Human monoclonal antibodies to the S glycoprotein and related proteins as potential therapeutics for SARS

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Polyclonal antibodies have a century-old history of being effective against some viruses and, recently, monoclonal antibodies (mAbs) have also shown some clinical success. Human mAbs to the severe acute respiratory syndrome (SARS) coronavirus spike glycoprotein have been developed by several research groups at an amazing pace. These antibodies potently neutralize infectious virus in tissue cultures and animal models, and, alone or in combination with vaccines and other drugs, may have potential for the prevention and treatment of SARS.

Keywords Antibodies, prophylaxis, SARS, S glycoprotein, S protein, therapy, treatment

Introduction

Serum-derived antibody preparations from humans or animals (mostly rabbits and horses) have been used for prophylaxis and therapy of viral diseases since the late 1800s [1•], although mostly for prophylaxis, either prior to an anticipated exposure or following exposure to an infectious agent [2-4]. Antibody products licensed in the US for the prevention or treatment of viral diseases include human immunoglobulin (Ig) for use against hepatitis A and measles, and virus-specific polyclonal human Ig against cytomegalovirus, hepatitis B, rabies, respiratory syncytial virus (RSV), vaccinia and varicella-zoster. Polyclonal Ig has also been used with varying success for diseases caused by other human viruses, including parvovirus B19, Lassa virus, West Nile virus, some enteroviruses, herpes simplex virus, Crimean-Congo hemorrhagic fever virus, Junin virus and HIV-1. Patients infected with the coronavirus (CoV), which causes severe acute respiratory syndrome (SARS), were treated with convalescent patient plasma containing polyclonal antibodies [5•,6•]. Improvements of the antibody preparations were suggested [7], and batches of virus-inactivated hyperimmune globulins containing 5- to 6-fold higher titers of SARS-CoV (SCV)-specific antibodies than convalescent plasma were produced [8].

Although serum polyclonal antibody preparations have been clinically effective in many cases, problems related to toxicity, including a risk for allergic reactions, lot-to-lot variation and uncertain dosing, have limited their use [9]. Monoclonal antibodies (mAbs), including chimeric animal-human, humanized and fully human mAbs (hmAbs), have lower or absent immunogenicity, toxicity and lot-to-lot variation. The molecular mechanisms of the therapeutic efficacy of such antibodies are easier to dissect and can be engineered to further improve their therapeutic properties. One such antibody, the humanized mAb palivizumab, was licensed by the US FDA for the prevention of RSV infections, but remains the only one approved for clinical use against an infectious disease. At an amazing pace of research, several research groups have recently developed human mAbs to the SCV spike (S) glycoprotein, which neutralize the virus and have potential for the therapy and prophylaxis of SARS. This review will mostly describe these SCV-neutralizing antibodies (nAbs) and analyze their potential as therapeutic agents.

Neutralizing antibodies elicited by SCV infection or immunization

Infections by many viruses, including CoVs, elicit potent nAbs that can affect the course of infection and help clear the virus; nAbs can also protect an uninfected host exposed to the virus. SCV is no exception and nAbs have been detected in SCV-infected patients [10-14,15•], mice [16], hamsters [17] and monkeys [18]. These antibodies also protected uninfected animals from SCV infection, for example, passive transfer of immune serum to naive mice prevented virus replication in the lower respiratory tract following intranasal challenge [16]. nAbs from serum targeted the S protein [15•], including epitopes containing portions of the receptor-binding domain (RBD) on S1 [19], conserved fragments from S2 (eg, Leu^{803} to Ala^{828} and Pro^{1061} to Ser^{1093}) [20], and a limited number of fragments from other regions of S [14]. Their epitopes appear to be both conformational and linear, one study found an association between linear epitopes and S protein C-terminal regions, and conformational epitopes with the S protein N-terminal domain [21].

Immunization with various antigens also induced nAbs in mice [19,22-29], hamsters [30], rabbits [27,31,32], ferrets [33], pigs [34] and monkeys [35,36]. The S glycoprotein, which alone can mediate entry of the SCV [15•,37•], has been mostly used as an immunogen. nAbs can be elicited with approximately equal efficacy by the soluble ectodomain and the full-length membrane-associated S protein using DNA immunization of mice without boosting with protein [X Xiao, A Biragyn, DS Dimitrov, unpublished data].

Mice were immunized for the production of neutralizing murine mAbs (nmAbs) [38,39]. Two of the S-specific mAbs (F26G18 and F26G19) demonstrated the highest *in vitro* neutralizing potency (in the sub-nanomolar range), with the nmAbs targeting predominantly conformational epitopes

[38]. Antibodies from convalescent SARS patients, but not normal human serum, have specifically competed with binding of these mAbs to whole SCV, indicating the existence of antibodies in infected humans targeting the same or overlapping epitopes as the mouse nmAbs. The most potent murine nmAbs, for example, F26G18, may have potential for the prevention and treatment of SARS, especially after their humanization to avoid possible immunogenicity effects.

Neutralizing hmAbs generated by Epstein-Barr virus transformed B-cells from a convalescent patient

hmAbs represent a promising alternative to hyperimmune sera and humanized mAbs. Such antibodies can be produced by Epstein-Barr virus (EBV)-immortalized B-cells, or selected from human antibody libraries, and hybridomas from immunized transgenic mice carrying human Ig genes. Recently, an improved method for EBV transformation of human B-cells based on a CpG oligonucleotide (CpG-2006; Coley Pharmaceutical Group Inc) that increases the B-cell immortalization efficiency from 1 to 2%, to 30 to 100% was developed and used for selection of hmAbs specific for SCV proteins [40••]. IgG+ B-cells were obtained from a convalescent patient whose serum contained SCV-specific antibodies (IgG1), which were detected by enzyme-linked immunosorbent assay (ELISA) of viral protein sodium dodecyl sulfate extract and staining of baby hamster kidney (BHK) cells transfected with the SCV S-encoding DNA. nAbs measured by an *in vitro* neutralization assay of SCV infection persisted for more than 12 months in the serum of this patient, although their titer began to decline after 8 months (from 1/128 to 1/64); interestingly, the S-specific antibody titer began to decline 2 months following the start of the infection.

Screening the culture supernatants of B-cell clones using an *in vitro* neutralization assay or by staining S transfectants resulted in the identification of 35 hmAbs capable of completely neutralizing SCV infection *in vitro* at concentrations ranging from 1 to 850 ng/ml. Some of these antibodies bound with high affinity to cell surface-associated S glycoprotein and exhibited neutralization titer proportional to the level of staining, while others stained S transfectant cells poorly but showed high neutralizing activity. One of these antibodies (S3.1) was approximately 500-fold more effective in neutralization than convalescent serum; it stained the S glycoprotein on the viral spikes, as measured by immunoelectron microscopy. In a mouse model of SCV infection, 200 and 800 µg of this antibody prevented viral replication in the lower respiratory tract, and reduced it in the upper respiratory tract at the highest concentration (800 µg). Unfortunately, data for the *in vivo* neutralizing activity of other neutralizing hmAbs (nhmAbs) selected in this study [40••], including the most potent antibody (S215.13), which has a neutralizing concentration (1 ng/ml) 300-fold lower than that of S3.1, have not been reported. The high neutralizing activities of these hmAbs in IgG1 format indicate possibilities for their use alone or in combination for the prophylaxis and treatment of SARS.

nhmAbs selected from naive phage-displayed antibody libraries

Phage-display technology has been increasingly used to produce high-affinity hmAbs from both naive and immune libraries. An advantage of using a naive library is that B-lymphocytes from an infected or immunized host are not required. Recently, two human, non-immune, single-chain, variable region fragment (scFv) libraries containing a total of 2.7×10^{10} members were developed from the B-cells of 57 unimmunized donors, and used for selection of antibodies against a purified S fragment containing residues 12 to 672 [41•]. Eight unique scFvs were identified, one of which (80R) exhibited neutralizing activity *in vitro*. To increase avidity and half-life *in vivo*, this scFv was converted to IgG1 and extensively characterized. The avidity, measured by surface plasmon resonance, increased by approximately 20-fold (K_d = 1.6 nM), which correlated with approximately the same fold increase in its *in vitro* neutralizing activity. IgG1 80R can neutralize 50% of the virus in a microneutralization assay at a concentration of 0.37 nM. It also blocked formation of syncytia, which could contribute to the spread of the virus *in vivo*, although at a significantly higher concentration (25 nM). Its epitope overlaps the binding site of the SCV receptor angiotensin-converting enzyme (ACE)-2, suggesting a possible mechanism of neutralization by preventing the virus attaching to its receptor [41•]. Further studies, including testing its neutralizing activity in animals, are required to determine the potential clinical utility of this antibody.

Three nhmAbs were also generated by screening a large naive antibody library [42••,43•]. All antibodies bound a recombinant S1 fragment comprising amino-acid residues 318 to 510, which includes the RBD [43•]. The most potent of these nhmAbs, IgG1 CR3014, required the residue N479 for binding [43•]. This antibody bound to S expressed on the surface of HEK293T cells and exhibited 50% neutralizing activity at approximately 1 µg/ml *in vitro* [42••,43•]. More importantly, this antibody showed neutralizing activity in ferrets, measured by two sets of experiments [42••]. In the first set of experiments, ferrets were inoculated either with virus at two doses, low (10^3 $TCID_{50}$) and high (10^4 $TCID_{50}$), or with virus pre-incubated with the antibody at 0.13 mg/ml for the low dose and 1.3 mg/ml for the high dose. Animals exposed to the virus-antibody mixture had almost undetectable SCV in the lung, showed no lung lesions on days 4 or 7, and did not shed virus in their throats, unlike control animals treated with irrelevant antibody. In a second set of experiments, 10 mg/kg of antibody was administered 24 h before challenge with virus and reached 65 to 84 µg/ml of serum concentration in three of the animals (< 5 µg/ml in the fourth animal). In the three ferrets with high antibody concentration, virus shedding in the throat was completely abolished, while in the fourth animal it was comparable to that of the control group. The CR3014-treated animals had 3.3-log lower mean virus titer than the controls, and were completely protected from macroscopic lung pathology. The antibody dose used (10 mg/kg) was less than the dose (15 mg/kg) used for prevention of RSV infections in infants, which is administered once a month. These results suggest a potential use of CR3014 for prophylaxis of SCV infections in

humans if it can reduce the virus replication to the same extent as in ferrets.

We have generated a panel of scFvs from phage libraries [MY Zhang, V Choudhry, X Xiao, DS Dimitrov, unpublished data]. Two of the scFvs, designated m301 and m302, exhibited high-affinity binding to purified S, as measured by surface plasmon resonance. The K_d for m301 was 16.4 nM (k_{on} = 4.2 x 10^4 $M^{-1}s^{-1}$, k_{off} = 6.9 x 10^{-4} s^{-1}) and for m302 was 60.8 nM (k_{on} = 9.7 x 10^3 $M^{-1}s^{-1}$, k_{off} = 5.9 x 10^{-4} s^{-1}). In an ELISA, these antibodies competed with ACE-2 for binding to the spike protein. They were converted to an IgG1 format and their potency in neutralizing SCV is being evaluated.

nhmAbs obtained from immunization of transgenic mice with human Ig genes

Recently, two hmAbs (201 and 68; Medarex Inc/University of Massachusetts Medical School) were derived from transgenic mice with human Ig genes and evaluated in a murine model of SCV infection [44•]. One of these antibodies (201) bound within the RBD of the S protein at amino-acid residues 490 to 510, and the other (68) bound to a region including residues 130 to 150. Mice that received 40 mg/kg of these antibodies prior to challenge with the SCV were completely protected from virus replication in the lungs, and doses as low as 1.6 mg/kg offered significant protection. These antibodies have potential as therapeutics and research tools, and further studies are planned to evaluate the nhmAb 201 for potential clinical use [44•].

Anti-ACE-2 nhmAbs, S protein fragments and soluble ACE-2 as potential therapeutics

nAbs directed to S inhibit SCV entry, either by interfering with S RBD-receptor interactions [41•] or by other mechanisms that remain to be elucidated. Such mechanisms could include steric hindrance that indirectly prevents virus attachment to receptors and binding to entry intermediates. Other mechanisms that could operate *in vivo*, which will not be discussed here due to lack of data, are related to the antibody biological effector functions conferred by the antibody Fc, for example, antibody-dependent cellular cytotoxicity.

S-ACE2 interactions can be blocked by antibodies targeting either S or ACE-2. Indeed, antibodies to ACE-2, but not an anti-ACE-1 antibody, blocked viral replication on Vero E6 cells [X Xiao, DS Dimitrov, unpublished data] [45•]. As the receptor is a host molecule that does not mutate, the use of antibodies targeting receptor molecules may prevent the generation of resistant mutants. However, it appears that for SCV infection, which is an acute infection, generation of resistant mutants may not be a significant problem. In addition, such anti-ACE-2 antibodies could deplete cells expressing ACE-2. Studies in animal models are required to investigate whether SCV infection in the presence of nhmAbs will lead to generation of neutralization escape mutants and whether anti-ACE-2 nhmAbs have deleterious effects on the host.

S-ACE-2 interactions can also be blocked by fragments containing the S RBD and by soluble receptor molecules. Fragments containing the N-terminal amino-acid residues 17 to 537 and 272 to 537, but not 17 to 276 bound specifically to Vero E6 cells and purified soluble receptor molecules. Together with data from binding inhibition by antibodies developed against peptides from S, these findings suggested that the RBD is located between amino-acid residues 303 and 537 [46•]. Two other research groups obtained similar results and found that independently folded fragments as short as 193 residues can specifically bind receptor molecules [47,48]. The 193-residue fragment blocked S protein-mediated SCV infection with an IC_{50} of less than 10 nM, whereas the IC_{50} of the S1 domain was approximately 50 nM [47]. Similar results were found for other fragments containing the RBD. S fragments containing residues 272 to 537 and 17 to 537 also displayed inhibitory effects on S-mediated cell fusion, albeit with lower activity (IC_{50} > 100 nM) [X Xiao, DS Dimitrov, unpublished data]. The S fragments showed higher neutralizing activity when fused to an Fc fragment. It is possible that Fc fused to S fragments helps to maintain the conformation of the RBD. Another possibility is that the dimerization conferred by the Fc could enhance binding of the S fragments to the ACE-2 molecules. Soluble receptor molecules have been tested as inhibitors for HIV-1 infection and it was suggested that such an approach could also be used for inhibition of SCV infections [45•,49•]; indeed, soluble ACE-2 (sACE-2) blocked viral replication in Vero E6 cells [45•]. Of note is that the sACE-2 form used was a fusion protein in which sACE-2 was joined to an Fc antibody that would confer long half-life *in vivo*.

Fragments from regions of the S protein other than the RBD can also inhibit SCV entry. Computer analysis suggested the existence of two heptad repeats; peptides from the N- and C-terminal regions of S2 (NP and CP, respectively) can form stable complexes (six-helix bundle structures), indicating that, as for other class I fusion proteins, such structures are an important intermediate in the fusion process [50-53]. The formation of these structures could be disrupted by NP and CP. Indeed, several recent studies demonstrated that SCV infection can be inhibited by CP, although in most cases the inhibitory peptide concentrations were in the micromolar range [50-53], except for one isolate, which appears to be inhibited at nanomolar concentrations [52]. In contrast, T20, which was the first virus entry inhibitor approved by the FDA for clinical use (except Igs), can inhibit HIV-1 infections much more efficiently. The underlying mechanism of these differences is unknown, but could be related to the different pathways of entry of these two viruses, endocytosis (SCV) and entry at the cytoplasmic membrane (HIV). However, we recently found that one of the CP peptides can efficiently inhibit cell fusion with an IC_{50} value of only 20 nM; the IC_{50} of the same peptide for infectious virus was in the micromolar range [X Xiao, CC Broder, DS Dimitrov, unpublished data]. The dominant mechanism of SCV spread *in vivo* is unknown and, therefore, the relevance of inhibiting cell-free virus entry to *in vivo* efficacy, versus cell fusion *in vitro*, remains to be elucidated. We have also expressed and purified full-size S2 fused to Fc [MY Zhang, DS Dimitrov, unpublished data]. Interestingly, S2-Fc is a monomer and remains soluble in phosphate buffered solution. It remains to be seen whether this full-size S2 fusion protein can efficiently block SCV entry into host cells.

In general, it appears that of all the entry inhibitors related to the S protein, hmAbs directed to the S protein are currently at the most advanced stage of development and offer the best hope as potential therapeutics. However, only further exploration of possible protein-based entry inhibitors and their evaluation in animal models would allow identification of the best candidates for clinical trials.

Conclusions

Human mAbs that neutralize SCV have been developed at an unprecedented speed, which is characteristic of SARS research in the last two years. These antibodies have potential for further development into a clinically useful product for prophylaxis and perhaps treatment of SCV infections. They are potent in the IgG1 form and the SCV infection is an acute infection that requires control of virus replication for relatively short periods of time, not to exceed a few weeks, after which the host immune system can clear the virus. In addition, these antibodies are cross-reactive, thus the problem of neutralization-resistant mutants able to evade their inhibitory activity and the immune response is not as significant as, for example, chronic infections caused by HIV. A note of caution is that careful examination of candidate antibody therapeutics is required due to the possibility of infection-enhancing effects and animal model-dependent effects, as well as toxicity in some cases, although this is rare. A recent study reported for the first time the possibility that neutralizing antibodies can enhance the entry of SCV by a mechanism that involves antibody interactions with conformational epitopes in the S RBD [54•].

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