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Review article

Perspectives on the development of neutralizing antibodies against SARS-CoV-2

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Abstract

SARS-CoV-2 gains entry to human cells through its spike (S) protein binding to angiotensin-converting enzyme 2 (ACE2). Therefore, the receptor binding domain (RBD) of the S protein is the primary target for neutralizing antibodies. Selection of broad-neutralizing antibodies against SARS-CoV-2 and SARS-CoV is attractive and might be useful for treating not only COVID-19 but also future SARS-related CoV infections. Broad-neutralizing antibodies, such as 47D11, S309, and VHH-72, have been reported to target a conserved region in the RBD of the S1 subunit. The S2 subunit required for viral membrane fusion might be another target. Due to their small size and high stability, single-domain antibodies might have the ability to be administered by an inhaler making them potentially attractive therapeutics for respiratory infections. A cocktail strategy combining two (or more) antibodies that recognize different parts of the viral surface that interact with human cells might be the most effective.

Statement of Significance: Neutralizing antibodies are being developed to treat COVID-19 by reducing SARS-CoV-2 infectivity. Broad-neutralizing antibodies targeting a conserved region on the spike proteins of SARS-CoV-2 and SARS-CoV might be useful for treating COVID-19 and future infections. A cocktail strategy combining two or more antibodies might be the most effective.

KEYWORDS: SARS-CoV-2; SARS-CoV; COVID-19; neutralizing antibody; spike (S) protein

INTRODUCTION

COVID-19 is caused by the new coronavirus SARS-CoV-2 (initially called 2019-nCoV) [1, 2]. As of May 16, 2020, there are 4,632,903 confirmed cases and 311,739 death worldwide with 188 countries affected (https://coronavirus.jhu.edu/map.html). It is widely believed that neutralizing antibodies can be used to treat COVID-19 by reducing SARS-CoV-2 infectivity [3]. In the current issue of *Antibody Therapeutics*, two articles related to COVID-19 have been published through a fast track peer review, revision, and production [4, 5]. While most publications in our journal have been focused on cancer therapies, it was not the first time that *Antibody Therapeutics* published papers that were relevant to antibody development for viral diseases. In one previous study, a human papillomavirus vaccine using a novel virus-like particle was shown to induce antibody response in animal models [6]. In another report, single domain antibodies that bind the spike (S) protein of MERS-CoV and SARS-CoV were isolated from a shark V_{NAR} single domain antibody phage display library [7].

THE ROLE OF THE S PROTEIN IN SARS-CoV-2 INFECTION

As an enveloped single strand RNA virus, SARS-CoV-2 enters into a human cell through its S protein binding to angiotensin-converting enzyme 2 (ACE2) [8, 9] (Fig. 1). After endocytosis of the virus, cell surface ACE2 is down-regulated. Activation of the renin–angiotensin–aldosterone system may cause lung injury to viral infection [8]. The genome sequence (~30 kilobases) of SARS-CoV-2 shares the highest level of genetic similarity (~96% identity) with

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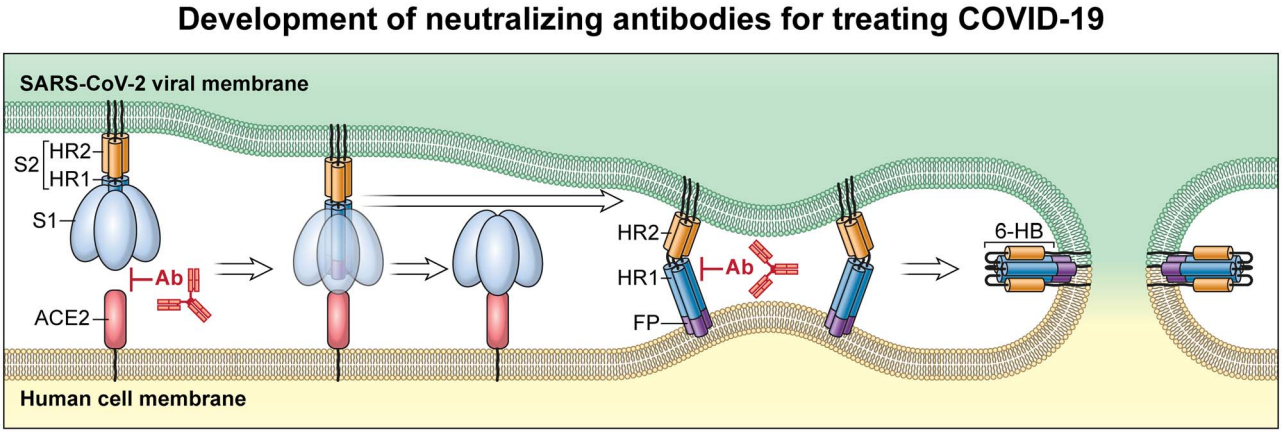

Figure 1. Development of neutralizing antibodies for treating COVID-19. In the receptor binding stage, the S1 subunit of SARS-CoV-2 binds human ACE2 on the host cell surface. Antibodies that bind the RBD domain on the S1 subunit might block the interaction of the RBD and the ACE2. Cross-reactive antibodies (e.g., 47D11, S309, and VHH-72) that bind highly conserved epitopes on the RBDs of SARS-CoV and SARS-CoV-2 could have broad neutralization activities against viral infection. In the viral fusion stage, after the cleavage of S1 subunit, the viral fusion peptide (FP) on the S2 subunit inserts into the host cell membrane, inducing the conformational change of the S2 subunit, which forms a six-helix bundle (6-HB) with the HR1 and HR2 trimers. Antibodies (e.g., 1A9 against SARS-CoV) that target the HR domains might block viral fusion. Ab, antibody.

the bat coronavirus RaTG13, indicating that the bat coronavirus might be the origin of the SARS-CoV-2 [10]. Furthermore, SARS-CoV-2 might be the result of a recombination between bat (RaTG13) and pangolin coronaviruses, as particularly indicated in the S protein sequence [10]. The receptor binding domain (RBD) of the SARS-CoV-2 S protein contains several novel residues that might be introduced through recombination with the pangolin coronavirus, indicating a possible critical step in the evolution of the ability of SARS-CoV-2 to infect humans [10]. The structures of SARS-CoV-2 S protein trimer [11] and human ACE2 [12] have been rapidly solved using modern cryo-electron microscopy (Cryo-EM). The affinity of the SARS-CoV-2 RBD for human ACE2 appears stronger than the SARS-CoV RBD. The structural analysis of the RBD-ACE2 complex reveals some of the key mutations on the RBD, such as F486 and N501, that form stronger contacts with human ACE2 [12]. Interestingly, these residues can be found in the pangolin coronavirus [10].

SEARCH FOR NEUTRALIZING ANTIBODIES TARGETING THE RBD

In the review paper from Dr Zhiqiang An's group in the University of Texas Health Science Center at Houston, Ku *et al*. summarized current findings on the structures and functions of SARS-CoV-2 viral proteins. They describe potential strategies for repurposing drugs for the treatment of COVID-19 and the current development of vaccines, plasma therapies, and neutralizing antibodies [4]. Their review highlights the potential viral targets, screening methods, *in vitro* and *in vivo* models, as well as discussing potential antibody-dependent enhancement (ADE) and Fc engineering for developing neutralizing antibodies for treating COVID-19 patients. In particular, their review describes major screening strategies for the discovery and development of SARS-CoV-2 neutralizing antibodies and provides several representative examples using these methods. Antibody sources may include memory or plasma B cells from recovered patients, phage, yeast and ribosome libraries, or from mouse, rabbit, monkey, and llama immunizations. Most antibodies are tested for their ability to block S protein (or RBD) binding to ACE2 and preventing spike-mediated membrane fusion. Antibody activity is tested either by using a pseudovirus-based neutralization assay or by a live virus-based neutralization assay. Animal models such as human ACE2 transgenic mice are also summarized in their review article.

In the research paper from Chengdu Medical College and ABLINK Biotech Co., Ltd in China, Zeng *et al*. isolated a human monoclonal antibody (named "rRBD-15") that inhibits the interaction of the RBD of SARS-CoV-2 and the ACE2 and neutralizes the pseudovirus infection [5]. The group used a competitive screening strategy to isolate human antibodies from a phage display library. In the first round of phage panning, they followed the standard procedure by screening phage on immobilized RBD. After the first-round enrichment of RBD binders, in the 2nd and 3rd rounds, they immobilized ACE2 and added the mixture of free RBD and a phage pool enriched from the 1st round. Phage that bind RBD at epitopes different from the ACE2-binding site were captured by the immobilized ACE2 to form a "sandwich" complex. Phage that competed with ACE2 for RBD were in the supernatant along with presumably unknown amounts of nonbinders or nonspecific binders. The phage that bind the ACE2 site on the RBD were then isolated by magnetic beads using the histidine tag on the RBD. The key for success using this strategy is the ratio of immobilized ACE2, free RBD, and phage concentrations in solution. Therefore, they used a low concentration of RBD (1 μg/ml) close to the EC_{50} value of RBD binding to ACE2 and a low concentration 1×10^{10} pfu of the phage they enriched from 1st round of panning on RBD. Standard phage panning protocols used about 100 times more phage or about 1×10^{12} pfu. In this way, they expected to reduce nonspecific binding of phage.

ISOLATION OF CROSS-NEUTRALIZING ANTIBODIES

Several important questions are raised in the development of neutralizing antibodies for treating COVID-19. Could such antibodies be cross-reactive with other SARS-related CoVs (SARSr-CoVs)? Could such cross-reactive antibodies have neutralizing activities for all SARSr-CoVs? What would be ideal targets or epitopes for cross-neutralizing antibodies? Selection of cross-neutralizing antibodies would be useful for treating not only current COVID-19 patients but also future SARSr-CoV infections.

The RBD of the S protein is the primary target for neutralizing antibodies. Many known neutralizing antibodies, including S230, m396, and 80R, are specific for SARS-CoV RBD but fail to bind SARS-CoV-2 even at the concentration up to 1 μM [11]. Polyclonal antibodies from mice immunized with a stabilized SARS-CoV S protein can inhibit SARS-CoV-2 entry into target cells. This suggests that immunity against one SARSr-CoV can potentially provide protection against related SARS-CoV [13]. In contrast, another study showed that polyclonal rabbit anti-SARS-CoV S1 antibodies (T62) inhibited entry of SARS-CoV, but not SARS-CoV-2 pseudovirus [14]. In addition, sera from recovered SARS and COVID-19 patients show only modest cross-neutralization, suggesting that recovery from one SARSr-CoV infection might not protect against the other. A recent report showed that none of the monoclonal antibodies isolated from SARS-CoV-2 infected individuals by single B-cell sorting were cross-reactive with the RBD of SARS-CoV [15]. In a research article published in our journal, Zeng *et al*. did not test the cross-reactivity of the rRBD-15 human antibody for SARS-CoV RBD [5]. In the review article, Ku *et al*. discusses two unique cross-reactive antibodies, CR3022 and 47D11, which bind highly conserved epitopes in the RBD [4]. CR3022 is an antibody that binds both SARS-CoV and SARS-CoV-2 RBDs, but it cannot neutralize SARS-CoV-2 as it does SARS-CoV [16]. More recently, Wang *et al*. identified the 47D11 monoclonal antibody that can neutralize both SARS-CoV and SARS-CoV-2 infection [17]. The 47D11 antibody was isolated from transgenic mice immunized sequentially with purified S proteins of different coronaviruses (HCoV-OC43, SARS-CoV, and MERS-CoV). The transgenic mice produce chimeric immunoglobulins with human variable regions and rodent constant regions. Four of 51 antibodies specific for the S protein of SARS-CoV show cross-reactivity with the S1 subunit of SARS-CoV-2. Among them, the 47D11 antibody exhibits the cross-neutralizing activity of SARS-CoV-2 and SARS-CoV in cell culture. Interestingly, 47D11 binds the RBD but does not block the interaction of RBD and ACE2, indicating that 47D11 might bind a highly conserved epitope of the RBD distinct from the ACE2 binding site. Previous studies indicate that SARS-CoV RBD antibodies that block the interaction of the RBD and ACE2 are not cross-reactive with SARS-CoV-2 RBD [11]. CR3022 also binds a highly conserved epitope on the RBD and binds both SARS-CoV and SARS-CoV-2 RBDs [16], but unlike 47D11, it does not have the cross-neutralizing activity against SARS-CoV-2. The structure complex of 47D11 and the RBD (or the S1/S protein) would reveal a novel conserved site on the RBD for broad-neutralizing antibodies against SARSr-CoVs. In addition to 47D11, another human antibody (S309) isolated from memory B cells of a SARS survivor infected in 2003 neutralizes SARS-CoV-2 [18]. Interestingly, S309 recognizes a glycan-containing epitope on the RBD in both the open and closed S states. The cryo-EM structure of the complex of S309 and SARS-CoV-2 S protein indicates that the antibody engages an epitope distinct from the ACE2 binding motif and would not clash with ACE2 for its binding to S protein. The glycan recognition of S309 implies the importance of the N-glycans in SARS-CoV-2 S protein. Furthermore, antibody cocktails containing S309 further improved SARS-CoV-2 neutralization and might be useful for preventing or mitigating virus escape mutants. This supports the idea that antibody cocktails could be more effective than single antibody therapy. Besides human neutralizing antibodies, a single domain camelid antibody (VHH-72), commonly called "nanobody", was isolated from a llama immunized with SARS-CoV S and MERS-CoV S. This nanobody showed reactivity with SARS-CoV-2 S protein by binding to a highly conserved epitope on the RBD partially overlapping the CR3022 binding site [19]. However, unlike CR3022, the bivalent VHH-72-Fc fusion protein not only prevents the binding of ACE2 but also has neutralizing activity against SARS-CoV-2 pseudovirus. The neutralizing effects of 47D11 and VHH-72 suggest that co-immunizing animals with S proteins from SARS-CoV-2 and other coronavirus may produce potent broad-neutralizing antibodies against SARS-CoV-2 by targeting the RBD. Single domain antibodies have unique binding features [20, 21], so they can bind novel viral conformational epitopes including highly conformational and/or buried sites unreachable by conventional antibodies. Besides their unique binding features, other advantages include the construction of multivalent/multispecific molecules and thermostability/chemostability [22]. For respiratory infection, single domain antibodies might be particularly attractive because they might be administered as an inhaler directly to the site of infection [19]. Both 47D11 and S309 are fully human IgG molecules, whereas VHH-72 is a single domain antibody derived from a llama heavy chain antibody that has not been humanized yet. For therapeutic applications, the camelid-specific sequences in the framework may need to be mutated to their human heavy chain variable domain equivalent [23]. Its suitability for prophylactic and therapeutic treatments remains to be determined.

It might be useful to analyze the mutations of SARS-CoV-2 as it spreads worldwide, so neutralizing antibodies can be effective for multiple strains of the virus [24–26]. Up to now, most of the mutations found in the RBD of SARS-CoV-2 are of low frequency [26]. The G476S mutation was located in the binding interface of the RBD with the ACE2, although it occurred in early samples and diminished in late samples, indicating that the virus with mutations in the critical functional site might not have advantage for its survival or spread.

NEUTRALIZING ANTIBODIES TARGETING NON-RBD REGIONS

While the RBD is the focus for the development of neutralizing antibodies against SARS-CoV-2, the function of non-RBD regions is poorly understood. Recently, an antibody (4A8) isolated from convalescent COVID-19 patients shows the binding on the N-terminal domain (NTD) of the SARS-CoV-2 S protein and exhibits high neutralization potency SARS-CoV-2. The structural analysis has confirmed its binding to the NTD, not the RBD which directly interacts with ACE2, demonstrating a new vulnerable epitope in the S1 subunit as a target of neutralizing antibodies for treating COVID-19 [27].

Antibodies that target the S protein beyond the S1 subunit have rarely been reported. The S2 subunit, in particular heptad repeat (HR) loops including HR1 and HR2 domains, required for membrane fusion might be another target. The 1A9 antibody is the only known monoclonal antibody that binds the HR2 domain on S2 subunit of SARS-CoV [28] (Fig. 1). A more recent study showed that SARS-CoV-2 had a superior plasma membrane fusion capacity compared with that of SARS-CoV [29]. The X-ray crystal structure of six-helical bundle (6-HB) core of the HR1 and HR2 domains in the SARS-CoV-2 S protein S2 subunit has also been solved [29]. A lipopeptide (EK1C4) that disturbs viral 6-HB formation by binding the HR1 domain can inhibit the fusion of SARS-CoV-2 as well as other human coronavirus including SARS-CoV and MERS-CoV, suggesting that a broad inhibitor targeting the HR region should be tested for the treatment of infection by current and future SARSr-CoVs.

Besides widely studied protein targets, glycan targets might be worth exploring as well. The S protein of SARS-CoV-2 is highly glycosylated [11]. Isolation of the S309 neutralizing antibody that recognizes a glycan-containing epitope on the RBD indicates that glycosylation on the S protein would affect the development of neutralizing antibodies targeting SARS-CoV-2 [18].

HOST CELL TARGETS

On the host cell, the primary target is the viral entry protein, ACE2. It has been proposed that recombinant ACE2 might be used as a potential inhibitor to block the virus entry [30]. The ACE2-human IgG1 Fc fusion has been engineered. The fusion protein can neutralize pseudoviruses that express the S proteins of SARS-CoV-2 or SARS-CoV in cell culture [31].

Heparan sulfate proteoglycans (HSPGs) provide the initial sites for the virus to make primary contact with the cell surface [32]. My laboratory research has been focused on the biology of HSPGs, in particular glypicans, and their roles as targets in cancer therapy [33]. Using one of our human antibodies (HS20) specific for heparan sulfate oligosaccharides with high affinity [34–36], we and collaborators previously showed that the HS20 antibody can block the attachment of pathogenic polyomaviruses on cells [37]. Interestingly, treatment of the cells with heparinase or exogenous heparin prevents the binding of the S protein to host cells and inhibits SARS pseudovirus infection [32], suggesting that in addition to ACE2, HSPGs might be essential for SARS-CoV entry into host cells. Blocking the HSPGs on human cells by therapeutic antibodies is worth investigating as another potential strategy for treating COVID-19.

PERSPECTIVE

Many antibodies capable of neutralizing specifically either SARS-CoV or SARS-CoV-2, but not both, have been identified and reported through many methodologies. A very few and very special SARS-CoV and SARS-CoV-2 cross-neutralizing antibodies have also been documented, including 47D11, S309, and VHH-72, among which the former two, which are human monoclonal antibodies, are ACE2 nonblockers, whereas the third one, which is a llama single domain antibody, is an ACE2 blocker.

Competitive phage display panning might be a new way to identify both ACE2 blockers and nonblockers. Even though Zeng *et al*. only reported the identification of one antibody, rRBD-15, that blocked ACE2 and neutralized SARS-CoV-2 [5], the same phage display competitive panning strategy could also be used to identify antibodies that do not block the interaction between S protein and ACE2 yet still neutralize viral infections. More importantly, with S proteins of both SARS-CoV and SARS-CoV-2 as competitors, this competitive panning strategy could be utilized to identify both ACE2 blocking and nonblocking antibodies against both viruses with functional profiles similar to 47D11, S309, and VHH-72, respectively.

In the last 20 years, human coronaviruses have infected humans and causes three major outbreaks due to SARS-CoV, MERS-CoV, and SARS-CoV-2. An urgent and important challenge in modern medicine is whether we could identify a so-called "universal" target or strategy for inhibiting SARSr-CoV or even all coronaviruses. The molecular mechanisms of SARS-CoV-2 infection are not yet fully understood. More research on the SARS-CoV-2 biology is urgently needed. Current challenges in developing neutralizing antibodies against SARS-CoV-2 include mutations in less conserved region of S1 subunit, possibly antigen drift, immunodominant epitope, ADE potentially induced by nonneutralizing antibodies, or increased affinity of viral S protein for ACE2 [26]. It is important to identify and develop neutralizing antibodies, such as 47D11, S309, and VHH-72, against highly conserved region of RBD or S1, to combat not only various strains of SARS-CoV-2 but also broadly against other SARSr-CoVs. Furthermore, a single monoclonal antibody therapy might not be enough. There might be different strains of the virus that cannot be recognized by the antibody. Mutations in the virus can lead to escape variants [38]. Multiple strategies and combination of multiple mechanisms are highly expected as described in MERS [39] and SARS [38] antibody development. A combination of two (or more) antibodies that recognize different parts including both neutralizing and nonneutralizing epitopes (e.g., RBD, NTD, HR, and glycan) of the viral surface that interact with human cells might be the most effective. Future therapeutic applications could include cocktail therapy by combining

antibodies (including multiple single domain antibodies) that target different epitopes via different mechanisms.

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CONFLICT OF INTEREST STATEMENT

None declared.

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