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Original article

A single amino acid substitution in the S1 and S2 Spike protein domains determines the neutralization escape phenotype of SARS-CoV

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Abstract

In response to SARS-CoV infection, neutralizing antibodies are generated against the Spike (S) protein. Determination of the active regions that allow viral escape from neutralization would enable the use of these antibodies for future passive immunotherapy. We immunized mice with UV-inactivated SARS-CoV to generate three anti-S monoclonal antibodies, and established several neutralization escape mutants with S protein. We identified several amino acid substitutions, including Y442F and V601G in the S1 domain and D757N and A834V in the S2 region. In the presence of each neutralizing antibody, double mutants with substitutions in both domains exhibited a greater growth advantage than those with only one substitution. Importantly, combining two monoclonal antibodies that target different epitopes effected almost complete suppression of wild type virus replication. Thus, for effective passive immunotherapy, it is important to use neutralizing antibodies that recognize both the S1 and S2 regions.

Keywords: Escape mutants; Neutralizing antibody; Passive immunotherapy; SARS-CoV

1. Introduction

Severe acute respiratory syndrome (SARS) is caused by a novel coronavirus (SARS-CoV) that emerged as a serious epidemic between late 2002 and early 2003, during which more than 8000 people were infected, nearly 10% of whom died. Coronaviruses are enveloped, positive-strand RNA viruses that encode the structural Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N) proteins, as well as non-structural proteins [1]. The 1255 amino acid (aa) viral S protein mediates both cell attachment and membrane fusion. The S1 and S2 domains of the SARS-CoV are defined, although the S protein of SARS-CoV does not appear to be cleaved [2,3]. The viral receptor binding domain (RBD) of the S protein, located between residue 318 and 510 of the S1 domain [4], interacts with angiotensin-converting enzyme 2 (ACE2), which has been identified as the SARS-CoV receptor [5].

Although no SARS has been reported since 2004, a protective vaccine and reliable diagnostic should be available to control any outbreak that might re-emerge. Convalescent serum has been reported to contain high titers of IgG antibody against SARS-CoV [6], suggesting that anti-SARS-CoV antibodies could be useful for passive immunization against SARS. Among the different protein candidates recognized by the anti-SARS-CoV antibodies, the S protein has emerged as a major target for vaccine development [7]. In particular, the RBD of S1 contains important epitopes for neutralizing

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antibodies, although a neutralizing mAb that recognizes the S1 N-terminal region (aa 130–150) was also reported [8]. These findings suggest that SARS-CoV infection could be inhibited by interfering with the interaction between S1 and ACE2, and specifically the binding of ACE2 with the RBD of S1.

The S2 domain contains a putative fusion peptide [9] and two heptad repeat regions (HR1 and HR2) that associate to form a six-helix bundled structure [3]. The human immunodeficiency virus type 1 (HIV) gp41 virus was neutralized by monoclonal antibodies (mAbs) that recognized epitopes proximal to the viral membrane [10]. Similarly, antibodies targeting the membrane-anchoring or HR2 domain in the S2 region were able to neutralize SARS-CoV [11–13]. Furthermore, many neutralizing epitopes mapped to the S2 region were identified in the antisera of convalescent SARS patients [14]. Together this indicates that SARS-CoV can be neutralized by antibodies that target either the S1 or S2 domains.

We previously reported that high levels of anti-S and anti-N antibodies were induced in mice immunized with UV-inactivated SARS-CoV [15]. We generated anti-S mAbs from these mice and demonstrated their efficient neutralization of SARS-CoV infection in Vero E6 cells [16]. To determine the regions of these mAbs that are biologically important for neutralization, we selected neutralization-escape mutant clones that dominantly grew under the selection pressure of these neutralizing mAbs. We determined the amino acid substitutions in the mutant S proteins and analyzed the level of viral resistance conferred against the neutralizing antibody.

2. Materials and methods

2.1. Cells and viruses

Vero E6 cells (ATCC) were grown in Eagle's minimal essential medium (MEM) with non-essential amino acids (Invitrogen, Carlsbad, CA) and supplemented with 5% FBS (Japan Bioserum), 2 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate and antibiotics. SARS-CoV (HKU-39849) was kindly provided by Dr J.S.M. Peiris (Department of Microbiology, University of Hong Kong). The viruses were propagated and assayed using Vero E6 cells. A20.2J B cells expressing SARS-CoV S protein (A20.2J/S6.2) [17] were maintained in RPMI1640 supplemented with 10% FBS, 2 mM L-glutamine, 0.5 μg/ml blasticidin (Invitrogen) and antibiotics.

2.2. Neutralization assay

For plaque titration, approximately 100 pfu of SARS-CoV were incubated with serially diluted anti-S mAbs or control IgG_1 for 1 h at 37 °C, and then added to confluent Vero E6 cells in 6-well plates in duplicate. After 1 h of incubation, cells were overplayed with 1% low melting agarose (SeaPlaque, FMC Corp., Rockland, ME) in MEM supplemented with 10% FBS. After 3 days, virus was inactivated with 10% formalin/PBS and samples were stained with 10% formalin/PBS containing 0.1% crystal violet (Sigma-Aldrich). The number of plaques obtained in the presence of mAb was calculated as a percentage relative to those obtained in control mouse IgG_1, which alone did not largely affect plaque formation, even at 100 μg/ml (data not shown).

2.3. Immunofluorescence microscopy

Vero E6 cells were spread onto 8-well Lab-Tek chamber slides (Nalge Nunc Inc. Napeville, IL) and either infected with SARS-CoV at MOI 0.1 or uninfected. When the cytopathic effect (CPE) was observed, slides were washed with PBS and fixed with methanol:acetone (1:1) for 20 min at −20 °C. The cells were permeabilized in PBS containing 0.05% Tween 20 and treated with SKOT3, SKOT19, or SKOT20 mAb, followed by FITC-conjugated anti-mouse IgG. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Coverslips were mounted onto slides using the anti-fade reagent Fluoromount G (Invitrogen), and samples were examined with an LSM510 confocal microscope (Carl Zeiss Co. Ltd.).

2.4. Flow cytometry analysis

A20.2J/S6.2 cells were suspended in staining buffer (PBS containing 2% FBS and 0.05% sodium azide) containing 30% goat serum, followed by incubation for 30 min with biotinylated SKOT3, SKOT19, or SKOT20 mAb (0.5 μg/ml) [16]. After three washes, cells were incubated with streptavidin–APC (eBio Inc., Tokyo, Japan) for 20 min on ice, washed and then analyzed using FACScalibur (BD Bioscience, San Jose, CA).

2.5. ELISA-based competition assay

The antibody competition assay was conducted using combinations of unlabeled and biotinylated mAbs. An ELISA-plate (Nunc) was coated with UV-inactivated SARS-CoV (0.8 μg/ml in 50 mM sodium bicarbonate buffer, pH 8.6) at 4 °C overnight, and blocked with StartingBlock™ (Pierce) at room temperature for 30 min. Unlabeled antibodies (5 μg/ml, PBS–Tween) were added and samples were incubated overnight at 4 °C; biotinylated antibodies were added to a final concentration of 1 μg/ml. The amount of bound biotinylated antibodies was quantitated with streptavidine–HRP (1/2000 dilution, SouthernBiotech) using *O*-phenylenediamine (Invitrogen) as a substrate.

2.6. Isolation of escape mutants

Isolation of escape mutants was performed as previously described [18]. In brief, SARS-CoV was incubated for 1 h at 37 °C in the presence of 0.2 μg/ml SKOT20, 0.5 μg/ml SKOT19 or 10 μg/ml SKOT3 mAb; these concentrations were estimated to be suboptimal concentrations of plaque inhibition based on the neutralization activity by a half tissue culture infectious dose (TCID) [16]. Virus and antibody incubations were then added to confluent Vero E6 cells in 6-well

plates. After 1 h of incubation, cells were overplayed with 1% agar noble (GIBCO, NY) in MEM containing 10% FBS and mAbs. After 3 days, five plaques from each sample that had formed in the presence of mAb were picked together with the supporting agar and soaked in 0.5 ml medium. Potential escape-mutant viruses were obtained after three rounds of plaque purification. During the first plaque purification, escape mutant viruses obtained in the presence of SKOT3, SKOT19 and SKOT20 were designated v3, v19 and v20, respectively, followed by the subclone number. These viruses were stored at −80 °C.

2.7. Sequence analysis

Viral RNA was extracted from the plaque-soaked medium using the QIAamp Viral RNA Mini kit (QIAGEN, GmbH, Germany). The cDNA fragments were then synthesized with the QIAGEN OneStep RT-PCR Kit (QIAGEN) using the primer sets spanning the entire S gene (Table 1). Nucleotide sequences were determined by BigDye Terminator v3.1 (Applied Biosystems).

3. Results

3.1. Characterization of anti-S mAbs

We previously established mouse mAbs of the IgG_1 subclass against the SARS-CoV S protein (SKOT3, SKOT19 and SKOT20). These mAbs demonstrated neutralizing activities, measured by TCID50, and were predicted to recognize conformational epitopes of the S protein [16]. To precisely determine the neutralizing titers, we first performed a SARS-CoV plaque assay. As shown in Fig. 1, SKOT20 inhibited plaque formation completely at 1 μg/ml, whereas plaque inhibition by SKOT19 was weaker, and approximately 5 μg/ml was required to achieve a similar level of plaque inhibition. In contrast, SKOT3 had no neutralizing activity at concentrations less than 1 μg/ml and at least 10 μg/ml was required.

Immunofluorescent analysis of SARS-CoV-infected Vero E6 cells revealed that all three mAbs reacted strongly with the cell membrane, as well as with the perinuclear ER/Golgi rich region (Fig. 2A). To further characterize these mAbs, murine A20.2J-derived S6.2 cells expressing S protein [17] were stained with biotinylated anti-S mAbs and analyzed using FACScalibur. SKOT19 stained the surface of S6.2 cells only marginally, whereas the staining signals obtained with SKOT3 and SKOT20 were stronger (Fig. 2B, upper panel). Importantly, these signals were substantially enhanced by combining SKOT3 and SKOT19 or SKOT19 and SKOT20 (Fig. 2B, lower panel). Of note, we previously showed that S expression in S6.2 cells was detectable at high levels using a mixture of all anti-S mAbs [17]. To further define the epitopes targeted by the mAbs, we next performed competition experiments. As shown in Fig. 2C, binding of SKOT-3 to virion was not inhibited either by SKOT19 or SKOT20, whereas binding of SKOT19 and SKOT20 was partially inhibited by each other and also by SKOT3. Together this indicated that the mAbs recognize two distinct epitopes of S protein, one epitope recognized by SKOT3 and the other by SKOT19 and SKOT20. Taken together, these anti-S mAbs likely recognize distinct conformational epitopes of S protein.

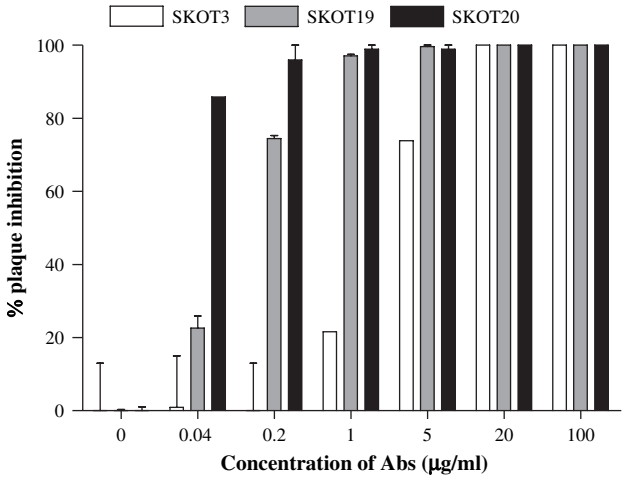

Fig. 1. The neutralizing activity of mAbs. Wild type virus were incubated for 1 h in the absence or presence of serially diluted control IgG_1 or anti-S mAbs, and then used to inoculate Vero E6 cells. Infected cells were overplayed with 1% low melting agarose and cultured for 3 days. The number of plaques obtained in the presence of mAb was calculated as a percentage relative to those obtained in the presence of control IgG_1. The means of two independent experiments are shown with standard deviation (SD).

Table 1
Primers used for the nucleotide sequence of Spike region[a]

a	5′-AATACACCTACTTTAGCTGTACCCACAAC-3′
b	5′-ATCCACCGACTGTGACTTG-3′
c	5′-TTGTCCBTBBTTGGGTTTTTGG-3′
d	5′-GTAATAAAGAAACTGTATGGTAACTAGCAC-3′
e	5′-CAGCTTGGCGCATATATTCTACTGGAAAC-3′
f	5′-AGCCGAGCCAAACATACC-3′
g	5′-GAAAGGGCTACCACCTTA-3′
h	5′-TCTGTAGACAACAGCAAGCACAAACAAGC-3′
i	5′-ATTGTTCTCAAAATCCACTTG-3′
j	5′-GATGCTACTTCAACTGGT-3′
k	5′-CAACAATTTGGCCGTGAT-3′
l	5′-CCTGACCCTCTAAAGCCA-3′
m	5′-AGGTGCTGATAGTTCAAT-3′
n	5′-CCTTCACGAGGGAAGTAT-3′
o	5′-ACGCTTCTGTCGTCAACA-3′
p	5′-GTCACACTAGCCATCCTTACTG-3′

[a] a–h primers were also used for the cDNA synthesis.

3.2. Target amino acids in S protein required for neutralization escape-mutant viruses

Since viral entry into cells is often accompanied by a conformational change in the S protein, the *in vivo* impact of neutralizing epitopes identified by *in vitro* assays needs to be further evaluated. Importantly, by deducing the amino acid substitutions in escape mutants of various neutralizing antibodies, a single amino acid has been implicated in the integrity of each antigenic site [18,19]. The identity of these amino acids represents important information with respect to the

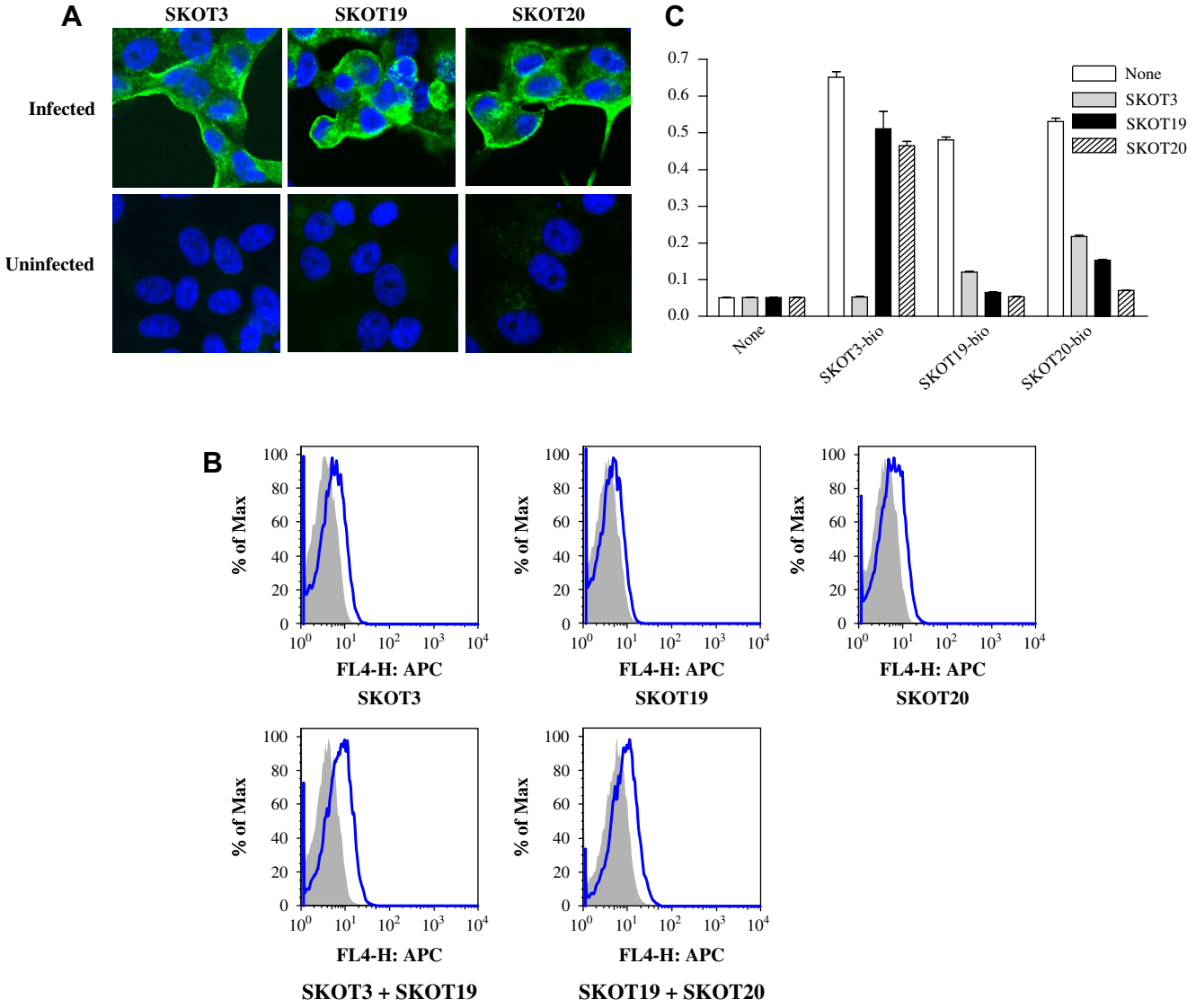

Fig. 2. Characterization of mAbs. (A) Immunofluorescence detection of SARS-CoV S protein. SARS-CoV-infected or uninfected Vero E6 cells were cultured for 40 h. Prior to development of an extensive CPE, cells were fixed, permeabilized and incubated with anti-S mAbs SKOT3, SKOT19 or SKOT20, followed by incubation with FITC-conjugated anti-mouse IgG. Nuclei were counter-stained with DAPI. The slides were examined using confocal microscopy. (B) Flow cytometric detection of SARS-CoV S protein. S6.2 cells expressing S protein were stained with biotinylated anti-S mAbs using one antibody (blue line), a combination (blue line), or control IgG (gray line), followed by incubation with streptavidin-APC. Stained cells were analyzed by FACScalibur: Upper panel: single staining patterns of SKOT3, SKOT19 and SKOT20. Lower panel: mixted staining patterns of SKOT3 and SKOT19 (left panel), or SKOT19 and SKOT20 (right panel). Control staining was depicted as shadows. (C) Competition assay of mAbs. The antibody competition assay was conducted with the combinations of unlabeled monoclonal antibodies (SKOT3, SKOT19, SKOT20) and biotinylated antibodies by ELISA using a plate coated with UV-inactivated SARS-CoV virion. The means of four independent experiments are shown with SD.

emergence of escape mutant viruses. Therefore, to generate SARS-CoV clones resistant to anti-S mAb neutralization, Vero E6 cells were infected with a wild type virus in the presence of a suboptimal concentration of each mAb. RNA from the escape mutant resistant viruses was then extracted, reverse-transcribed and sequenced.

The deduced amino acid substitutions for each mutant clone are summarized in Table 2. We detected an aspartic acid to asparagine substitution at aa 757 (D757N) in the S2 domain in the three of the four clones that escaped from neutralization with SKOT3. In addition, we identified a tyrosine to phenylalanine substitution at aa 442 (Y442F) in the S1 RBD of clones that escaped from SKOT3-, SKOT19-, and SKOT20-mediated neutralization. Other changes included a valine to glycine at aa 601 (V601G) in the S1 domain and an alanine to valine at aa 834 (A834V) in the S2 domain of a SKOT3 and a SKOT20 escape mutant, respectively. Thus, it is likely that residues 757 in S2 and 442 in S1 play important roles in the interactions between neutralizing antibodies and SARS-CoV. No S protein mutations were found in one of the four SKOT3 selection clones or four of the five SKOT19 selection clones.

Table 2
Amino acid changes in the S1 and S2 domains of neutralization-escape mutants

Viral clone	S1 domain	S2 domain
v3-1	V601G	D757N
v3-3	Y442F	D757N
v3-4	–	D757N
v3-5	–	–
v19-1	–	–
v19-2	–	–
v19-3	–	–
v19-4	–	–
v19-5	Y442F	–
v20-2	Y442F	–
v20-3	Y442F	–
v20-4	Y442F	A834V

Deduced amino acid changes due to mutations are shown. –, no substitution.

3.3. Escape mutants exhibit resistance to the neutralizing effect of anti-S mAbs

We examined the ability of these plaque-purified viruses to grow under the selection pressure of neutralizing mAbs. Vero E6 cells were infected with each wild type or escape mutant virus clone in the presence of various concentrations of the respective mAb used for selection. We compared the number of plaques of escape mutant and wild type viruses (Fig. 3A).

Three viruses containing the D757N substitution (v3-1, v3-3 and v3-4) exhibited substantial resistance to the neutralizing effect of SKOT3, even at high concentrations (100 μg/ml). The v3-1 escape mutant was also sensitive to the neutralizing effect of SKOT19 or SKOT20. The growth of clone v3-5, which lacked a mutation in the S protein, was inhibited completely at 100 μg/ml of SKOT3, indicating that v3-5 is not an escape mutant. A greater level of resistance to mAb was shown by clones with two aa substitutions (v3-1 and v3-3) compared to the clone with only a single aa modification (clone v3-4). These results indicate that aa 757 in the S2 domain may play a major role in neutralization by this mAb.

The S1 domain substitution of Y442F was identified in only one v19 clone (v19-5). This clone was relatively resistant to SKOT-19 at concentrations of 1 to approximately 5 μg/ml compared with wild type virus (Fig. 3A). Although other v19 subclones were weakly resistant to much lower concentrations of SKOT19 (approximately 0.04–0.2 μg/ml), we did not

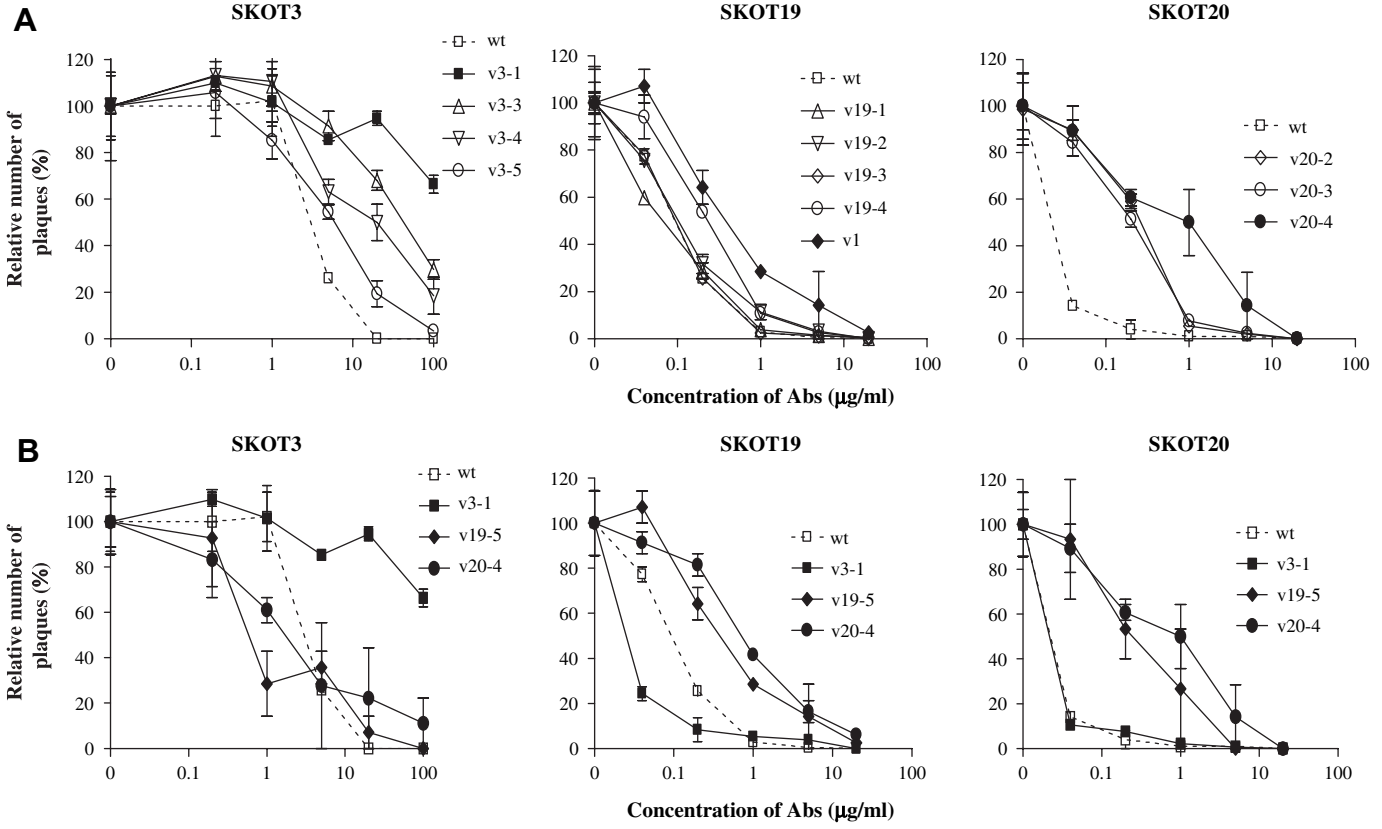

Fig. 3. The neutralization resistant phenotype of escape mutant clones. Wild type and plaque-purified virus clones were incubated for 1 h in the presence of serially diluted anti-S mAbs or control IgG_1, and then used to inoculate Vero E6 cells. Infected cells were overplayed with 1% low melting agarose and cultured for 3 days. The percentage of plaques obtained in the presence of mAb was determined relative to those obtained in the absence of selection mAb. The plaque count was carried out in duplicate and means of two wells are shown. (A) Comparison between wild type and v3 clones (left panel), v19 clones (middle panel) or v20 clones (right panel). The experiment was repeated with consistent results. (B) Wild type and plaque-purified virus clones were incubated for 1 h in the presence of serially diluted anti-S mAbs or control IgG_1 that were different from the mAb used for selection. V3-1, v19-5 and v20-4 were neutralized by SKOT3 (left panel), SKOT19 (middle panel) or SKOT20 (right panel). A titration curve of a wild type virus was depicted with a dotted line. The means of two independent experiments are shown with SD.

detect any mutation in the S region in these clones. The neutralizing curve shown in Fig. 1 indicates that the concentration of SKOT-19 used (0.5 μg/ml) was not sufficient for the selection of escape mutants, other than v19-5 with Y442F mutation. Alternatively, it is possible SKOT19 is a low affinity antibody and requires many molecules for exerting a clear neutralizing effect in this assay.

Three SKOT20-resistant clones (v20-3, v20-3 and v20-4) contained the same Y442F substitution. Thus, the Y442F substitution, which occurs in the RBD of the S1 region, appears to be important for viral escape from the neutralizing effect of both SKOT19 and SKOT20. The additional aa alteration that was identified in v20-4 (A834V) appeared to give an advantage for this clone to escape from the most powerful neutralizing effect of SKOT20 (approximately 0.2–1.0 μg/ml). Thus, the double substitution may have provided an additive effect to SKOT20 resistance, as in the case of v3 clones.

We further analyzed the resistance of representative subclones of v3, v19 and v20 to mAbs not used for their selection (Fig. 3B). As expected, v19-5 and v20-4 did not grow well in the presence of SKOT3 (left panel), and v3 did not grow in the presence of SKOT19 or SKOT20 (middle and right). In contrast, v19-5 and v20-4 were similarly resistant to both SKOT19 and SKOT20 (right). The resistance of v20-2 and v19-5 to SKOT19 (middle) was similar to that of v20-4 and only the growth of v20-3 was affected at a minimum concentration of SKOT19. Therefore, the contribution of individual or combined aa changes was not as obvious compared to the clear difference in v3 subclones.

These results indicate that substitution at aa 442 plays an important role in neutralization by both SKOT19 and SKOT20, while aa 757 is within a major epitope of SKOT3. Taken together, these findings indicate that a stronger resistance to neutralization is conferred by substitution of critical residues in both the S1 and S2 domains.

3.4. Neutralization escape is efficiently inhibited by the combination of mAbs

Next, we examined the effect of selection using a combination of two anti-S mAbs. Wild type virus was incubated with a single mAb or a mixture of SKOT3 (1, 2, or 4 μg/ml), SKOT19 (0.05, 0.1 or 0.2 μg/ml), and SKOT20 (0.025, 0.05, or 0.1 μg/ml). In Fig. 4, A represents the lowest concentration used, followed by B and C. In C, the concentration of each mAb can neutralize virus to half of the maximum. In any condition, the combination of two mAbs dramatically inhibited viral growth (Fig. 4). A combination of any two mAbs recognizing the S1 and S2 region, but not S1 only, was quite effective for neutralization, indicating that at least two types of neutralizing antibody (i.e. targeting both the S1 and S2 domains) should be used for effective passive immunotherapy against SARS.

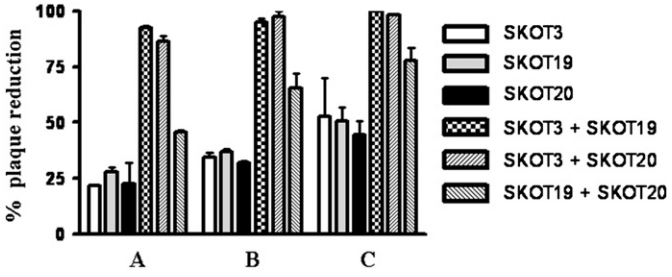

Fig. 4. Complete neutralization by a combination of any two mAbs. Wild type virus was incubated with or without SKOT3 (A = 1.0, B = 2.0, C = 4.0 μg/ml), SKOT19 (A = 0.05, B = 0.1, C = 0.2 μg/ml), SKOT20 (A = 0.025, B = 0.05, C = 0.1 μg/ml) or a mixture of two mAbs. Following infection of Vero E6 cells, the plaques were counted and the percent reduction calculated. The means of two independent experiments are shown with SD.

4. Discussion

Previously, we generated anti-S mAbs from mice immunized with UV-inactivated virus [16]. In this study, we further characterized three mAbs (SKOT3, SKOT19 and SKOT20) that possessed strong neutralization activity but different affinities towards the S protein. Immunofluorescence assays indicated that these mAbs reacted similarly to the cytoplasmic and ER/Golgi membranes of SARS-CoV-infected Vero E6 cells, but did not recognize purified virion that was blotted onto a membrane [16]. Flow cytometry analysis suggested that three mAbs recognize distinct conformational epitopes of S protein. Furthermore, the competition assay using these mAbs indicated that the epitope recognized by SKOT3 and that by SKOT19 and SKOT20 do not overlap. We generated neutralization escape viral mutants against these mAbs, and detected substitutions at positions 442 and 601 in the S1 domain, as well as 757 and 834 in the S2 domain, indicating that the major antigenic determinants of SKOT3 include aa 757 in the S2 domain, whereas those of SKOT19 and SKOT20 include aa 442 in the S1 RBD. We speculate that SKOT19 and SKOT20 may block virus entry by interfering with the interaction between the RBD and ACE2 receptor. Although SKOT3 binds outside of the RBD and with low affinity, it can also inhibit virus entry, presumably by interfering with the conformational change in the S2 region that is induced by binding.

The crystal structure of RBD bound to ACE2 revealed a loop termed the receptor-binding motif (RBM: aa 424–494) that interacts with ACE2 [2]. The major antigenic epitopes identified thus far have been shown to localize to this region [20–22]. For example, Yi et al. identified a positively charged region (aa 422–463) within the S protein that is critical for the induction of neutralizing antibodies in the DNA immunization of mice [22]. They found that pseudoviruses containing the R441A substitution failed to induce neutralizing mAbs, indicating that the Y442F substitution we identified here reflects the virological importance of this region. Interestingly, Tyr442 is well conserved in SARS-CoV isolated from humans and related viruses in civets and palm civet cats, but not those in bats (Ser442). Whether or not our neutralizing mAbs recognize SARS-CoV-related isolates from bats needs to be studied.

It was hypothesized that upon ACE2 binding with the RBD, the interaction between HR1 and HR2 would lead

to a conformational change in S2, resulting in membrane fusion for viral entry [23]. In fact, some neutralizing epitopes were localized in the HR2-containing region [11,12] and a further N-terminal region in S2 (aa 803–828) [13]. Of note, the neutralizing titers of murine mAbs recognizing aa 1091–1192 ranged from 13 to approximately 100 μg/ml [12], which is a similar level to that observed for SKOT3. Although the D757N and A834V substitutions in v3 and v20-4, respectively, do not correspond to residues in the HR2 domain, our data is consistent with others in suggesting that a critical determinant for neutralization is present also in the S2 region.

High titers of neutralizing IgG antibodies are found in SARS patients [24] and accumulating evidence has indicated that neutralizing antibodies are critical for protection against SARS-CoV infection. Thus, passive immunization with neutralizing antibody could be an effective SARS therapy. Human mAbs have been developed using a variety of techniques, and similar to the findings with murine mAbs, many of the epitopes were located in the RBD (review in [7]). In this context, Coughlin et al. immunized the XenoMouse, in which mouse immunoglobulin genes are replaced with human genes, with baculovirus-derived S protein to produce various human mAbs with neutralizing activity [25]. Twenty-seven reacted to a variety of epitopes within the S1 domain, whereas an additional 57 mAbs did not. Three mAbs that reacted with the HR2 domain did not exhibit neutralizing activity. Importantly, recently developed human neutralizing mAbs are potently cross-reactive to many clinical isolates, including zoonotic viruses [26,27].

In a previous study, the escape mutation from a human neutralizing mAb CR3014 was shown to bind a recombinant S1 fragment (aa 318–510), and a single P462L substitution was found in all 5 isolated viruses [28]. Recently, Rockx et al. extensively characterized 23 human mAbs and identified at least six distinct neutralizing profiles in S proteins with respect to specificity to various human and zoonotic isolates [26]. The authors identified several escape mutant viruses from the broad-spectrum mAbs. Surprisingly, all the escape variants had a single amino acid substitution: a single escape variant against one mAb with an L443R substitution, and two escape variants, with T332I and K333N mutations, against the other mAb. A combination of mAbs recognizing distinct epitopes worked synergistically in both reports, consistent with our results.

We demonstrated that a combination of mAbs strongly neutralizes SARS-CoV at much lower concentrations than that required for neutralization using a single mAb. The virus v20-4 contained substitutions in both the S1 and S2 regions, and exhibited a growth advantage over wild type virus in the presence of SKOT20 and SKOT19, while it was efficiently neutralized by SKOT3, which recognizes an epitope in S2. Thus, we predict that treatment with a combination of mAbs targeting both S1 and S2 could largely reduce the risk that escape mutants will grow. Such a combination of humanized neutralizing antibodies should be effective in the treatment against any potential re-emergence of SARS.

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