278-312}$ were derived from immunodominant sites I and III, respectively, whereas peptides $S_{511-552}$, $S_{536-566}$, and $S_{603-634}$ overlapped with sequence of the major immunodominant site IV (Table I). One peptide ($S_{368-419}$) derived from immune silence region was included as a control. As shown in Fig. 6, all of the peptides derived from the immunodominant sites were highly reactive with most convalescent SARS sera, whereas control peptide from the immunosilent region had only weak reactivity with some of sera. Peptide $S_{603-634}$ derived from site IV reacted significantly with all 42 SARS serum samples, but did not react with any of control sera from healthy blood donors, suggesting that this peptide may serve as an ideal Ag for SARS diagnosis. To further verify the specificity of these longer peptides, we tested 25 serum samples from hepatitis patients who were infected by either hepatitis B virus or hepatitis C virus. None of these peptides cross-reacted with the sera from the hepatitis patients (data not shown).

Computational analysis of antigenic sites on the S protein

The antigenic sequences on the S protein were predicted by a semiempirical method based on a statistics of appearance frequency of each amino acid in known segmental epitopes. Interestingly, all of the immunodominant sites (sites I to V) contain multiple predicted antigenic sequences (Table II). Sites I, IV, and V contain three predicted antigenic sequences, and sites II and III have two antigenic sequences. The longest predicted antigenic sequence is aa 590–620, which is located in site IV, the major immunodominant epitope.

Discussion

In the present study, we demonstrated that the recombinant SARS-CoV S1 protein reacted with all of the convalescent sera from the SARS patients, but were not reactive with any serum samples from the healthy blood donors (Fig. 1*B*), consistent with results obtained with the commercial kit containing the mixture of proteins purified from SARS-CoV lysates (Fig. 1*A*). These suggest that SARS-CoV S protein, among other SARS-CoV proteins, is a major Ag that induces Ab responses in SARS patients. To determine the immunodominant epitopes on the S protein, we used Pepscan analysis against the convalescent sera from SARS patients. We identified five linear immunodominant sites on the S protein (Fig. 2). Sites I to IV are localized in the S1 domain, whereas site V is located in the S2 domain. These results indicate that the S protein of SARS-CoV contains multiple linear immunodominant sites that are capable of inducing site-specific Ab response during infection. Its antigenic sites are mainly located in the S1 domain, because it is the peripheral fragment of the viral envelope glycoprotein which is exposed to the immune system, thereby being able to induce high levels of Ab response. The S2 domain is the membrane-spanning fragment that may be buried in the S1 peripheral region in the native state. Therefore, it may not be accessible to immune effector cells, resulting in low immunogenicity or immunosilence.

Among the five immunodominant epitopes, site IV located in the middle region of the S protein (aa 528–635) reacted with >80% of the convalescent sera from SARS patients, suggesting that it is a major antigenic determinant on the S protein that elicits a strong Ab response in SARS-CoV infected individuals. This is consistent with the reports by Lu et al. (28) and Wang et al. (29), in which they demonstrated that the fragments of aa 441–700 and 599–620, which overlap with sequence of site IV, also reacted with most SARS sera tested. We further showed that several peptides derived from site IV were strongly reactive with the antisera from mice and rabbits immunized with inactivated SARS-CoV (Figs. 4 and 5), suggesting that this site is an immunodominant epitope shared by humans and animals. Therefore, the peptides overlapping the site IV sequence may be used as Ags to develop diagnostic tests for detecting S-specific Abs in the sera of SARS-CoV-infected individuals. To verify this assumption, we synthesized five peptides overlapping the sequences of sites I, III, and IV, and tested their reactivity with serum samples from SARS patients and healthy blood donors. We found that only the peptide $S_{603-634}$, which overlaps the site IV sequence, is positive to all the SARS sera tested, but negative to all of the normal control sera (Fig. 6), confirming that the peptides or recombinant proteins corresponding to the sequences of the immunodominant site IV can be used as Ags to develop immunodiagnostics. It appears that the longer peptide $S_{603-634}$ has greater reactivity with antisera than the shorter peptides that were used in the Pepscan analysis. This may be due to the potential additive effects or conformational effects. Indeed, $S_{603-634}$ mixed with either $S_{278-312}$ or $S_{511-552}$ had better reactivity with SARS convalescent sera than $S_{603-634}$ alone (data not shown), suggesting that the sensitivity of peptide-based assay for detecting Abs to SARS-CoV could be improved by using peptide mixture. Because this immunodominant epitope is shared by humans and animals, the above peptides and recombinant proteins can be used for immunizing animals to produce polyclonal Abs or mAbs to the major immunodominant epitope. These Abs may be used for designing sandwich ELISAs to detect Ags containing the

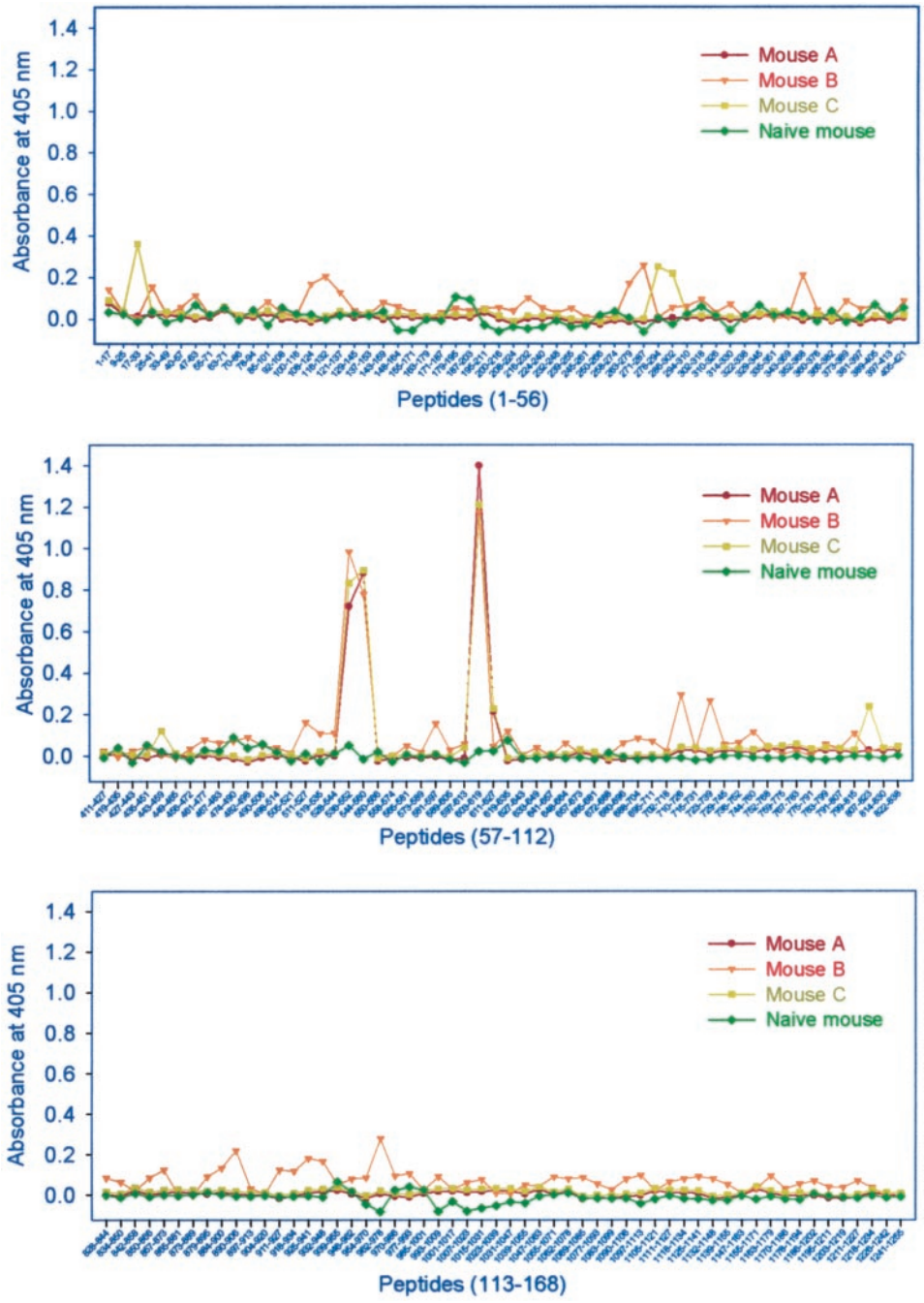

FIGURE 4. Pepscan analysis against the antisera from mice immunized with inactivated SARS-CoV. Reactivity of the mouse antisera at 1/100 dilution with the peptides overlapping the entire sequences of the S protein was measured by ELISA.

sequence of site IV. The ELISA-based diagnostic kits, if well-developed, may be used for early diagnosis of SARS-CoV infection before emergence of Abs to SARS-CoV in serum.

It was reported that a candidate vaccine containing inactivated SARS-CoV has been tested in phase I clinical trial in China, but concerns remain over the risk of such a vaccine enhancing, instead of inhibiting, SARS infection (30). It is expected that the S protein is the major Ag in the vaccine to induce protective immune response. However, the antigenicity and immunogenicity of the S protein have not been thoroughly studied. In this study, we showed that sera from mice and rabbits that were immunized with the inactivated SARS-CoV reacted significantly with the S protein (Fig. 3), confirming that this protein is a major Ag in the virus that induces substantial Ab responses in the immunized animals. Pepscan analysis against mouse antisera indicated that only the peptides overlapping the sequence of major immunodominant site IV could induce Ab response in the mice immunized with the inactivated SARS-CoV. The rabbit antisera could react with the peptides derived from the immunodominant sites I, III, and IV. None of the peptides derived from S2 domain reacted with mouse and rabbit antisera. These results indicate that, although some immunodominant epitopes in human do not induce humoral immune response in small animals, the major immunodominant epitope (site IV) is capable of eliciting site-specific Abs in different species. However, it is unclear whether these Abs have virus-neutralizing activity or not. Some immunodominant epitopes on the S protein S1 domain of murine hepatitis virus can induce virus-neutralizing Abs (21, 22). However, we previously demonstrated that rabbit antisera directed against the peptides derived from the immunodominant domain on the HIV-1 envelope glycoprotein did not neutralize, but rather enhanced HIV-1 inhibition (31). We are now using the peptides derived from the immunodominant sites to immunize animals

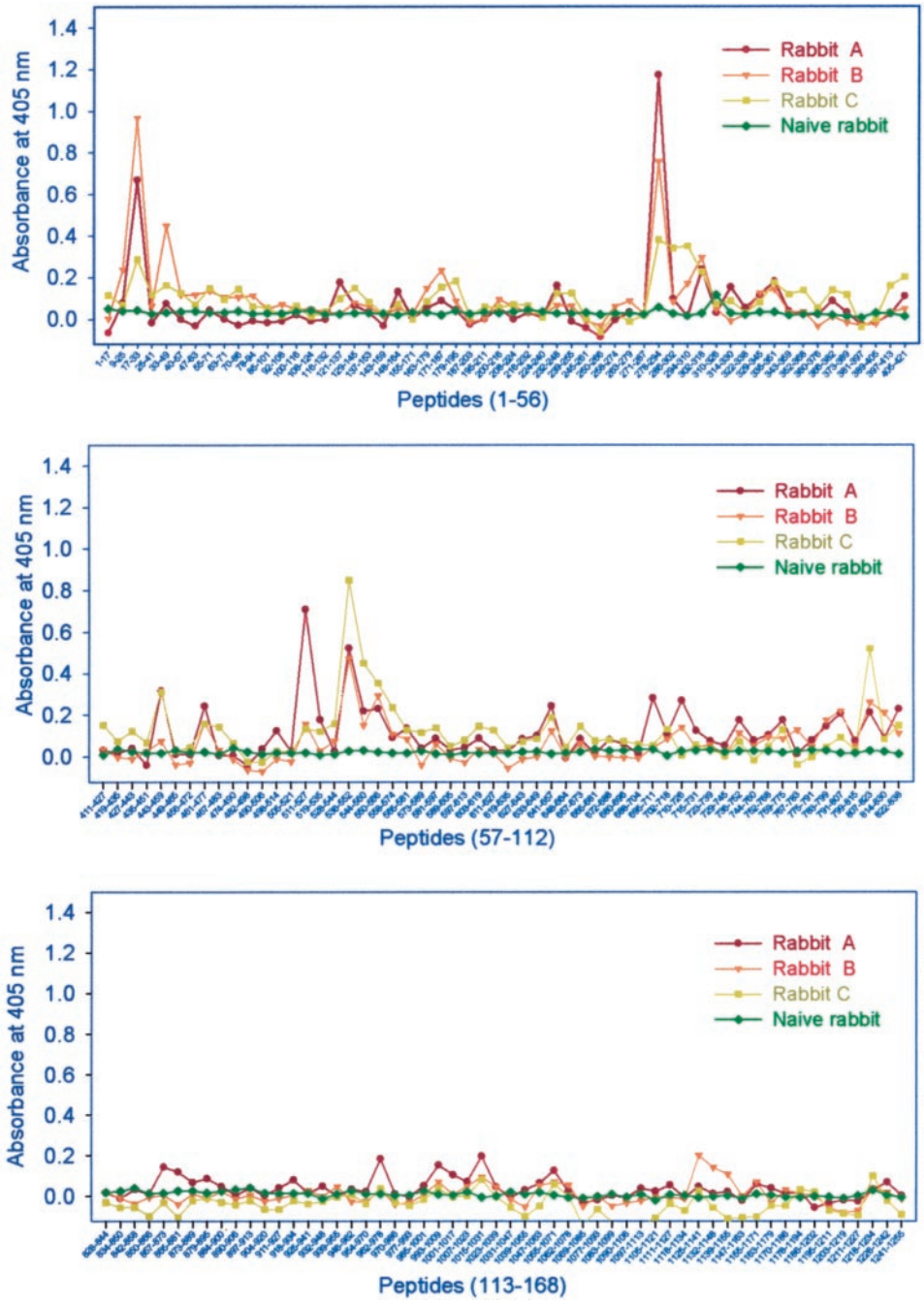

FIGURE 5. Pepscan analysis against the antisera from rabbits immunized with inactivated SARS-CoV. Reactivity of the rabbit antisera at 1/100 dilution with the peptides overlapping the entire sequences of the S protein was measured by ELISA.

for producing polyclonal Abs and mAbs, and will test their neutralizing or enhancing activity against SARS-CoV. The sequences of the immunodominant domains that elicit virus-neutralizing Abs will be included in a candidate subunit vaccine. However, the sequence of the immunodominant domains that induce nonneutralizing or enhancing Abs should be eliminated from any potential vaccines.

Although the pathogenesis of SARS has not been defined, lung injury, the major pathological change in SARS patients (32), may be caused by harmful inflammatory and immune responses induced by some Ags in SARS-CoV. This was the rationale of using immunosuppressants (e.g., steroids) for the early treatment of SARS-CoV infection, although controversies of using these drugs remain (33, 34). It is interesting to study the role of the immunodominant epitopes on the S protein and Abs elicited by these epitopes in the pathogenesis of SARS. For example, these peptides or recombinant proteins containing the immunodominant epitopes or Abs directed against these epitopes may be used as probes to investigate the potential effects on the inflammatory factors or immunoregulators. These may provide some clues for understanding the pathogenesis of SARS.

Table I. *Peptides derived from the immunodominant sites of the S protein*

Peptides	Sequences
S_{19-48}	CTTFDDVQAPNYTQHTSSMRGVYYPDEIFR
$S_{278-312}$	CSQNPLAELKCSVKSFEIDKGIYQTSNFRVVPSGD
$S_{511-552}$	CGPKLSTDLIKNQCVNFNFNGLTGTGVLTPSSKRFQPFQQFG
$S_{536-566}$	GVLTPSSKRFQPFQQFGRDVSDFTDSVRDPK
$S_{603-634}$	CTDVSTAIHADQLTPAWRIYSTGNNVFQTQAGC
$S_{368-419}$	CFSNVYADSFVVKGDDVRQIAPGQTGVIADYNYKLPDDFMGC

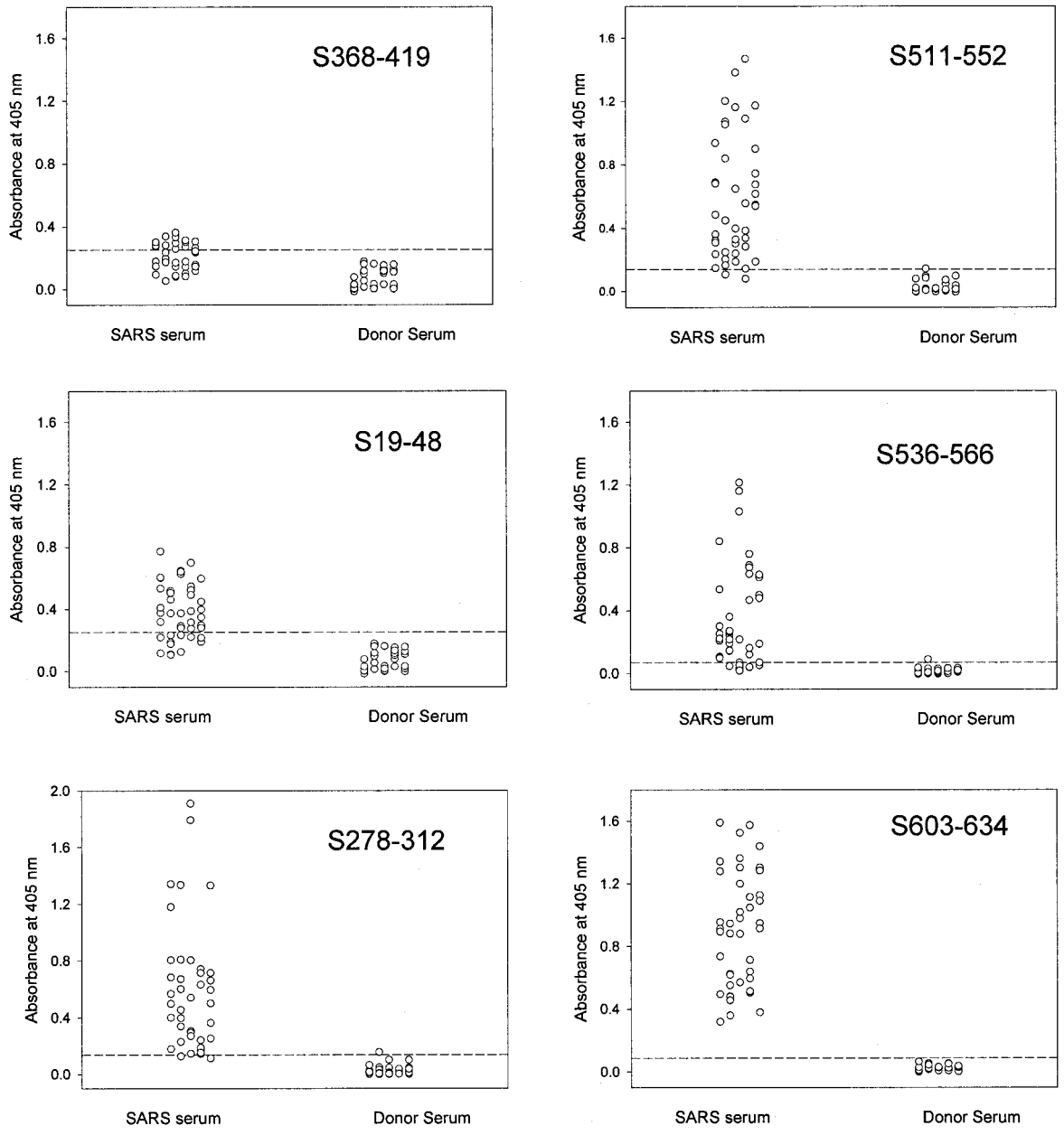

FIGURE 6. Detection of Abs in the convalescent sera from SARS patients against the synthetic peptides derived from the immunodominant epitopes on the S protein by ELISA. Sera from 42 SARS patients and 30 healthy blood donors were tested at 1/50 dilution. The dashed lines represent the cutoff values (the mean absorbance at 405 nm of sera from healthy blood donors plus 3 SDs).

Table II. *Computational prediction of antigenic sequences on the S protein of SARS-CoV*

Immunodominant Sites	Predicted Antigenic Sequences
Site I (aa 9–71)	TFDDVQAPN (aa 21–29) MRGVYYPD (aa 37–44) RSDTLYLTQDLFLPFYSNVTG (aa 48–68)
Site II (aa 171–224)	GFLYVYKGYQPIDVVRDL (aa 192–209) TLKPIFKLPL (aa 215–224)
Site III (aa 271–318)	ITDAVDCSQNPLAELKCSVKS (aa 274–292) SNFRVVPSGDVVRF (aa 303–316)
Site IV (aa 528–625)	TGTGVLTP (aa 533–540) EILDISPCSFGGVSVIT (aa 569–585) ASSEVAVLYQDVNCTDVSTAIHADQLTPAWR (aa 590–620)
Site V (aa 842–913)	IAAYTAALVSG (aa 852–862) AGAALQIPF (aa 872–880) VTQNVLY (aa 893–899)

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