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Original Article

Monoclonal neutralizing antibodies against SARS-COV-2 S protein

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Abstract: Novel coronavirus pneumonia, also known as coronavirus disease 2019 (COVID-19), is caused by subsevere acute respiratory syndrome type 2 coronavirus (SARS-CoV-2) infection. The spike (S) protein of SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) receptors widely expressed on the surface of human cells leading to life-threatening respiratory infections. A serious hazard to human health is posed by the lack of particular treatment medications for this virus infection. We advocate the creation of high-affinity antibodies using the receptor binding domain (RBD) of S protein as a specific antigenic epitope to develop a drug that can precisely target therapy COVID-19 because SARS-CoV-2 infection of the host cells is dependent on S protein binding to ACE2. Finally, we obtained high-affinity antibodies 14F4HL and 14E3HL that have high affinity with RBD and well-drug-forming properties, suitable for further humanization studies. Thus, monoclonal antibodies that neutralize the S protein were identified in our study, which may provide new insights for the development of COVID-19 therapeutic drugs.

Keywords: SARS-CoV-2, COVID-19, ACE2, monoclonal antibody, hybridoma fusion

Introduction

COVID-19, caused by SARS-CoV-2 infection, gradually spread worldwide in 2019, and up to March 2022, there are more than forty million infections with mortality up to 1.36%, which has become one of the biggest threats to global health [1, 2]. Droplets and fomites are the main transmission routes of COVID-19, but it also has other routes of transmission infection pathway, such as the fecal-oral route [3, 4]. The diversity of dissemination channels may correlate with the distribution location of ACE2. The S protein of SARS-CoV-2 binds to ACE2 receptors, which is also the main pathogenic mechanism of the virus as well as virulence [5, 6]. Multi-country has already started the study of COVID-19, yet to date, comprehensive anti-SARS-CoV-2 antibodies are still not found [7-9].

The S protein is located on the envelope of SARS-CoV-2 and, during infection of the cell, can bind to ACE2 and be cleaved into S1, and S2 subunits by furan proteases of the host cell. After the dissociation of the S1 subunit and S2 subunit, the conformation of the S1 subunit will change. The RBD contained in the N-terminal and C-terminal will bind to the ACE2 receptor, leading to a significant change in the conformation of the S2 subunit, thus, SARS-CoV-2 could bind to ACE2 and cause damage to hosts [10]. The receptor-binding motif (RBM) of S1 consists of amino acids from 424 to 494, which is easy to induce the generation of neutralizing antibodies. Based on the structure of SARS-CoV-2 and the characteristics of the invasion routes, animals immunized with RBD or RBM can induce the production of corresponding neutralizing antibodies and play an important role in virus neutralization [7, 11].

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In the present study, we constructed a recombinant SARS-CoV-2 S RBD protein sequence through the gene engineering method. Next, we obtained RBD protein with high affinity to ACE2 by expressing it in Expi293F and purifying through Ni-NTA. We immunized BALB/c mice with recombinant RBD protein, then splenocytes of hyper-immunized mice were fused with myeloma cells. The selected hybridomas were subcloned by limited dilution and screened by ELISA. We finally obtain 3 monoclonal cell lines that can stable secret anti-RBD antibodies. After transfection, antibody purification, and screening, monoclonal antibodies that can specifically bind to RBD protein were obtained. Here, we will study two antibodies 14F4HL and 14E3HL with high affinity to RBD protein, and observe their binding epitopes and mutants. The stability and biological activity were verified by pharmacokinetic tests and pseudovirus neutralization assay. We hoped that after further engineering of the IgG heavy chain coding sequences of the screened monoclonal antibodies, potent humanized antibody molecules will be obtained that will contribute to the clinical treatment of COVID-19.

Methods and materials

Mice

Male wild-type (BALB/c) mice, aged 6-8 weeks and weighing 18-20 g were raised with free access to food and water, and all procedures were carried out in compliance with the Regulations of Experimental Animal Administration issued by the Committee on Ethics of Medicine, Naval Medical University, PLA.

ELISA quantification for RBD binding ability

SARS-CoV-2 RBD protein at 1 μg/ml was coated on the ELISA board overnight at 4°C. After washing and blocking, 200 μL of configured ACE2 was added to each well and incubated overnight at 4°C. Plates were washed three times and incubated with anti-human IgG (H+L)/HRP (Abcam) for 1 h at 37°C. TMB (Beyotime) substrate was added and reacted under dark and optical density (OD) was measured at 450 nm.

Competitive ELISA assay

RBD proteins (0.5 μg/ml, 100 UL per well) were coated onto ELISA plates and incubated overnight at 4°C. The plates were washed with PBST followed by blocking with 5% defatted milk for 1 h at 37°C. Meanwhile, a solution containing 6.4 μg ACE2-Fc fusion protein by volume was mixed with a solution containing 50 μg IgG antibody or 26.7 μg VHH-FC volume of solution mixed well and diluted in PBS. A mixture of 200 μL of antibodies was tested with biotin labeled ACE2-Fc fusion protein and was then added to the loading wells. After 1 h of incubation and three times washing, the plates were incubated with HRP conjugated Streptavidin (1:5000, Beyotime) for 1 h at 37°C. Next, the plates were washed with PBST and treated with the TMB buffer. After 10 min, 50 μl stop the buffer to stop the reaction, and read absorbance at OD450 nm.

Analysis of pharmacokinetics

Male BALB/c mice were selected and randomly divided into Group A and Group B. Mice in Group A and Group B were injected with 14F4HL, 14E3HL, and LYCoV555 at the dose of 15 mg/kg. After the injection of the drug, the orbital blood samples were collected from the mice of Group A and Group B, which were placed at room temperature for 30 min, centrifuged at 4°C, 9000 rpm, 10 min. The serum was taken into 1.5 ml EP tubes (Axygen) and sent to the company for testing. The pharmacokinetic parameters of rhTPO in mice were calculated using a non-atrioventricular model from Pheonix WinNonlin 6.4. Serum rhTPO concentrations and sampling standard times were used to estimate pharmacokinetic parameters for each individual. The area under the serum concentration-time curve (AUC) was calculated using the trapezoidal method. Determine the extrapolation from the last sample collection point to the AUC at infinity by eliminating the concentration measured at the last to eliminate the rate constant (ke). $AUC_{0-\infty}$ was calculated from the sum of the time of the last measurement (AUClast) and the extrapolated area. The k-value is determined by the slope of the final phase of the logarithmic conversion concentration-time curve fitted by the least squares method. The maximum serum drug concentration (C_{max}) and the time to reach C_{max} (t_{max}) were both measured. Data collation and mapping were performed using GraphPad Prism 8.3.

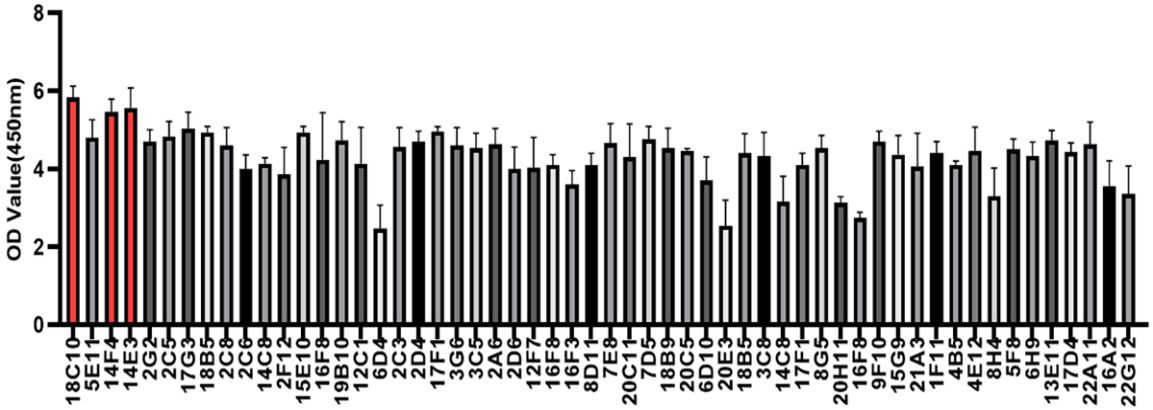

Figure 1. Screened partial cell lines from 96-well plates. Screening some cell lines with the high binding ability to RBD protein from hybridoma cell lines through a 96-well plate. OD value >5 was considered positive.

Pseudotyped virus neutralization assay

An in vitro neutralization assay was used to Assess the pseudovirus-neutralizing activity of neutralizing antibodies. 24 hours before the determination, HEK293-ACE2 cells were treated with 1.0 × 10^4/well density was inoculated in a 96-well plate. Serum was diluted 3-fold serially in DMEM containing 2% FBS, after which 5 μl of the diluted antibody sample was mixed with 50 μl of pseudovirus solution (Vazyme) diluted in DMEM containing 2% FBS and incubated at 37°C for 1 h. 100 μL mixture was added into the well, supplemented with DMEM 100 μl complete medium 12 h later, and incubated at 37°C for 48 h. Bio-LiteTM Luciferase Assay System was used to measure the expression of Luciferase (RLU). Three duplicate pores were made at each dilution, and the controlled pore of pseudovirus was cultured in DMEM with 2% FBS. GraphPad Prism 8.3 was used to calculate the RLU value and 50% inhibition concentration (IC50).

Statistical analysis

Student's t-test was used to determine the difference between the two groups. GraphPad Prism 8.3 was used to calculate IC50 defined as a 50% reduction in the dilution of RLU values compared with a pseudovirus control pore. Non-atrioventricular model in Pheonix WinNonlin 6.4 to calculate pharmacokinetic parameters of rhTPO in mice. GraphPad Prism 8.3 were used for data processing.

Results

Preparation of monoclonal antibodies

The gene sequence of the SARS-CoV-2 S protein was obtained from the NCBI database (www.NCBI.com). We designed and synthetic the SARS-CoV-2 RBD plasmid transiently transfected the constructed RBD plasmid into Expi293F cells, and purified the RBD protein. Using the same method we also purified various mutant proteins of the RBD: The British mutant strain (B.1.1.7), South African mutant strain (B.1.351), Brazilian mutant strain (P.1), India mutant strain (B.1.617.1), and California mutant strain (B.1.429).

ELISA was used to screen for cell lines and 56 clones were obtained, OD value >5.0 was considered positive (**Figure 1**). Limiting dilution was used for subcloning, culture, and selection. Three positive monoclonal cell lines with the ability to stably secrete anti-RBD antibodies and the highest affinity for RBD were obtained and designated as follows: 14E3, 14F4, 18C10.

To obtain monoclonal antibodies 14E3HL, 14F4HL, and 18C10HL, both heavy chain and light chain were transiently transfected into Expi293F cells. The monoclonal antibodies were bound to RBD measured by ELISA. Results showed that the affinity between 14F4HL and 14E3HL with RBD is higher than that of 18C10HL after repeated experiments. Therefore, 14F4HL and 14E3HL were chosen for further studies, LY-CoV555 and JS016 were used as a positive control (**Figure 2A-C**) [12, 13].

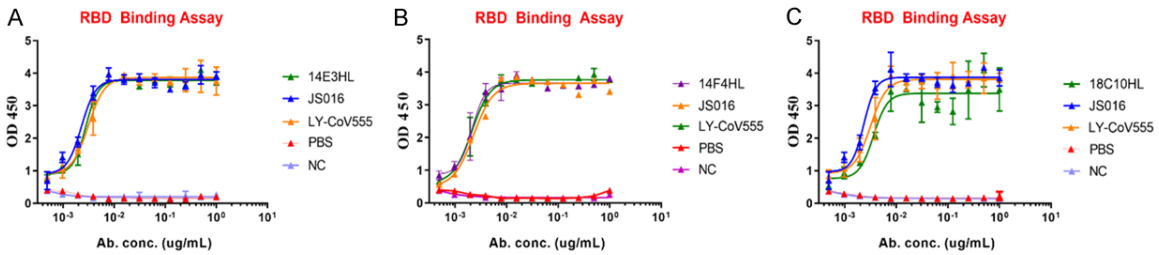

Figure 2. Antibodies affinity assays with RBD. A. Affinity abilities of 14E3HL to SARS-CoV-2 RBD. LY-CoV555 and JS016 were used as positive control. B. Affinity abilities of 14F4HL to SARS-CoV-2 RBD. LY-CoV555 and JS016 were used as positive control. C. Affinity abilities of 18C10HL to SARS-CoV-2 RBD. LY-CoV555 and JS016 were used as positive control.

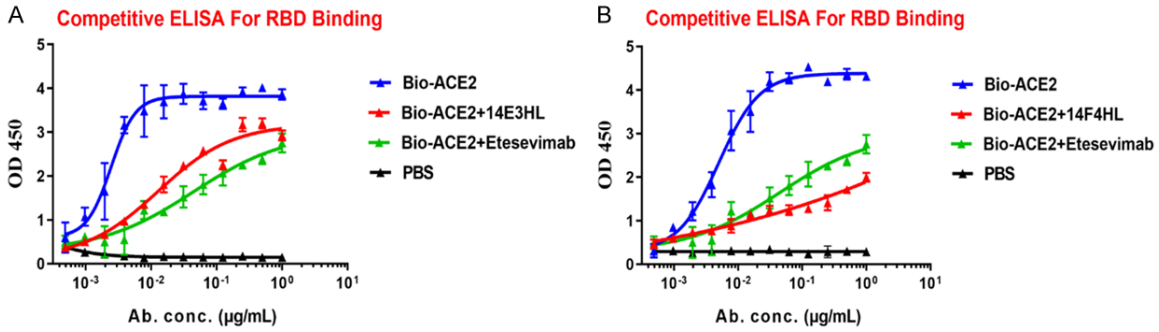

Figure 3. Competition characteristics of 14E3HL and 14F4HL. A. The competitive capacity of 14E3HL to ACE2. Etesevimab was used as a positive control. B. The competitive capacity of 14F4HL to ACE2. Etesevimab was used as a positive control.

Binding activities of antibodies to RBD

Competitive ELISA was used to further validate the properties of 14E3HL and 14F4HL, biotin-labeled ACE2 mixed with the antibody at a molar ratio of 1:10 was added as a primary antibody to the microplate plates that had been coated with RBD proteins. Etesevimab, which has been reported [14], was used as a positive control. The results showed no significant change in absorbance with decreasing concentrations of antibodies, suggesting that they target novel epitopes on the S protein that target specific antigenic sites with decreasing concentrations of antibodies, providing a foundation for the further design of antibody-based COVID-19 therapeutic (**Figure 3A**, **3B**).

Neutralization properties of antibodies

To better assess the neutralization properties of monoclonal antibodies, we further measured the inhibition of 14F4HL and 14E3HL against wild-type and mutant pseudoviruses to determine the neutralization activity of antibodies. Antibodies whose inhibition rates are more than 50% after dilution are considered effectively neutralizing. IC50 was obtained. The results showed that compared with Etesevimab, 14F4HL was inhibitory to WT (IC50 = 0.047 μg/ml vs IC50 = 0.026 μg/ml, P<0.0001), B.1.617.1 (IC50 = 0.049 μg/ml vs IC50 = 0.018 μg/ml, P<0.0001), B.1.429 (IC50 = 0.042 μg/ml vs IC50 = 0.032 μg/ml, P<0.05) and P.1 (IC50 = 0.061 μg/ml vs IC50 = 0.021 μg/ml, P<0.0001), instead of B.1.17 (IC50 = 0.034 μg/ml vs IC50 = 0.061 μg/ml, P<0.0001), and B.1.351 (IC50 = 0.061 μg/ml vs IC50 = 0.082 μg/ml, P<0.0001) (**Figures 4A-F**, **6**). 14E3HL had lower neutralizing activity to WT and B.1.617.1 (IC50 = 0.047 μg/ml vs IC50 = 0.069 μg/ml, P<0.0001) and B.1.617.1 (IC50 = 0.049 μg/ml vs IC50 = 0.065 μg/ml, P<0.001) (**Figures 5A-F**, **6**).

Pharmacokinetic parameters of monoclonal antibodies in mice

To determine whether 14E3HL and 14F4HL are of medicinal value, we studied their pseudo-

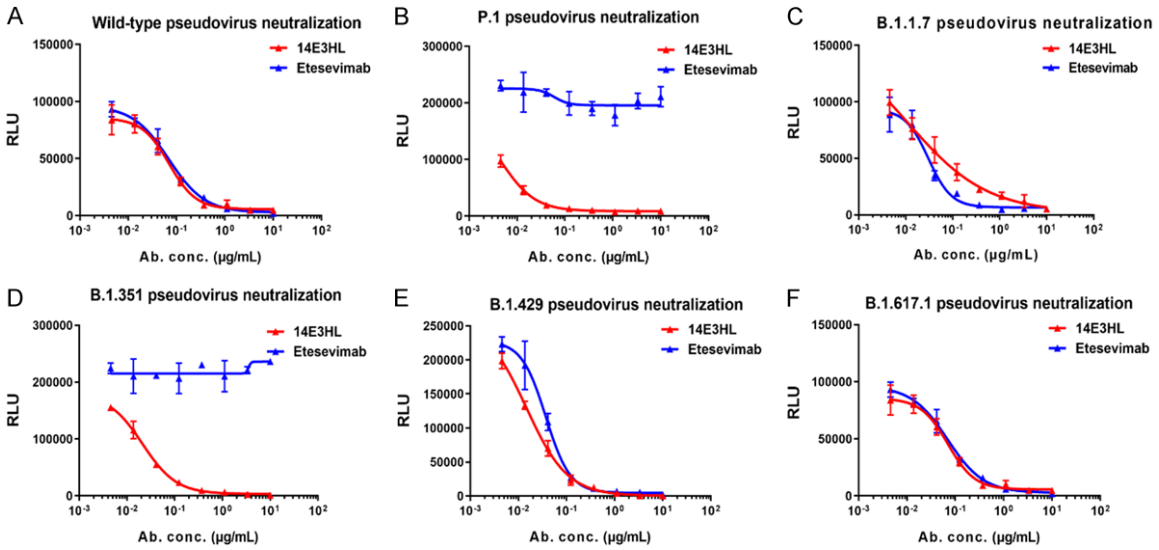

Figure 4. Neutralizing capacities of 14E3HL for each mutant strain. Neutralization of 14E3HL to: A. Wild-type pseudovirus. B. P.1 pseudovirus. C. B.1.1.7 pseudovirus. D. B.1.351 pseudovirus. E. B.1.429 pseudovirus. F. B.1.617.1 pseudovirus.

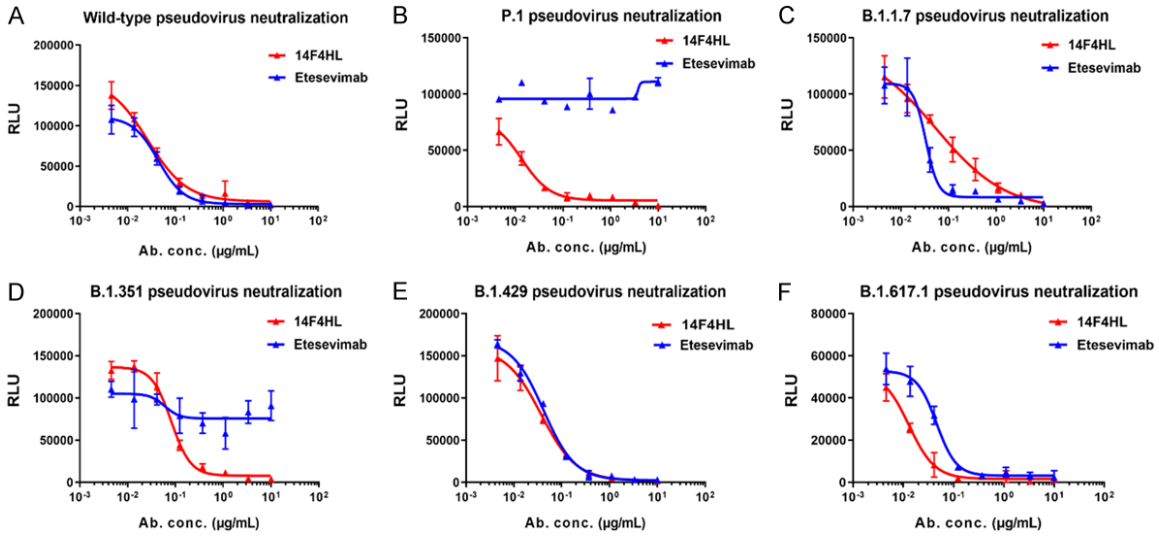

Figure 5. Neutralizing capacities of 14F4HL for each mutant strain. Neutralization of 14F4HL to: A. Wild-type pseudovirus. B. P.1 pseudovirus. C. B.1.1.7 pseudovirus. D. B.1.351 pseudovirus. E. B.1.429 pseudovirus. F. B.1.617.1 pseudovirus.

virus neutralization activity and pharmacokinetics. Neutralizing antibodies could theoretically neutralize epitopes of the RBD on pseudoviruses by rendering the pseudovirus incapable of binding to ACE2 on the cell surface, losing the ability to infect cells [15-17].

Healthy mice received a single tail vein injection of 14F4HL or 14E3HL. After the collection of blood, three samples at each time point of 0-330 h were sent for inspection. Pharmacokinetic parameters were calculated from the plasma concentration versus time curves. We found that $AUC_{0\text{-}192}$ hr of all drugs was higher, of which 14F4HL was twice as high as positive control LY-CoV555, indicating that the total amount of 14F4HL and the elimination half-life (T1/2) were higher in mice during 0-330 h (**Figure 7**).

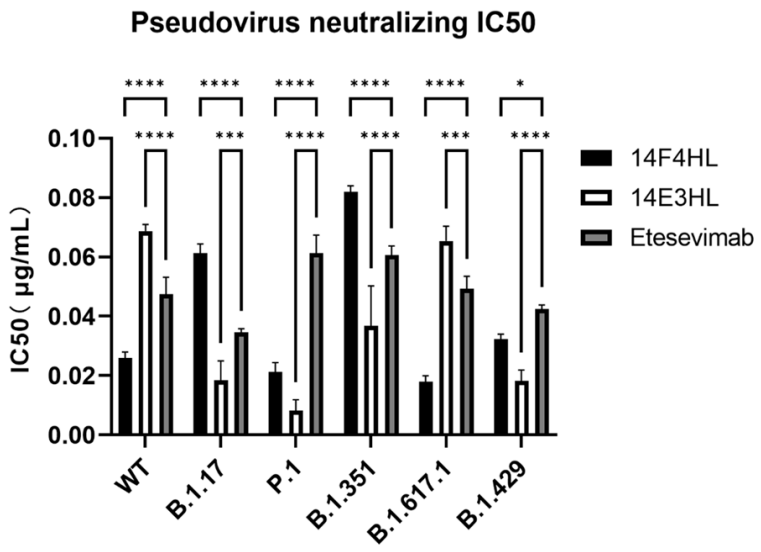

Figure 6. IC50 of 14F4HL and 14E3HL for each mutant strain. The inhibition of 14F4HL and 14E3HL against wild-type and mutant pseudoviruses was measured to determine the neutralizing activity of the antibodies. Data are representative of three independent experiments and shown as means ± SD. *P≤0.05, ***P≤0.001, and ****P≤0.0001 (Student's t-test).

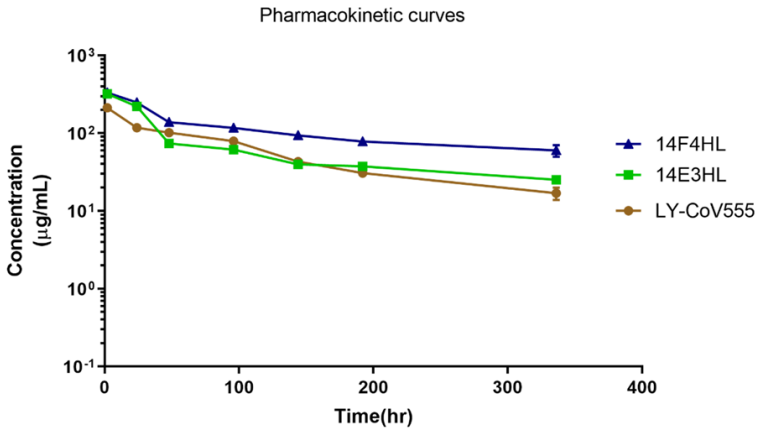

Figure 7. Pharmacokinetic curves of 14F4HL, 14E3HL, and LY-CoV555. Metabolic profiles of mice in blood during 0-330 h after injection of 14E3HL, LY-CoV555, and 14F4HL antibodies.

Discussion

To date, a variety of epidemic coronavirus infections have occurred, which pose a serious threat to human life and health, including SARS-CoV, Ebola virus, Middle East respiratory syndrome coronavirus, and recently SARS-CoV-2 [7, 18]. The global spread of SARS-CoV-2 has had an unprecedented impact on medical, research, and economic aspects [19, 20]. To control the outbreak, it is urgent to develop effective treatments to deal with the current clinical rescue and treatment needs [21, 22]. At present, most monoclonal antibodies against COVID-19 come from the B cells of patients infected with COVID-19. The anti-SARS-CoV-2 antibody sequence was obtained by RBD-specific B-cell screening, single-cell cloning, and sequencing; or immunizing animals with the susceptible site on the surface of nucleocapsid protein on the virus surface as the target to obtain monoclonal antibodies [23-25]. This study is to prepare RBD monoclonal antibody and verify its performance based on the ACE2 receptor-mediated SARS-CoV-2 entry into cells.

Detection of monoclonal antibodies by ELISA showed that the affinity of extracted monoclonal antibodies to RBD was different. Therefore, two monoclonal antibodies 14F4HL and 14E3HL with better stability were selected in the later study. Analysis of their binding specificities with the mutants revealed that the affinity of the two antibodies to RBD protein and mutant protein is similar, and their EC50 is about 0.02-0.03 μg/ml. However, it has no affinity to the South Indian mutant. Compared with the positive control antibody Etesevimab, the affinity of the Brazilian mutant and South African mutant is not high, and it has certain advantages in terms of immune escape [26, 27]. Competitive ELISA uses biotin assays to measure competitive reactions between excess streptavidin-HRP and solutions of known biotin concentrations or sample solutions and to measure the residual activity of free-form streptavidin-affinity catalase associated with initial biotin concentrations. The process includes an additional step for antibody-antigen interaction [28]. In this study, biotin-labeled ACE2 mixed with the antibody at a molar ratio

of 1:10 was added as a primary antibody to the microplate plates that had been coated with RBD proteins, and the plates were washed after incubation for 1 h. The results showed that competitive inhibition could be generated at a concentration less than 1 stage when we used the competition ELISA assay of 1:10 of ACE2 with the molar mass of the antibody. Increasing the molar ratio of ACE2 to antibody resulted in competitive inhibition in the region of a higher concentration, from which we can conclude that there is a competitive relationship between 14E3HL, 14F4HL, Etesevimab, and ACE2. Pseudovirus entry would then drive cells to express Fluc proteins that could react with luminescent substrates [15]. Bio-LiteTM Luciferase Assay System was used to detect the expression of RLU in the luciferase of fireflies and to compare the luciferase expression with that of the control group. The ID50 (antibody concentration when 50% of the pseudovirus was suppressed) was calculated to indicate the neutralizing activity of the antibody against the pseudovirus [29]. The results showed that our 14F4HL and 14E3HL antibodies blocked the binding of RBD protein and its common mutants to human ACE2 receptors in both wild and all mutant strains, and had good pseudovirus neutralization activity. Pharmacokinetic assays are designed to assess the pharmacokinetics of monoclonal antibodies after a single injection in mice and to visualize their plasma concentration-time profiles [30]. We found that $AUC_{0\text{-}192}$ hr of all drugs was higher, of which 14F4HL was twice as high as positive control LY-CoV555, indicating that the total amount of 14F4HL and the elimination half-life (T1/2) were higher in mice during 0-330 h. It can be seen that our alternative antibody molecule metabolizes slowly in vivo. Since we injected the antibody to be tested into the mice, there was no absorption peak and it reached C_{max} directly after injection.

In this study, the affinity of 14F4HL and 14E3HL antibodies to SARS-CoV-2 has been verified, but there are still inadequate studies on the stability and no more detailed study on the stability of protein aggregation tendency. During the course of the pharmacokinetic study, only the drug concentration in blood was investigated, and the pharmacokinetics of drug distribution in tissues was not explored [31]. The distribution and metabolism of drugs in different tissues are of great significance for the study of SARS-CoV-2 with different target organ damage in human infection [32, 33]. In addition, the antibody is currently a murine antibody. If it is directly used in human clinical treatment, it may activate the patient's immune system, resulting in the rapid elimination of murine antibodies by the body, which will also produce many side effects [34, 35]. Therefore, in further study, we need to prepare full-human monoclonal antibodies according to the mouse monoclonal antibody sequence through affinity maturation, glycosylation modification, Fc receptor modification, and other methods [36, 37]. ELISA, competitive ELISA, pseudovirus neutralization activity and other experiments showed that 14E4HL and 14E3HL could block the binding of RBD protein to human ACE2 protein. It has neutralizing activity against RBD protein and a variety of common mutants at the pseudovirus level. It has a slow metabolism and good medicinal properties, which is suitable for further humanization research. In addition, a wide variety of mutations in the Fc region will also be characterized for monoclonal antibodies under development to enhance or eliminate effector functions or improve the half-life of monoclonal antibodies and be used as monotherapy or cocktail therapy. The success and failure of these tests will be key to the development of more effective anti-respiratory virus antibodies.

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Disclosure of conflict of interest

None.

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