

Characterization of neutralizing monoclonal antibodies recognizing a 15-residues epitope on the spike protein HR2 region of severe acute respiratory syndrome coronavirus (SARS-CoV)

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Summary

The spike (S) glycoprotein is thought to play a complex and central role in the biology and pathogenesis of SARS coronavirus infection. In this study, a recombinant protein (rS268, corresponding to residues 268–1255 of SARS-CoV S protein) was expressed in *Escherichia coli* and was purified to near homogeneity. After immunization with rS268, S protein-specific BALB/c antisera and mAbs were induced and confirmed using ELISA, Western blot and IFA. Several BALB/c mAbs were found to be effectively to neutralize the infection of Vero E6 cells by SARS-CoV in a dose-dependent manner. Systematic epitope mapping showed that all these neutralizing mAbs recognized a 15-residues peptide (CB-119) corresponding to residues 1143–1157 (SPDVLDLGDISGINAS) that was located to the second heptad repeat (HR2) region of the SARS-CoV spike protein. The peptide CB-119 could specifically inhibit the interaction of neutralizing mAbs and spike protein in a dose-dependent manner. Further, neutralizing mAbs, but not control mAbs, could specifically interact with CB-119 in a dose-dependent manner. Results implicated that the second heptad repeat region of spike protein could be a good target for vaccine development against SARS-CoV.

Introduction

It is now well-documented and strong evidence supporting that a new emerging coronavirus (CoV) is etiologically linked to the outbreak of Severe Acute Respiratory Syndrome (SARS) in Asia and Canada last year [1–3]. SARS associated CoV has several major structural proteins: membrane (M) and spike (S) glycoprotein, and nucleocapsid (N) and envelop (E) proteins [4]. Of these structural proteins, it is thought that the S protein

is a multifunctional protein that plays complex and central role in the biology and pathogenesis of SARS infection [4]. Major known properties and functions of the coronavirus S protein had been found to include: (a) binding to specific receptor glycoproteins on the surface of host cells; (b) after binding to receptor the S protein possible conformational changes causing induction of fusion of the viral envelop with the cell membrane; (c) cell fusion facilitating viral RNA entry into host; (d) a target for induction of the neutralizing antibody and (e) elicitation of cell-mediated immunity [4]. It had been demonstrated the N-terminal 600 amino acids (S1 domain) associated with altered

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antigenicity and virulence [5,6]. Actually, there were considerable diversity in both the lengths and nucleotide sequences of the S1 derived from different groups of coronaviruses, and sometime even within different strains of a single family of coronavirus [7–10]. Alignment of the amino acid sequences of the SARS-CoV S (GI 29836496) and HCoV-229E spike proteins (GI 13604334) had revealed only 29% identity. This indicated that there was very considerable diversity of S proteins between SARS-CoV and other human coronavirus (HCoV-229E and HCoV-OC43).

A rapid treatment of SARS patient is urgently needed. Therefore, it is very necessary to develop an effective and safe vaccine to prevent the infection of SARS-CoV. In this study, we evaluated the neutralization effect of mAbs to the spike protein of SARS-CoV, and mapped the interaction region of spike protein with neutralizing mAbs. It is anticipative to find out conserved neutralizing epitope(s) of spike protein that can be used as a vaccine target or a therapeutic agent against SARS-CoV.

Materials and methods

Bacterial strains and expression vectors

Escherichia coli TOPO10 was the plasmid used in the cloning SARS spike protein, and *Escherichia coli* BL21 Star (DE3) was used for over expression of proteins under the control of phage T7 *lac* promoter. The plasmid vector pET101/D-TOPO (QIAGEN, K101-01) was used to express the histidine-tagged fused at the carboxyl-terminus of S protein to generate recombinant plasmid, rS_{268–1255}.

Construction of plasmid expressing recombinant spike protein of SARS-CoV

The mRNA was extracted from Vero E6 cells infected with SARS-CoV isolated from suspected-SARS patients using the QIAamp Viral RNA Mini Kit (Qiagen) according to the instruction of manual. Extracted mRNA was resuspended into TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) and used as a template in the RT-PCR for amplification of amino acids 268–1255 of the SARS coronavirus. Oligo-dT-18 (5'-TTTTTTTTTTTTTTTTTTT-3')

and one pair of primers corresponding to nucleotides 25241–25215 (22278–22295 and 25241–25215 (CoV268pET: 5'-CACC-ATggAAAATggTACAATCACA-3'; and CoV25241pET: 5'-TgTgTAA TgTAATTTgACACCCTTgAg-3') of the SARS-CoV spike protein-encoding sequences were designed based on the published sequences (GenBank accession no. NC_004718). The PCRs were carried out with an initial denaturation step of 94 °C for 5 min followed by 30 cycles of denaturation (94 °C for 1 min), annealing (50 °C for 1 min), and extension (68 °C for 4 min), with a final prolonged extension step (68 °C for 10 min). The amplified coding sequences were inserted into the pET101/D-TOPO vectors to generate the plasmid, pET101/D-TOPO-S₂₆₈. Recombinant plasmid DNA was sequenced and correct coding sequence was confirmed.

Virus neutralization assay

The ability of antisera from immunized mice to inhibit SARS-CoV virus infection of Vero E6 cells was assessed by virus neutralization assay. To prepare virus stock solution for the neutralization assays, Vero E6 cells were infected with SARS-CoV (GenBank accession no. GI 29836496) and incubated at 37 °C in 5% of CO₂ for 3 days. The infectious virus stock with TCID₅₀, was calculated using the method of Reed and Muench (1938) [11], and 1×10⁷ TCID₅₀ of the virus stock solution was aliquoted into individual tubes and stored at -70 °C. For virus neutralization testing, 2×10⁵ Vero E6 cells/ml was inoculated onto a 12-well tissue culture plate (Falcon #3043, 96-well flexible plate) at 37 °C in 5% of CO₂ overnight. Pre-immune serum and antiserum of BALB/c raised against the recombinant SARS-CoV spike protein were pre-treated at 56 °C for 30 min to destroy heat-labile, non-specific viral inhibitory substances, and diluted to the beginning dilution 1/20 with DMEM maintenance medium, then added into a well containing 2×10⁴ TCID₅₀ of the virus in a volume of 0.15 ml. MAbs were diluted to 50, 20, 5, and 1 µg per 0.15 ml. Equal volumes of the serum or mAb solution and the test-virus dilutions were mixed and incubated at 37 °C for 1 h. Then, the serum-virus mixtures and virus controls (no sera) were inoculated into Vero E6-containing culture plates, which had been pre-washed with DMEM maintenance medium and emptied just

prior to the addition of serum–virus mixtures into culture plates. After absorption at 37 °C (or at 4 °C for virus entry-inhibition assay) for 2 h, the wells were washed with DMEM maintenance medium and then 2% of fetal calf serum/DMEM buffer was added. After 24 h incubation at 37 °C, the wells were washed twice with PBS (pH 7.4), lyses buffer was added. The microplates were stored at –70 °C until SDS-PAGE/Western blot analysis for the presence of virus replication. The 90% virus neutralization titer is calculated when the SARS-CoV viral protein were not detected in SDS-PAGE/Western blot analysis.

Western immunoblotting analysis

For the identification of purified rS₂₆₈ protein, equal amounts of lysates from *Escherichia coli* BL21 containing mock or induced recombinant spike protein, and purified protein were boiled and loaded onto an SDS-PAGE as described below. Purified fusion proteins were detected with mouse antihistidine antibody (1/1000; Amersham Pharmacia Biotech) by Western immunoblot analysis. For confirmation of the neutralization effect of antiserum against the recombinant SARS-CoV spike protein, equal amounts of virus-infected cell lysates (1/100 of total lysates, 10 µl) were boiled in a sample buffer (125 mM Tris–HCl, [pH 6.8], 100 mM DTT, 2% SDS, 20% glycerol, 0.005% brophenol blue) for 5 min, and then loaded onto an 8% SDS-polyacrylamide gel. After electrophoresis of SDS-PAGE, specific proteins were detected with BALB/c anti-rS₂₆₈ polyclonal antibody (our laboratory) or mouse anti-β actin monoclonal antibody (Sigma, cat. no. SI-A5441) by Western blotting described previously [12].

Purification of recombinant protein

Recombinant proteins were expressed and purified using the denature method, as described previously with some modification [12]. In brief, *Escherichia coli* BL21DE3 bacteria, containing the plasmid pET-S_{268–1255}, were used for prokaryotic expression and purification of histidine-tagged proteins. After dialysis and brief centrifugation in a Sigma 3K12 centrifuge 5402 (14,000 rpm for 5 min) at 4 °C, the supernatant was quickly frozen in liquid nitrogen and stored at –80 °C.

Preparation of antisera against recombinant SARS-CoV spike protein

Normal healthy BALB/c and rat sera were obtained from the Animal Center of the Institute of Preventive Medicine of the National Defense Medical Center, Taiwan. All animals were confirmed healthy by a licensed veterinarian. Antisera against spike protein were prepared by three subcutaneous inoculations (15, and 5 µg of recombinant proteins mixed with Freund's adjuvant for rat, and BALB/c, respectively; complete adjuvant and incomplete adjuvants for the first, and for the second and third inoculations, respectively, at an interval of 1 month). One month after the last immunization, the animals were bled and the sera centrifuged at 3000 × g for 10 min at 4 °C in a Sigma 3K12 centrifuge with a Nr. 12154 rotor after 2 h of agglutination at room temperature. Antisera were collected and mixed with 50% glycerol and stored at –20 °C.

Preparation of monoclonal antibodies

For immunization, BALB/c mice received an intraperitoneal (i.p.) injection of 5 µg of recombinant spike protein (rS₂₆₈) in 100 µl of PBS emulsified with an equal volume of complete Freund's adjuvant. After an interval of 2 weeks and 4 weeks, booster injections were given as above, except that we used incomplete Freund's adjuvant instead. Three weeks after the third injection, final boosters containing 5 µg of antigen were administered via i.p. injection. Fusion was performed 5 days after the last injection with the spleen cells of the donor mouse. Hybridomas secreting anti-spike antibodies were generated according to the standard procedure [13]. Hybridoma colonies were screened by ELISA, and selected clones were subcloned by the limiting dilution method. Immunoglobulin classes and subclasses were determined using subtyping kit (Roche Diagnostics, Penzberg, Germany). Ascitic fluids were produced in pristane-primed BALB/c mice. Monoclonal antibodies were purified using protein A affinity chromatography, and stored in 1 µg mAb per ml at –80 °C.

Indirect immunofluorescent assay (IFA)

To check the SARS-CoV spike protein-recognizing capabilities of mAbs against recombinant spike

protein, an IFA [14] was performed. Briefly, the Vero E6 monolayer cells infected with SARS-CoV were washed three times with PBS and then fixed in acetone–Methanol mixture (1:1) for 3 min at room temperature. After blocking with 3% skim milk in PBS for 1 h at room temperature, an IFA assay was performed. Each stained monolayer was viewed with an immunofluorescent microscope (LEICA, DMIRB).

ELISA

The 96-well microtiter plates (Falcon, #3912) were coated with antigens (0.2 µg/ml purified recombinant rS268-truncated protein [268–1255 amino acid] in 0.05 M carbonate buffer, pH 9.6) at 4 °C overnight. Bound mAbs were detected using secondary antibody (goat anti-mouse IgG-HRP, 37 °C for 1 h). After washing, ABT (Boehringer) or (TMB or OPD (*o*-phenylenediamine, Sigma, P-6787)) substrate was added and stayed for 30 min (ABT, 15 min for TMB, and 30 min for OPD) at room temperature. Reaction was stopped with 1N H₂SO₄, and specific SARS-CoV IgG was detected by OD_{405nm} (ABT, OD_{405nm} for TMB) endpoint reading. Experiments were performed as described previously [12]. Sera were always assayed in duplicate; each plate also included an air blank, as well as negative control and positive controls.

Synthetic peptides competitive-inhibition assay

For the competitive-inhibition assay, different concentrations of synthetic peptides (CB119, CB119IA, SP-SGNCD, SP-SGIAA, SP-DLG, and SP2; as illustrated in Table 1) were mixed with mAbs before being transferred to the rS268 recombinant protein-coated plates and incubated for 1 h. After washing, the plates were incubated with HRP-conjugated goat anti-mouse Ig antibodies (1:2000 in PBST) (ICN Biomedicals) at 37 °C for 30 min, and the procedures described above were followed.

Labeling and purification of peptide probe

Synthetic peptide (SP_{1143–1172}, 100 µg) was labeled with Digoxigenin (Roche, Digoxigenin-3-*O*-methylcarbonyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide ester, cat. no. 1 333 054) according to the

instruction of manual. Labeled peptide was purified by passage through a NucTrap purification column (Stratagen, cat. no. 400702) according to the manufacturer's instructions. It was then concentrated to 100 µl (1 µg/µl) using CENTRICON centrifugal filter devices (Millipore, cat. no. YM-3000) according to the instruction of manual.

Gel migration shift assay (EMSA)-western immunoblotting analysis

For gel migration shift assay, 1 µl of Digoxigenin-labeled probe (SP_{1143–1172}-Dig) was incubated with mAbs (1 µl of Mab 5, 1/25 and 1/100; or Mab 6, 1/25) in the presence or the absence of cell lysates (Vero cell lysates, 1/100 of total lysates; or SARS-CoV virus-infected cell lysates, 1/500, 1/200, and 1/100 of total lysates), in the reaction buffer (20 mM Hepes, pH 8.0, 25 mM KCl, 10% glycerol, 2 mM MgCl₂, 0.5 mM dithiothreitol) for 30 min at room temperature. Then 2 µl of 10 × sample dye (0.5 × TBE, 10% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) was added in and probe–protein complexes were separated from the free probe by electrophoresis at 4 ° on a 5% native polyacrylamide gel. After electrophoresis, the gel was transferred to Hybond-C extra membrane. The membrane was blocking and further incubated with Anti-Digoxigenin-POD, Fab fragment (Roche, cat. no. 1 207 733). After finally washing with blocking buffer, the membrane was developed with ECL Western Blotting Reagent as described above in Western Immunoblotting Analysis.

Epitope mapping of SARS-CoV spike protein

Microtiter plates coated with different fusion proteins or synthetic peptides (D2-TM, S2-Fc, S3, rRBD2, D1 and D2 long peptide, CB116–CB123, CB124–131, CB132–138, SP1, SP2, PEP508, PEP509, and NP; 1 µg/well in carbonate buffer at 4 °C for overnight) were incubated with diluted mAbs (1–1, 3–2, 5–1, and 8–1; 1:5000 in PBS) at 37 °C for 1 h after blocking. Bound mAbs were detected using secondary antibody (goat anti-mouse IgG-HRP, 37 °C for 1 h). After washing, ABT (Boehringer) or TMB substrate was added and stayed for 30 min (ABT, 15 min for TMB) at room temperature. Reaction was stopped with 1 N H₂SO₄, and specific SARS-CoV IgG was detected by OD_{405nm} (ABT, OD_{405nm} for TMB) endpoint

Table 1. Fusion proteins and synthetic peptides used in this study.

Peptides	Sequences	Residues
D1-TM	D1(74–253)-GGGGGGGG-TM(1130–1255)	(74–253)-8G-(1130–1255)
D2-TM	D2(294–739)-GGGGGGGG-TM(1130–1255)	(294–739)-8G-(1130–1255)
S1-Fc	S1(1–333)-human IgG1 Fc (baculovirus expressed)	(1–333)-IgG1 Fc
S3	S3(667–999) (<i>Escherichia coli</i> expresses)	667–999
RBD2	RBD2(294–739) (<i>Escherichia coli</i> expresses)	294–739
CB116	DSFKEELDKYFKNHT	1128–1142
CB117	ELDKYFKNHTSPDVD	1133–1147
CB118	FKNHTSPDVDLGDIS	1138–1152
CB119	SPDVDLGDISGINAS	1143–1157
CB120	LGDISGINASVVNIQ	1148–1162
CB121	GINASVVNIQKEIDR	1153–1167
CB122	VVNIQKEIDRLNEVA	1158–1172
CB123	KEIDRLNEVAKNLNE	1163–1177
CB124	LNEVAKNLNESLIDL	1168–1183
CB125	KNLNESLIDLQELGK	1173–1188
CB126	SLIDLQELGKYEQYI	1178–1193
CB127	QELGKYEQYIKWPWY	1183–1198
CB128	YEQYIKWPWYVWLGF	1188–1203
CB129	KWPWYVWLGFIAGLI	1193–1208
CB130	VWLGFIAGLIAIVMV	1198–1213
CB131	IAGLIAIVMVTILLC	1203–1218
CB132	AIVMVTILLCCMTSC	1208–1223
CB133	TILLCCMTSCCSCLK	1213–1228
CB134	CMTSCCSCLKGACSC	1218–1233
CB135	CSCLKGACSCGSCCK	1223–1238
CB136	GACSCGSCCKFDEDD	1228–1243
CB137	GSCCKFDEDDSEPV	1233–1248
CB138	FDEDDSEPVLKGVKL	1238–1253
SP1	NQCVNFNFNGLTGTGV	522–537
SP2	SSNFGAISSVLNDILSRDKV	949–969
PEP508	GDYSHCSPLRYYPWWKCTYPDP	ACE2 inhibitor
PEP509	TDAVDCSQNPLAELKCSVKSF	273–293
CB1191A	SPDVDLGDASGINAS	1151I → A
CB119GA	SPDVDLGDISAINAS	1153G → A
SP-DLGIA	DLGDASGINASVVNI	1151I → A
SP-DLG	DLGDISGINASVVNI	1147–1161
SP-SGNCD	SGNCDSGNCDSGNCDSGNCD	SGNCD, 4 repeats
SP-SGIAA	SGIAASGIAASGIAASGIAA	SGIAA, 4 repeats
SP _{1143–1172}	SPDVDLGDISGINASVVNIQKEIDRLNEVA	1143–1172
SP _{917–946}	QESLTTTSTALGKLQDVVNQNAQALNTLVK	917–946

reading. Each plate also included blanks and negative controls.

ELISA cutoff value

Background reactivity and possible cross-reactivity were assessed by analyzing pre-immune serum

specimens from healthy rabbits, mice, and rats. The cutoff values were set at: $OD_n + 3 SD$, where OD_n is the mean of ODs recorded or the pre-immune serum or mock specimens. Those ODs greater than the calculated threshold ODs regarded as positive sera and all others regarded as negative.

Synthetic peptides and recombinant S fragments used in monoclonal antibodies mapping

The recombinant S fragments are: D1-TM (D1 fragment is residues 74–253 then linked with 8 Gly as linker to TM fragment residues 1130–1255) expressed in *Escherichia coli*; D2-TM (D2 fragment is residues 294–739 then linked with 8 Gly as linker to TM fragment residues 1130–1255) expressed in *Escherichia coli*; S1-Fc (S1 fragment is residues 1–333 linked to human Fc fragment of IgG1) expressed in baculovirus system; S3 (residues 667–999) expressed in *Escherichia coli*; rRBD2 (residues 294–739) expressed in *Escherichia coli*. CB116–CB138 peptide sequences are from residues 1128–1255, 15-mer overlapping by 10 residues (Kelowna, Taipei, R.O.C.) (Table 1).

Results

Purification and characterization of recombinant SARS-CoV spike protein

The coding sequence, corresponding to amino acids 268–1255 of the S protein of SARS coronavirus, was amplified using RT-PCR and cloned into the prokaryotic expressional vector containing a 6-histidine tag coding sequence, pET101/D-TOPO, to produce pET101/D-TOPO-S₂₆₈ plasmid. The expression of recombinant protein was induced by IPTG and the recombinant protein was purified to near homogeneity using Ni²⁺-NTA agarose affinity chromatography (Figure 1a, lane 5 for rS₂₆₈). Purified recombinant protein was confirmed with mouse antihistidine antibody as described in Materials and Methods. As shown in Figure 1b, purified rS₂₆₈ protein indeed to be a histidine-tagged fusion protein. To determine whether *Escherichia coli* expressed recombinant spike protein could conserve its immunological and biological properties, SARS CoV-specific antibodies from SARS patients were used to detect the purified recombinant with ELISAs. Using rS₂₆₈ as coating antigen, ELISAs were performed to measure SARS-specific IgG in the sera of controls and suspected SARS patients. Recombinant protein rS₂₆₈ was recognized by the IgG of the SARS patients (S4, #0612, S45, S25, S18, Pt-Shi, and Pt-Yea), but not by control sera (S3, S31 and #284) (Figure 1c). In short, the recombinant protein could be detected by SARS-specific

IgG in SARS-CoV infected patients implicated that rS₂₆₈ might conserve its antigenic characterization and epitopes.

Characterization of animal antibodies against recombinant spike proteins

The polyclonal antibodies from rat, and BALB/c sera raised against purified rS₂₆₈ protein, were tested and confirmed to recognize SARS coronavirus spike proteins using Western blot, and indirect immunofluorescent assay (IFA) (Figure 2, panel a, and b). As illustrated in Figure 2a, mouse anti-rS₂₆₈ antiserum recognized the spike glycoprotein in SARS-CoV-infected Vero E6 cell lysate (lane 2). The antibody reactivity titer against SARS-CoV spike protein by ELISA was found to be above 25,600 (for rat anti-rS₂₆₈ IgG), and 68,260 (for BALB/c anti-rS₂₆₈ IgG) after the last immunization (data not shown).

When mAbs raised against rS₂₆₈ were assayed for their capabilities to recognize SARS-CoV spike protein using an IFA assay. As illustrated in Figure 2b, mAbs (Mab 1, 3 and 5) specifically recognized SARS-CoV in virus-infected Vero E6 cells (panels E, D, and F). To characterize monoclonal antibodies, the isotype of each mAb was analyzed using IsoStrip mouse mAb isotyping kit (Roche). As shown in Table 2, most of mAbs were IgG1.

SARS patient's serum also recognized SARS-CoV infected Vero cells in the IFA assay (Figure 2b, panel H), while sera from mouse pre-immune or control patient did not (panels A, G). Taken all results together, the antisera against rS₂₆₈ could recognize and bind to the spike protein of SARS-CoV.

Biological activities of antisera and monoclonal antibodies

In order to characterize the neutralization of antisera raised against recombinant SARS spike proteins, virus-neutralization assays were performed to test the efficacy of BALB/c mice mAbs raised against rS₂₆₈. As shown in Figure 3a, some of mAbs (Mab 1, 3, 5, 7, 8) had significant virus neutralizing effect (< 10 µg per 10⁴pfu/10⁵ cells), some (Mab 2, 4, and 6) had a little virus neutralizing effect at this concentration (lanes 2, 4, 6), while some (Mab 9, 11, 12, and 13) had no

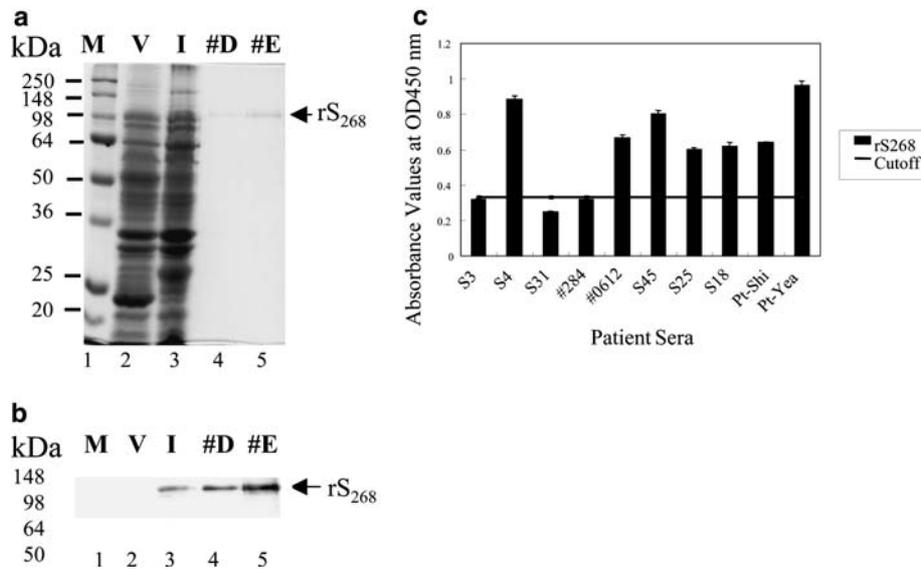


Figure 1. Purification and Characterization of recombinant SARS-CoV spike protein in *Escherichia coli*. The coding sequence for spike protein of SARS-CoV virus was amplified and cloned into prokaryote expression vector pET101/D-TOPO, then the spike protein was induced to express in the BL21 DE3 *Escherichia coli* strain. (a) Purification of recombinant truncated spike protein (rS₂₆₈, a.a. 268–1255). M, marker; V and I, total cell lysates from *Escherichia coli* transformed with vector and spike protein-coding sequences respectively; #D and #E, fractions eluted with buffer D and E respectively. Histidine-tagged spike proteins were purified by nickel chromatography as described in Materials and Methods. Proteins were resolved by SDS-8% PAGE and stained with Coomassie blue. (b) Detection of recombinant spike protein (rS₂₆₈) by Western blot. Purified rS₂₆₈ protein was confirmed to be histidine-tagged fusion protein with mouse antihistidine antibody as described in Materials and Methods. M, V, I, #D and #E is the same as mentioned above. (c) Detection of SARS-CoV IgG from patient sera by recombinant spike protein-base ELISA. Microtiter plates were coated with recombinant proteins (rS₂₆₈). After blocking, diluted control (S3, S31, and #284) and confirmed (S4, #0612, S45, S25, S18, Taichung-Shi, and Yanming-Yea) patient sera were incubated at 37 °C for 30 min. Specific SARS-CoV IgG was detected by ELISA as described in Materials and Methods. Error bars indicate standard deviations of duplicate tests. Each plate also included an air blank, as well as a negative and a positive control. The cutoff values were set at OD_n+3 SD, where OD_n is the mean of ODs of normal human serum.

neutralizing effect (lanes 9–12). Further studies indicated that these neutralizing mAbs could neutralize SARS CoV infection in Vero cell in a dose-dependent manner (Figure 3b, lanes 1–6 for Mab 1, 3, and 5), while others could hardly inhibit virus infection (Figure 3b, lanes 7–8 for Mab 13). Related result was summarized and tabled in Table 2. Results revealed that recombinant rS₂₆₈ protein maintained some conserved antigenic epitopes of SARS-CoV spike glycoprotein, so that antisera and mAbs raised from this recombinant protein were capable of neutralizing the infection of Vero E6 cells by SARS-CoV.

Monoclonal antibodies bind to the heptad repeat region of spike protein

To elucidate the mechanism of neutralization as well as to map the neutralizing epitope of spike

protein, a systematic epitope-mapping assay was performed using synthetic peptides derived from S protein and mAbs by ELISA. As shown in Figure 4a, neutralizing mAbs (Mab 1, 3, 5, and 8) bound to recombinant S protein fragments common with the trans-membrane domain (D1-TM and D2-TM). Further epitope mapping shown that all these neutralizing mAbs recognized peptides covering residues 1128–1168 (CB 116–123), as illustrated in Figure 4b. Actually, all these neutralizing mAbs specifically bound to an individual synthetic peptide, CB119, which only comprised of a 15-amino acid (SPDVDLGDIGINAS) and was located at the tip of one heptad repeat region (HR2) of the SARS-CoV spike protein (Figure 4c). The binding of antibodies to this heptad region was specific and sufficiently to neutralize the infection of VERO E6 cells by SARS-CoV, since all strong neutralizing mAbs

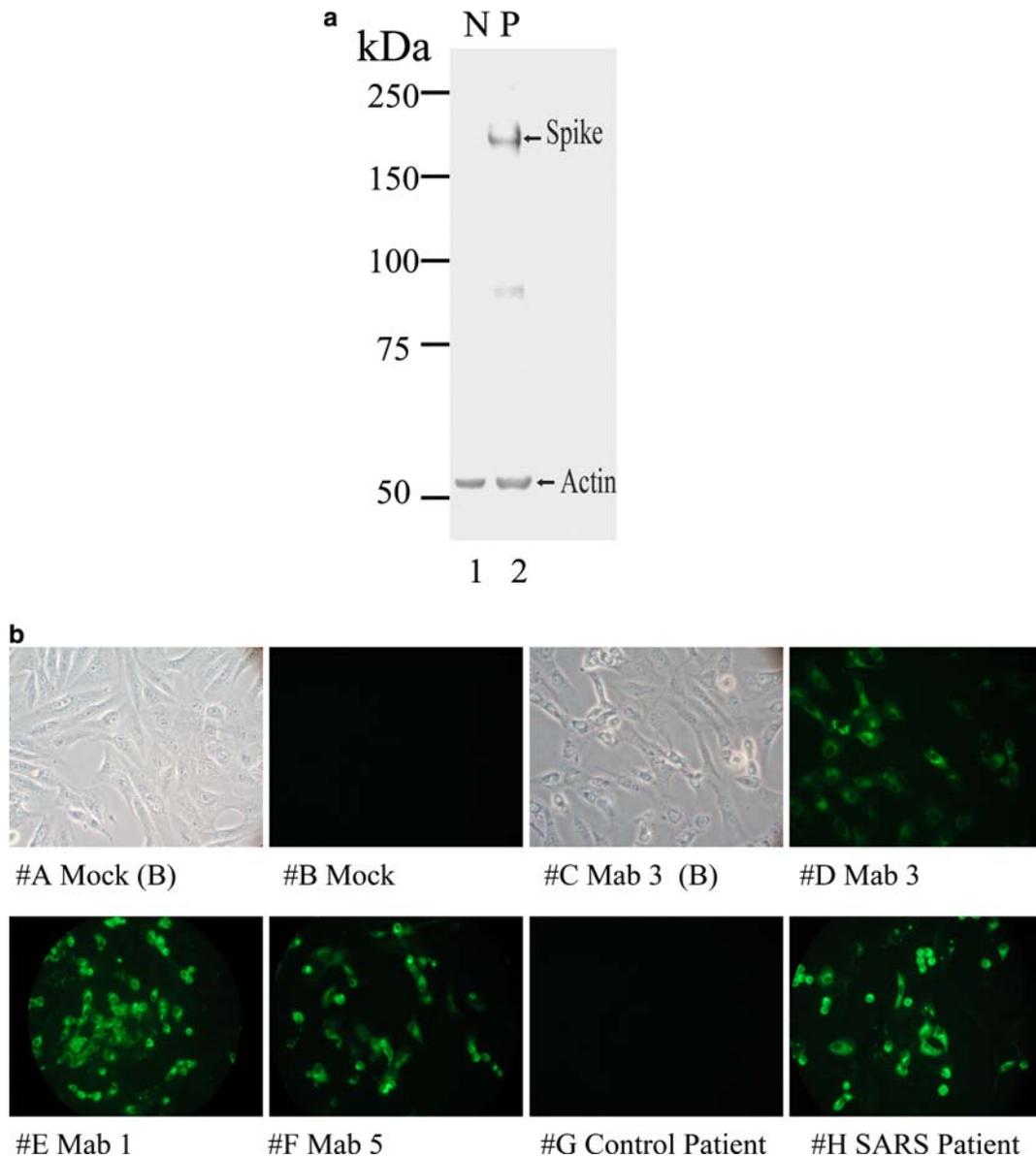


Figure 2. Detection of SARS coronavirus using mice antisera and monoclonal antibodies against recombinant SARS-CoV spike proteins. (a) Mouse antiserum against recombinant spike proteins recognized SARS coronavirus. After immunizing with purified recombinant rS₂₆₈ proteins, polyclonal antibodies of small animals were obtained and reacted with SARS-CoV spike protein by Western blot, as described in Materials and Methods. N denotes Vero E6 cell lysate, and P denotes the lysate of Vero E6 cells infected with SARS-CoV. (b) Vero E6 cells-infected with SARS-CoV (48 h post infection) was assayed by immunofluorescent assay (IFA), as described in Materials and Methods. #A. Mock (B), bright field; #B. Mock, IFA; #C. Mab 3 (B), bright field; #D. Mab 3, IFA; #E. Control Patient; #F. Mab 1; #G. Mab5; #H. SARS patient. Virus-infected cells were stained with mice pre-immune serum (1:200), mAb Mab 1 (1:1000), mAb Mab 3 (1:1000), mAb Mab 5 (1:1000), control patient serum (1:200), and SARS patient serum (1:200), respectively. Each stained monolayer was examined by an immunofluorescence microscope (LEICA, DMIRB) at original magnifications $\times 400$.

(Mab 1, 2, 3, 5, 7, and 8) bound to CB119 peptide but not for the weak or non-neutralizing mAbs (4, 6, 9, 12, and 13) (Figure 5a). Results from this

study revealed that mAbs binding to the heptad repeat region of spike protein could sufficiently neutralize the infection of SARS-CoV.

Table 2. Immunological characterization of monoclonal antibodies.

mAbs	Isotype	ELISA titer	Neutralization
Mab 1	IgG1	> 64,000	800
Mab 2	IgG1	> 64,000	50
Mab 3	IgG1	> 64,000	> 200
Mab 4	IgG1	> 64,000	< 50
Mab 5	IgG1	> 64,000	200
Mab 6	IgG2a	> 64,000	–
Mab 7	IgG1	> 64,000	< 200
Mab 8	IgG1	> 64,000	< 200
Mab 9	IgG1	> 64,000	–

Isotypes of monoclonal antibodies against spike protein of SARS-CoV was determined using IsoStrip mouse monoclonal antibody isotyping kit (Roche). Numbers in parentheses mean serum titers that can neutralize the infection of Vero E6 cells by SARS-CoV (10^4 pfu/ 10^5 cells). ‘–’ means monoclonal antibody had no virus neutralization activity per 10^3 pfu/ 10^5 cells.

Synthetic peptides mimicking the neutralization epitope compete with the interaction of monoclonal antibodies and spike protein

In order to reveal the neutralization mechanism of mAbs against spike protein of SARS-CoV, different synthetic peptides mimicking the neutralization epitope (CB119, corresponding to 1143–1157 amino acid of spike protein), its mutant, and other controls were used to test their competition abilities for inhibiting the interaction of mAbs and spike protein. As illustrated in Figure 5b, CB119 peptide, but not control peptide SP2, inhibited neutralizing mAbs (Mab 1 and 2) binding to spike protein in a dose-dependent manner. Meanwhile, CB119 peptide could not efficiently inhibit non-neutralizing mAb (Mab 6) binding to spike protein. The competitive inhibition of mAbs-spike protein interaction by CB119 was specific, since control peptides (SP2, SP-SGNCD, SP-SGIAA, SP-DLG) had no inhibition effect (Figure 5c). Further characterization of mAb-HR2 interaction by ELISA, as illustrated in Figure 5d, neutralizing mAbs have differential features for their binding to synthetic peptides mimicking various amino acid residues of heptad repeat region of spike protein. For example, some mAbs (Mab 1, 5, 7, and 8) bound to CB119IA peptide almost equally to wild type peptide (CB119); some did not (Mab 2 and 3). Results from these data implicated that neutralizing mAbs inhibiting the infection of SARS-CoV through specially binding

to the amino acid residues 1143–1157, located on the HR2 region, of spike protein.

Neutralizing monoclonal antibodies bind to synthetic peptides mimicking amino acid residues of HR2 region of spike protein in a dose-dependent manner

Aforementioned results implicated that mAbs might neutralize the infection of cells by SARS-CoV through specially binding to HR2 region of spike protein. To test this hypothesis, an EMSA assay was performed to examine the binding characterization of mAbs to synthetic peptides. This experiment used a Digoxigenin-labeled synthetic peptide (SP_{1143–1172}-Dig) as a probe. When there is no binding protein, it should migrate faster in polyacrylamide gel. Once there is binding protein (or antibody), the peptide-antibody complex should migrate slower than free peptide. Both free peptide and peptide-antibody complex should bind to POD-conjugated Anti-Digoxigenin secondary antibody and be detected by ECL substrate. As illustrated in Figure 6, synthetic peptide probe (SP_{1143–1172}-Dig) representing HR2 heptad repeat region specially bound to neutralizing mAb (Mab 5) in a dose-dependent manner (lanes 8–9), but not to non-neutralizing mAb (Mab 6) (lane 10). When lysates of SARS-CoV infected cells was added in, the peptide-mAb complex was competed by SARS-CoV virus-infected cell lysates in a dose-dependent manner (lanes 4–6). Result from this study showed that mAbs specifically recognizing the heptad repeat region might neutralize the infection of cells by SARS-CoV in a dose-dependent manner with differential features. Thus, it implicates that it might be practicable to prevent human infection of SARS-CoV by recombinant subunit vaccine or synthetic peptides mimicking amino acid residues of heptad repeat region of spike protein.

Discussion

The SARS pandemic had great impacted on the health and economic integrity of countries all over the world associated with transmission of this novel pathogen. Effective vaccines and drugs are important research candidates for the prevention and therapy of this novel viral disease.

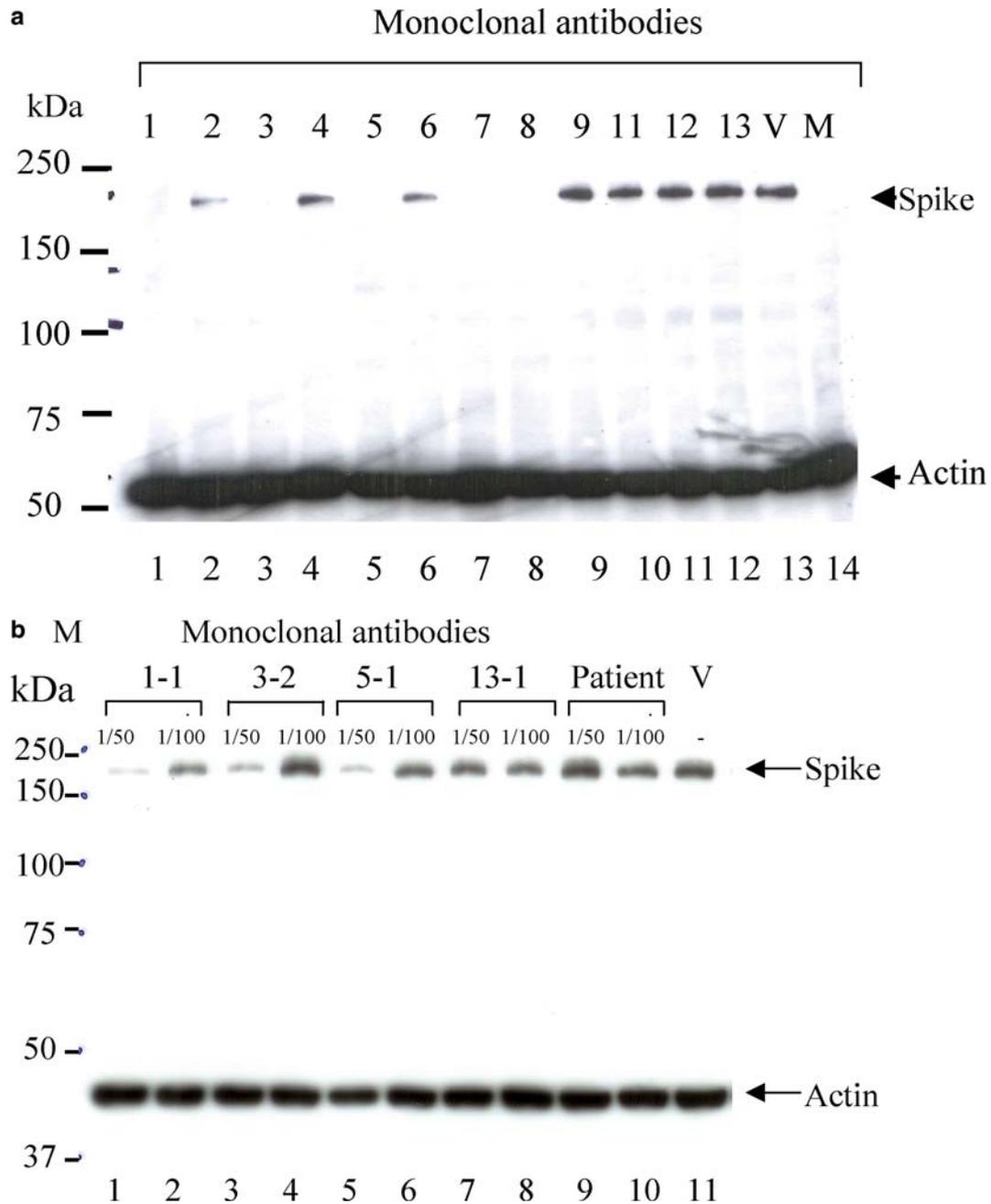


Figure 3. Neutralizing SARS-CoV infection in Vero E6 cells using monoclonal antibodies. Random selected mAbs (1/100 = 10 μ g of mAb) were mixed with equal volumes of virus dilutions, and neutralization was performed as described in Materials and Methods. Viral spike glycoprotein was resolved using SDS-PAGE (8%) and assayed by Western blotting. (a) Neutralizing SARS-CoV infection using mAbs against recombinant SARS-CoV spike protein. Lanes 1–12, different mAbs against recombinant spike protein (rS₂₆₈); lane 13, SARS-CoV-infected cell lysates; lane 14, Vero E6 cell lysates. (b) Monoclonal antibodies inhibit SARS-CoV entering into Vero E6 cells. Equal volumes of serum–virus dilutions were mixed and inoculated to Vero E6-containing culture plates for absorption at 4 °C for 2 h, and following procedures were performed as described in Materials and Methods. Lanes 1–8, serial dilutions of different monoclonal antibodies; lanes 9–10, serial dilutions of SARS patient serum; lane 11, SARS-CoV-infected Vero E6 cell lysates. The ‘1/50’ and ‘1/100’ denotes sera dilutions of 1:50 and 1:100 in PBS, respectively. The ‘-’ denote no antiserum was added. For comparisons of viral spike protein, β -actin was included as an internal control.

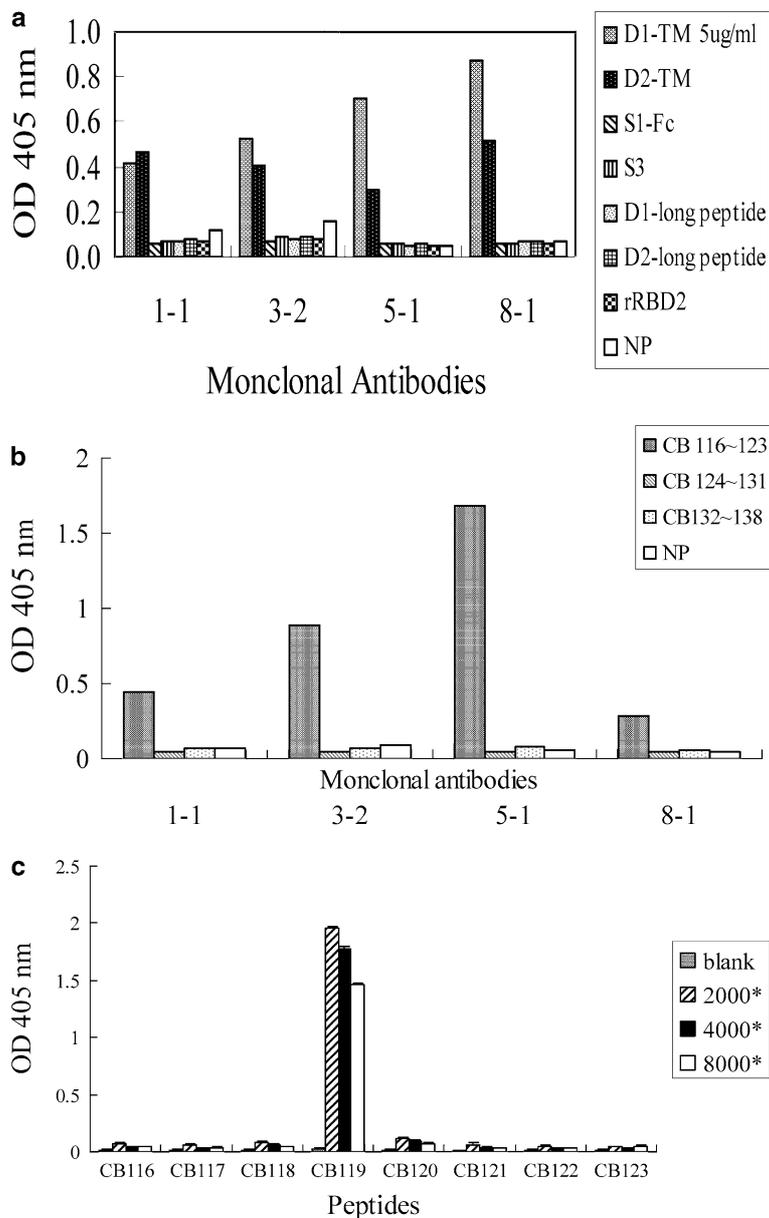


Figure 4. Epitope mapping of SARS-CoV spike protein with monoclonal antibodies by ELISA. (a) Mapping the interaction region of SARS-CoV spike protein with mAbs using fusion proteins or long synthetic peptides (D2-TM, S2-Fc, S3, rRBD2, D1 and D2 long peptide, and NP). (b) Mapping the interaction region of SARS-CoV spike protein with mAbs using synthetic peptides covering TM region (CB116–CB123, CB124–131, CB132–138, and NP). (c) Mapping the interaction region of SARS-CoV spike protein with mAbs using individual synthetic peptides (CB116–CB123). Microtiter plates were coated with different fusion proteins or synthetic peptides as described in Materials and Methods. The wells were then incubated with diluted mAbs (1–1, also for 3–2, 5–1, and 8–1; 1:500, 1:1000, and 1:2000 in PBS, respectively) at 37 °C for 1 h after blocking. Bound mAbs were detected by ELISA, as described in Materials and Methods. Each plate also included blanks and negative controls.

Therefore, the infection and the viral entry mechanism is important target for these works. In the present studies, we examined the biological properties of *Escherichia coli* expressed recombinant spike protein and the immunological prop-

erties of small animal antisera raised against recombinant rS₂₆₈ proteins compared to those obtained from SARS patients. Mouse mAbs generated from immunizing recombinant spike proteins were evaluated for their biological

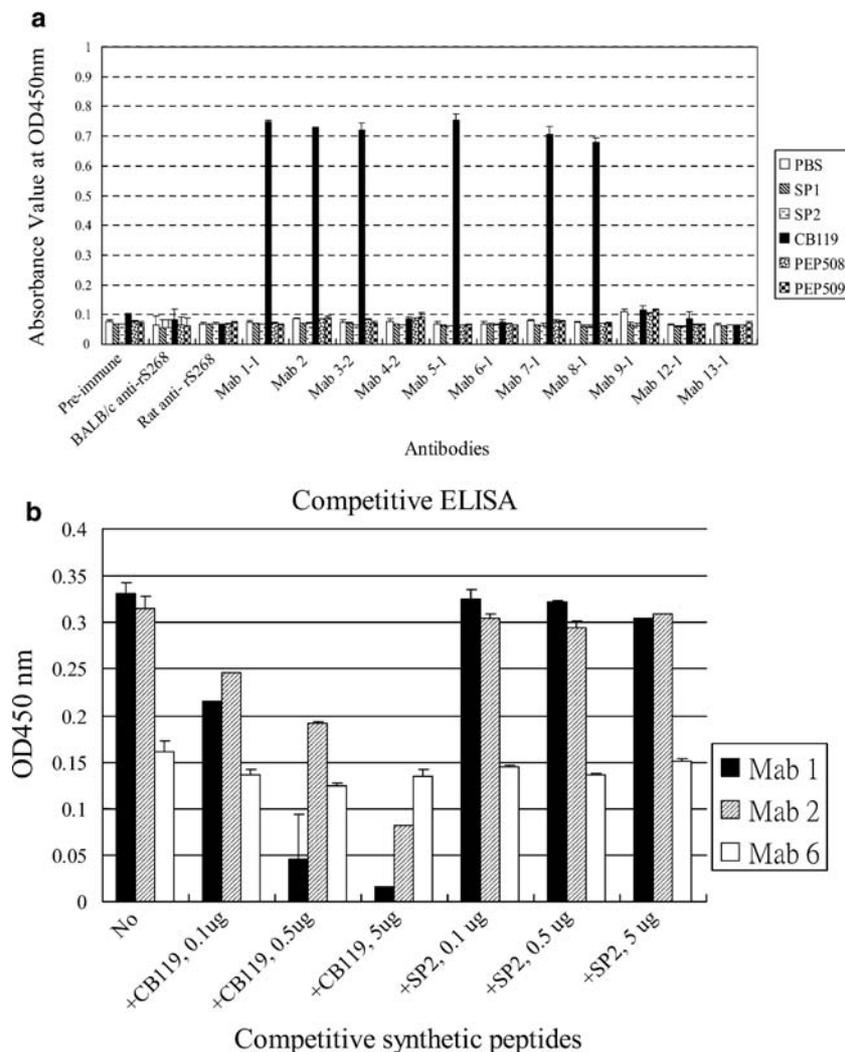


Figure 5. Neutralizing monoclonal antibodies bind to a specific synthetic peptide mimicking heptad repeat region of SARS-CoV spike protein. (a) Neutralizing mAbs bind to a specific peptide antigen mimicking HR-C region of spike protein. Microtiter plates coated with different synthetic peptides (SP1, SP2, CB119, PEP508, and PEP509; 1 μ g/well in carbonate buffer at 4 $^{\circ}$ C for overnight) were incubated with diluted mAbs (1/1000 in PBST) at 37 $^{\circ}$ C for 1 h after blocking. (b) Synthetic peptide mimicking neutralizing epitope specifically competed the binding of neutralizing mAbs to spike protein. Different concentrations (0.1, 0.5, and 5 μ g) of synthetic peptides CB119 and SP2 were mixed with mAbs before being transferred to the rS268 recombinant protein-coated plates and incubated for 1 h. (c) Synthetic peptide CB119 competed the binding of neutralizing mAbs to spike protein in a dose-dependent manner. Different concentrations (0.05, 0.1, 0.2, 0.4, and 0.8 μ g) of synthetic peptides CB119IA, SP-SGNCD, SP-SGIAA, and SP-DLG were mixed with mAbs before being transferred to the rS268 recombinant protein-coated plates and incubated for 1 h. (d) Monoclonal antibodies differently bind to HR2 region of spike protein. Microtiter plates coated with different synthetic peptides (as illustrated in Table 1) were incubated with diluted mAbs (1/1000 in PBST) at 37 $^{\circ}$ C for 1 h after blocking. All bound mAbs [in (a) (b) (c) (d)] were detected by ELISA, as described in Materials and Methods. Error bars indicate standard deviations of duplicate tests. Each plate also included blanks, as well as negative controls.

activities in virus neutralization against SARS-CoV, and then mapped out and characterized the virus neutralization epitope(s).

Since SARS-CoV spike protein was a glycoprotein, it could be argued that lack of protein modification in prokaryotic cell expression systems

would not retain the native conformation and folding of S protein. However, as shown in Figure 1c, the purified recombinant proteins appeared to be recognized by antibodies from the sera of SARS-CoV infected patients. Further, the antisera induced by recombinant rS₂₆₈ proteins did

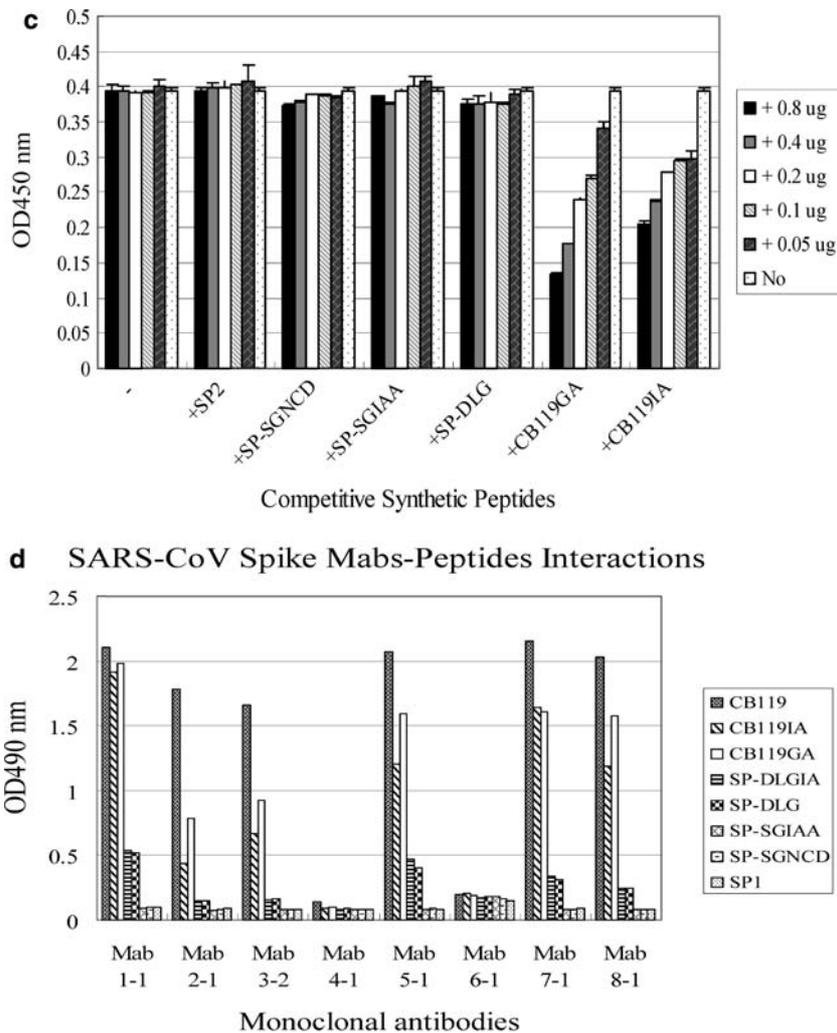


Figure 5. Continued.

recognize and captured SARS-CoV spike glycoprotein in Western blot, IFA, and capture sandwich ELISA (Figure 2a, b, and data not shown). Actually, several lines of evidence demonstrated that spike protein of SARS-CoV interacted with human ACE2 receptor in a glycosylation-independent manner [15]. A recombinant spike protein expressed in *Escherichia coli* could induce protective immunity against SARS-CoV [15]. Therefore, the recombinant rS₂₆₈ proteins were valuable and their induced antisera actually recognized the major characteristics and biological functions of spike protein (as shown in Figures 1, 2), even though they might lose some minor conformation-dependent antigenic epitopes related to glycosylation.

Small-animal antisera raised against recombinant spike proteins were evaluated for their neutralization effect in Vero cells infection by SARS-CoV. Rabbit antisera generated from recombinant S_{full} immunization had potent virus neutralization activity comparable to SARS patients' sera (data not shown). Meanwhile, mouse immunized with rS₂₆₈ did generate virus neutralizing antibodies against SARS-CoV. In the present studies, some of mAbs against recombinant spike protein had strong neutralization effect as seen in the virus neutralization assay; but some of mAbs had little or no neutralization effect (Table 2, Figure 3a, b). It suggested that different virus neutralizing antibodies recognized different biological important regions of spike protein. This

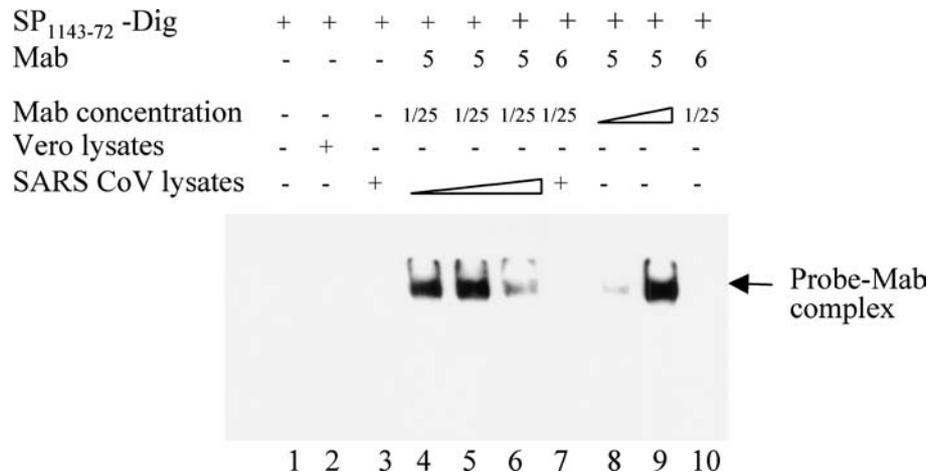


Figure 6. Neutralizing monoclonal antibodies bind to synthetic peptides mimicking amino acid residues of HR2 region of spike protein in a dose-dependent manner. Neutralizing monoclonal antibodies specially bind to HR2 region of spike protein in a dose-dependent manner. A Digoxigenin -labeled synthetic peptide (SP₁₁₄₃₋₁₁₇₂-Dig) was used as a probe in the gel migration shift assay (EMSA). The presence (+) or absence (-) of synthetic peptide probe (SP₁₁₄₃₋₁₁₇₂-Dig) and mAbs (Mab 5 and 6) are indicated above each lane of the photograph. For the interaction, 1 μ l of SP₁₁₄₃₋₁₁₇₂-Dig probe and mAbs (Mab 5, 1/25 for lanes 4-6, 9 and 1/100 for lane 8; or Mab 6, 1/25 for lane 7 and 10) were incubated at room temperature for 20 min in the presence or the absence of cell lysates (mock for lane 1; Vero cell lysates, 1/100 for lane 2; or SARS-CoV virus-infected cell lysates, 1/500 and 1/200 for lanes 4-5, and 1/100 for lanes 3, 6-7). The probe-antibody complex was separated from the free probe by electrophoresis on a 5% non-denaturing polyacrylamide gel. After this, Western blotting was finished as described in the Materials and Methods. Position of the probe-antibody complexes are indicated on the right.

was further supported that different mAbs have various binding activity for synthetic peptides mimicking spike protein (Figure 5c and d). As 10 μ g per 10^4 pfu/ 10^5 cells monoclonal antibodies [1,5,8,16,17] recognized the epitope (residues 1143-1157), could sufficiently neutralize SARS-CoV infection through blocking the virus entry into Vero E6 cells. The neutralization epitope (residues 1143-1157) mapped in this study was located at the tip of the heptad repeat region of spike protein, the HR-C domain. Thus, these mAbs likely neutralized the infection of SARS-CoV through inhibiting virus entry into Vero E6 cells with a mechanism that was different from virus-ACE2 receptor binding blocking mechanism reported by others [15]. Because viral-cellular membrane fusion mediated by several type I viral fusion proteins (for example, HIV gp160) could be inhibited by peptide mimicking either the HR-N or HR-C regions [17-27]. Author suggest that the HR1 and HR2 regions are crucial in the entry of SARS-CoV, and support the assumption that SARS-CoV may employ the same fusion mechanism as HIV-1 and other viruses with class I envelope proteins [28]. Studies indicate that after

SARS-CoV binding to the target cell, the transmembrane spike protein might change conformation by association between the HR1 (residues 902-952; 915-950; 889-926) and HR2 (1145-1184; 1151-1185; 1161-1178) regions to form an oligomeric structure, leading to fusion between the viral and target-cell membranes [28-31]. Therefore, HR2 has been suggested to use as a potent inhibitor peptides or a target for the development of therapeutic drug for SARS-CoV [28-33]. In this study, mAbs bound to HR-C regions and might inhibit the HR-N and HR-C forming coiled-coil structure that was required for viral-cellular membrane fusion, so neutralized the infection of SARS-CoV by blocking virus entry into Vero cells. However, our data showed rabbit antisera immunized with recombinant spike protein did not react with the CB119 peptide (data not shown). These rabbit antisera could have different virus neutralization effects. Recently, angiotensin-converting enzyme 2 (ACE2) was reported as a functional receptor for the SARS coronavirus in Vero E6 cells by Li et al. [34]. ACE2 receptor-associated epitope on SARS-CoV spike protein had been identified to be within residues 310-527

[15]. Further studies should be investigated to map the neutralization epitopes recognized by the rabbit antisera.

In summary, mouse mAbs raised against recombinant spike protein were effective and sufficient for neutralization of SARS-CoV infection against Vero E6 cells. Result from this study showed that mAbs, specifically recognizing the heptad repeat region of spike protein, might neutralize the infection of cells by SARS-CoV through binding to the neutralizing epitope located on the HR-C of spike protein. Further, monoclonal antibodies differently bind to synthetic peptides mimicking HR2 region implicated presence of several potential neutralizing epitopes. Thus, it might be practicable to prevent human infection of SARS-CoV by recombinant subunit vaccine or synthetic peptides mimicking optimal amino acid residues, such as virus neutralization

epitope SPDVDLGDIGINAS, of heptad repeat region of spike protein.

Acknowledgments

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Appendix 1

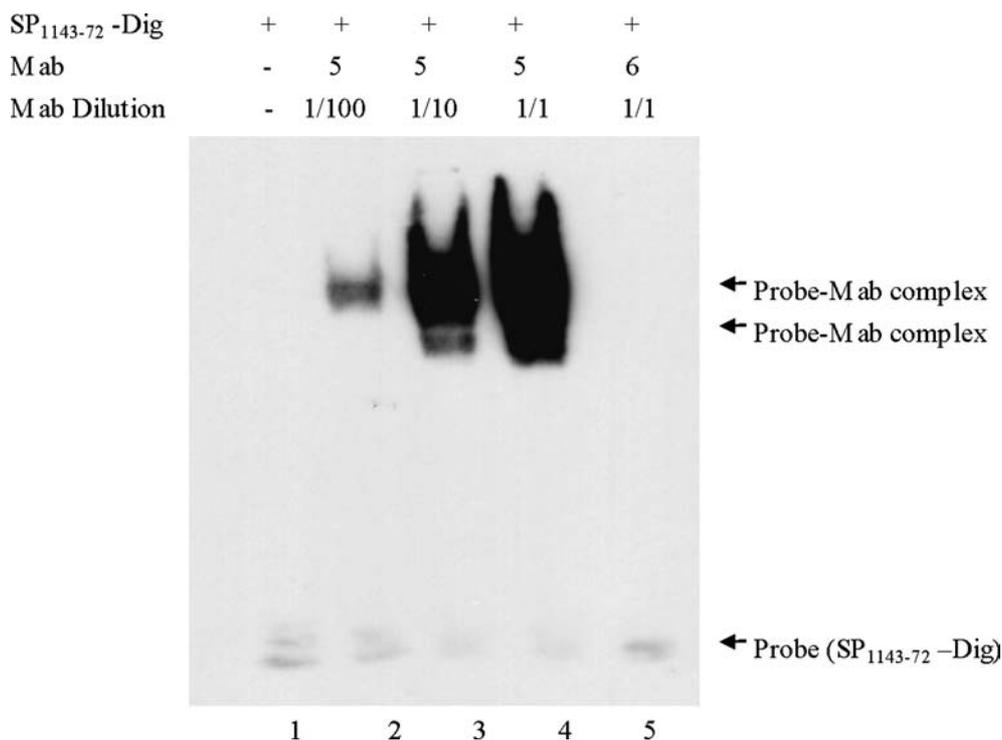


Figure A.1. Neutralizing monoclonal antibodies bind to synthetic peptides mimicking amino acid residues of HR2 region of spike protein in a dose-dependent manner. Neutralizing monoclonal antibodies specially bind to HR2 region of spike protein in a dose-dependent manner. A Digoxigenin-labeled synthetic peptide (SP₁₁₄₃₋₇₂-Dig) was used as a probe in the gel migration shift assay (EMSA). The presence (+) or absence (-) of synthetic peptide probe (SP₁₁₄₃₋₇₂-Dig) and mAbs (Mab 5 and 6) are indicated above each lane of the photograph. For the interaction, 1 μ l of SP₁₁₄₃₋₇₂-Dig probe and mAbs (Mab 5, 1/100, 1/10, and 1/1 for lane 2-4; or Mab 6, 1/1 for lane 5) were incubated at room temperature for 20 min. The probe-antibody complex was separated from the probe by electrophoresis on a 5 nondenaturing polyacrylamide gel. After this, Western blotting was finished as described in the Materials and Methods. Position of the probe-antibody complexes are indicated on the right.

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