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A SARS-CoV-2 nanobody that can bind to the RBD region may be used for treatment in COVID-19 in animals

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ABSTRACT

Coronavirus disease 2019 (COVID-19) caused by an infectious virus, severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2), poses a threat to the world. The suitable treatments must be identified for this disease in animals. Nanobody have therapeutic potential in the COVID-19. In this study, SARS-CoV-2 Spike RBD protein was used to make the nanobody. Nanobodies binding to the SARS-CoV-2 Spike RBD protein was obtained. Interestingly, the nanobody could bind to SARS-CoV-2 Spike S protein and RBD protein at the same time. Nanobodies were validated with a neutralizing antibody detection kit. The use of pseudoviruses confirmed that nanobodies could prevent pseudoviruses from infecting cells. We believe the nanobody are very valuable and could be used in the treatment of COVID-19. SARS-CoV-2 nanobodies can be rapidly mass-produced from microorganisms to block SARS-CoV-2 infection in vitro and in vivo with preventive and therapeutic effects.

1. Introduction

COVID-19 is a highly contagious disease caused by severe acute respiratory coronavirus 2 (SARS-CoV-2) (Ezzikouri et al., 2020). Vaccines are effective preventive strategies to prevent COVID-19. However, vaccine breakthrough and vaccine escape make it is urgent to treat COVID-19's disease (Sun et al., 2020; Xu et al., 2021). There are few approved effective treatments for SARS-CoV-2 (Esparza et al., 2020). The treatment of SARS-COV-2 still has many problems to solve (Valenzuela Nieto et al., 2021). Low-cost treatments have important implications for the COVID-19 pandemic (Ye et al., 2020). At the same time, SARS-COV-2 neutralizing antibody is very important as a COVID-19 drug (Pymm et al., 2021).

The pandemic of COVID-19 highlights the serious consequences of animal virus leakage on public health, economy and society (Olival et al., 2020). A worrying problem is that SARS-COV-2 may spread to local wild species, which may make the virus popular by setting up a second host (Franklin and Bevins, 2020). The widespread of SARS-COV-2 in human beings increases the theoretical risk of reversal of zoonotic events in wild animals, that is, SARS-CoV-2 is introduced into animals that are not allowed to be domesticated (Griffin et al., 2021). This phenomenon exists in pets with therapeutic value.

SARS-CoV-2 binds to host cell receptors through spike (S) protein (Wrapp et al., 2020). Spike protein is the key protein for SARS-CoV-2 to enter the host cell (Schoof et al., 2020a, 2020b). This protein provides a target for the preparation of therapeutic antibodies (Schoof et al., 2020a, 2020b). In this study, a nanobody directly binds to SARS-CoV-2 Spike RBD protein was prepared, and the potential of this nanobody in treating COVID-19's disease was assessed.

2. Materials and methods

2.1. Materials

SARS-CoV-2 spike S1 + S2 ECD-His recombinant protein (S protein) (No. 40589-V08B1) and SARS-CoV-2 spike RBD-His recombinant protein (No. 40592-V08B) were purchased from Sino Biological Inc (Beijing, China). A SARS-CoV-2 surrogate virus neutralization test kit was purchased from GenScript (Nanjing, China). Pseudovirus was provided by Southern Medical University. Bright-Glo™ luciferase assay reagent was purchased from Promega (Wisconsin, United States).

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2.2. Analysis of COVID-19 protein by SDS-PAGE

Three micrograms SARS-CoV-2 Spike RBD protein and S protein was added to the protein loading solution. The mixture was boiled for 10 min. Then, 12% separating gel and 5% concentrated gel were used for Sodium dodecyl sulphates (SDS) - polyacrylamide gel electrophoresis (PAGE). The SDS was dyed with Coomassie Brilliant Blue (Solarbio, Beijing, China) and then decolorized with a mixture of 30% glacial acetic acid (Solarbio, Beijing, China) and 70% alcohol.

2.3. Screen of the nanobody

To a 96-well plate, 10 μg SARS-CoV-2 spike RBD protein was added. Phosphate buffered saline (PBS) milk was used to cultivate the virus at 4 °C. The selected nanobody-phage was added and incubated for several hours. 0.1% Tween20-PBS was used to wash the sample 10 times. It was then eluted with 0.2 M glycine hydrochloric acid (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). The eluted phages were collected and used for the next screening, and the screening conditions were the same as above.

SARS-CoV-2 spike RBD protein (200 ng/well) was added to ELISA wells (Solarbio, Beijing, China) and placed in a refrigerator overnight at 4 °C, and then sealed with skimmed milk for 1 h. Screened nanobody-phage was added and incubated for 1 h, then eluted with PBS. Anti-phage Horseradish Peroxidase (HRP) antibody (HBindBiotech, Wuhan, China) was added, and then chromochrome solution (Solarbio, Beijing, China) was added for 15 min. Termination solution was added to terminate the reaction and the absorbance was measured at 450 nm wavelength.

2.4. Preparation of nanobody

The prokaryotic expression vector was pET28a-SUMO (HBindBiotech, Wuhan, China). *E. coli* BL21(DE3) (HBindBiotech, Wuhan, China) strain was used to express the nanobody. SDS-PAGE was used to verify the correct expression of the protein.

2.5. Determination of affinity

SARS-CoV-2 spike RBD protein and SARS-CoV-2 S protein (200 ng/well) were coated on 96-well ELISA plates, and samples without coating served as controls. Nanobody was used for ELISA detection. Mouse anti-HA polyclonal antibody (Solarbio, Beijing, China) was used as the secondary antibody, and HRP-conjugated Goat Anti-Mouse IgG (Solarbio, Beijing, China) was used as the last antibody. Chromochrome solution was added for 15 min. The termination solution was added to terminate the reaction and the absorbance was measured at 450 nm wavelength.

2.6. Neutralizing antibody detection experiment

The sample and HRP-SARS-CoV-2 Spike RBD protein were mixed in a volume ratio of 1:1. The sample was incubated at 37 °C for 30 min. Then, 100 μL mixture was added to the microplate. The 96-well plate was incubated at 37 °C for 15 min. The plate was washed four times. Next, 100 μL 3,3′,5,5’-Tetramethylbenzidine (TMB) solution was added to each well and incubated at 25 °C in the dark for 15 min, and 50 μL stop solution was added to the well. The absorbance was measured at 450 nm with a microplate reader.

2.7. Pseudovirus neutralization test

Dulbecco’s modified eagle medium (DMEM) was added to 100 μL/well solution and allowed to culture. The virus control was added 50 μL/well DMEM. Then, 90 μL/well medium was added to the third column, and 50 μL/well medium was added to the remaining wells. Nanobody was added to the third column with 10 μL/well and pipetted 6–8 times. All wells were diluted twice. SARS-CoV-2 pseudovirus was added to columns 3 through 11 at a concentration of 1.28×10^4 $TCID_{50}$/mL and volumes of 50 μL/well. The 96-well plate was placed in a 37 °C cell incubator and incubated in 5% CO_2 for 1 h. ACE2-293 T (GenScript, Nanjing, China) cells were added to each well with 3×10^4, and then incubated for 48 h. The supernatant was removed with 100 μL, and then 100 μL of luciferase detection reagent was added. After the 96-well plate was reacted at room temperature and protected from light for 5 min. Pipetting was repeated, and 200 μL of liquid was transferred to the white plate. The fluorescence was read using a microplate reader. The antibody concentration and neutralizing antibody titer were calculated when the inhibition rate was 50%.

2.8. Statistical analysis

The results are here expressed as the mean ± standard deviation (SD). Significant differences were confirmed using a Mann-Whitney *U* test $^*P < 0.05$, $^{**}P < 0.01$. Statistical analyses were conducted using GraphPad Prism software (GraphPad Software, California, United States).

3. Results

3.1. Verification of COVID-19 proteins

SARS-CoV-2 Spike S protein was expressed using an insect cell expression system. SARS-CoV-2 Spike RBD protein was expressed in a 293-cell expression system. SARS-CoV-2 Spike RBD protein has a single band around 30 kDa, and SARS-CoV-2 S protein has three bands with molecular weights around 60 kDa, 100 kDa, and 150 kDa (Fig. 1A), which is consistent with the literature. The protein has been shown to form nanobodies.

3.2. Screening of nanobodies

Four rounds of screening were conducted (Fig. 1B). In the second screening, the enrichment concentration was significantly increased (Fig. 1C). The specific nanobody was sequenced, and the sequencing primer was MP57 (TTATGCTTCCGGCTCGTA TG). The results of electrophoresis showed that the gene had been cloned correctly (Fig. 1D). After analysis and arrangement of the sequencing sequence, a number of nanobody sequences were produced. All fragments were amplified effectively, and the fragment size was about 400 bp. The pET-28-SUMO vector was used to express the protein (Fig. 1E). The expressed nanobodies were validated using SDS-PAGE (Fig. 1F).

3.3. Nanobody antibody ELISA tests

The results showed that nanobodies could bind to both SARS-CoV-2 S protein and SARS-CoV-2 Spike RBD protein (Fig. 1G). The nanobody has shown binding activity to two proteins: SARS-CoV-2 S protein and SARS-CoV-2 Spike RBD protein. SARS-CoV-2 Spike RBD protein has more affinity to nanobody than SARS-CoV-2 Spike S protein.

3.4. Virus neutralization test

The nanobody was also different considerably from the negative group with respect to OD450. Using the kit, we were able to see that the nanobodies exerted a neutralizing effect (Fig. 1H).

3.5. Neutralization of SARS-CoV-2 pseudovirus

Nanobodies have the ability to inhibit the entry of pseudoviruses into cells (Fig. 1I). As the concentration of nanobodies increased, their inhibitory ability also improved. When the quantity reached 100 μg, the rate of pseudovirus inhibition was 85%.

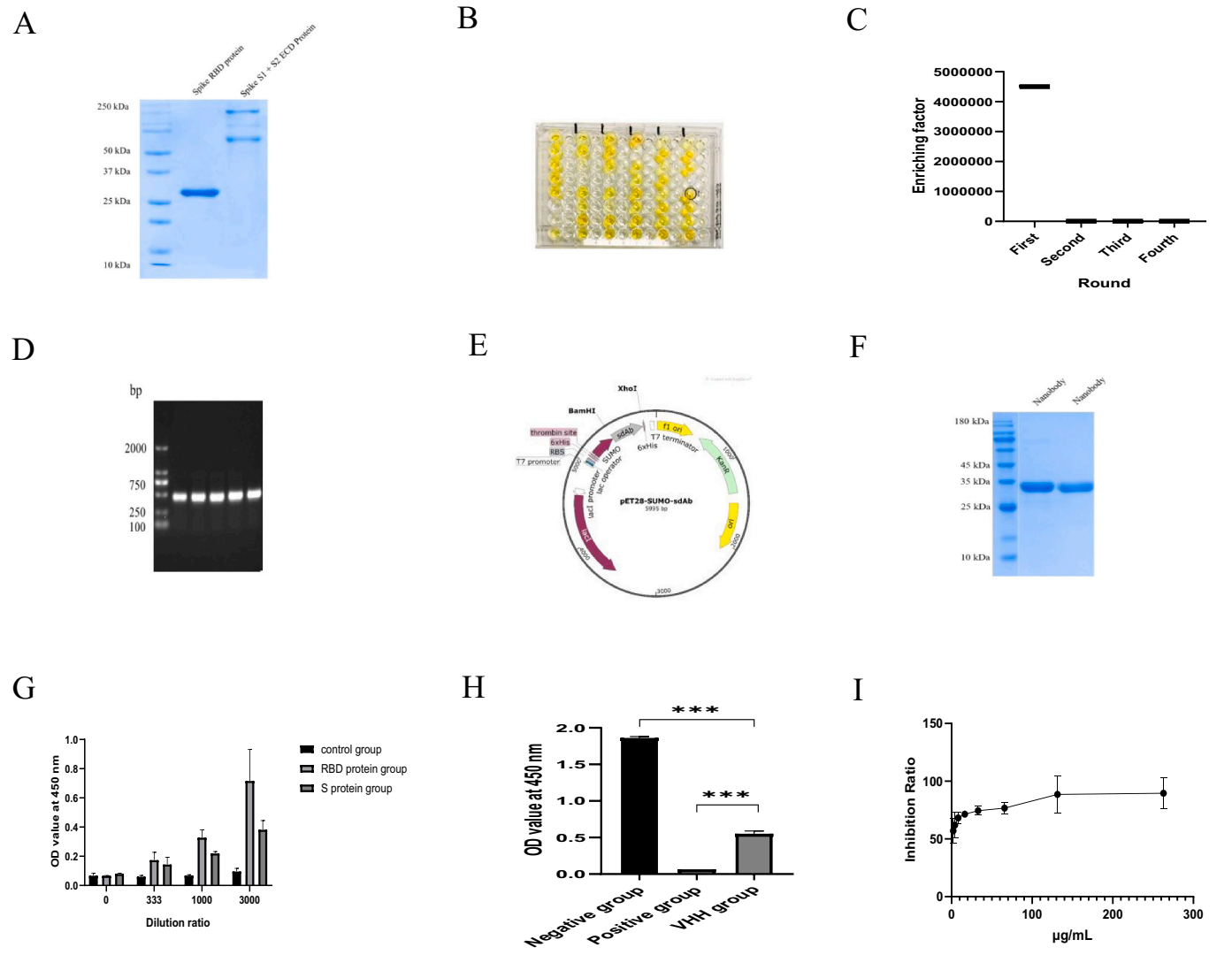

Fig. 1. Nanobody binds to SARS-CoV-2. (A) SDS-PAGE electrophoresis verification of SARS-CoV-2 S protein and SARS-CoV-2 Spike RBD protein. (B) Nanobody was screened using ELISA. (C) The nanobody was screened four times. (D) Nanobody gene was cloned for prokaryotic expression. (E) The pET-28-SUMO vector was used to express the protein. (F) The expressed nanobodies were validated using SDS-PAGE. (G) Nanobodies bind to SARS-CoV-2 Spike S protein and SARS-CoV-2 Spike RBD protein. (H) The neutralizing ability of nanobody was tested. (I) Inhibition ratio of the nanobodies.

4. Discussion

Nanobodies are very similar to human VHs and have potential applications in immunotherapy reagents (Ahmadvand et al., 2008). Nanobodies has the advantages of strong nano-affinity, easy modification, easy penetration into tissues and so on (Kong et al., 2014). Nanobodies are superior to traditional antibodies in high affinity and specific binding with different antigens (Kijanka et al., 2017). It is also easy to express in the prokaryotic expression system, which greatly reduces the production cost (He et al., 2018). The size of the nanobody is very small (15KDa), the cost is very low and easily penetrated the tissues such as lungs (Koenig et al., 2021). Nanobody has great potential in the treatment of the COVID-19 (Czajka et al., 2021).

The COVID-19 has severely affected the health of people and animals around the world (Xu et al., 2021). The COVID-19 pandemic is still very serious (Mast et al., 2021). It is of great significance to develop safe and effective methods to treat COVID-19 infection (Lu et al., 2021). Neutralizing antibodies are a promising method to fight the virus (Chi et al., 2020; Saied et al., 2021). However, the literature on antibody therapy is extensive. We believe that the nanobody could effectively bind to the neutralizing site of RBD. It doesn't allow the tissue virus to enter the cell. The nanobody demonstrated in this study can directly bind to RBD, and can also bind to S protein. This means that our nanobodies can directly bind to RBD regardless of the different structure of the S protein (Jia et al., 2021).

5. Conclusions

The nanobodies prepared in this study showed very high affinity to the structures on the SARS-CoV-2. The nanobody we discovered can make direct contact with SARS-CoV-2 Spike RBD protein and is not affected by other structures on the SARS-CoV-2 Spike S protein. Nanobodies can be prepared in large quantities, which involves low production costs and renders commercialization viable. Based on the results obtained from this study, nanobodies have significant potential for the treatment of COVID-19 especially under scenarios of pandemics.

Declaration of Competing Interest

None.

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References

Ahmadvand, D., Rahbarizadeh, F., Vishteh, V.K., 2008. High-expression of monoclonal nanobodies used in the preparation of HRP-conjugated second antibody. Hybridoma 27, 269–276.

Chi, X., Liu, X., Wang, C., et al., 2020. Humanized single domain antibodies neutralize SARS-CoV-2 by targeting the spike receptor binding domain. Nat. Commun. 11 (1), 4528.

Czajka, T.F., Vance, D.J., Mantis, N.J., 2021. Slaying SARS-CoV-2 one (single-domain) antibody at a time. Trends Microbiol. 29 (3), 195–203.

Esparza, T.J., Martin, N.P., Anderson, G.P., Goldman, E.R., Brody, D.L., 2020. High affinity nanobodies block SARS-CoV-2 spike receptor binding domain interaction with human angiotensin converting enzyme. Sci. Rep. 10 (1), 22370.

Ezzikouri, S., Nourlil, J., Tsukiyama-Kohara, K., Kohara, M., El Ossmani, H., Windisch, M.P., Benjelloun, S., 2020. Nanobodies: an unexplored opportunity to combat COVID-19. J. Biomol. Struct. Dyn. 1–3.

Franklin, A.B., Bevins, S.N., 2020. Spillover of SARS-CoV-2 into novel wild hosts in North America: a conceptual model for perpetuation of the pathogen. Sci. Total Environ. 733, 139358.

Griffin, B.D., Chan, M., Tailor, N., Mendoza, E.J., Leung, A., Warner, B.M., Duggan, A.T., Moffat, E., He, S., Garnett, L., Tran, K.N., Banadyga, L., Albietz, A., Tierney, K., Audet, J., Bello, A., Vendramelli, R., Boese, A.S., Fernando, L., Lindsay, L.R., Jardine, C.M., Wood, H., Poliquin, G., Strong, J.E., Drebot, M., Safronetz, D., Embury-Hyatt, C., Kobasa, D., 2021. SARS-CoV-2 infection and transmission in the north American deer mouse. Nat. Commun. 12, 3612.

He, T., Zhu, J., Nie, Y., Hu, R., Wang, T., Li, P., Zhang, Q., Yang, Y., 2018. Nanobody technology for mycotoxin detection in the field of food safety: current status and prospects. Toxins 10, 180.

Jia, Z., Liu, C., Chen, Y., Jiang, H., Wang, Z., Yao, J., Yang, J., Zhu, J., Zhang, B., Yuchi, Z., 2021. Crystal structures of the SARS-CoV-2 nucleocapsid protein C-terminal domain and development of nucleocapsid-targeting nanobodies. FEBS J. 10 (19), 1–13. https://doi.org/10.1111/febs.16239.

Kijanka, M., Van Donselaar, E.G., Müller, W.H., et al., 2017. A novel immuno-gold labeling protocol for nanobody-based detection of HER2 in breast cancer cells using immuno-electron microscopy. J. Struct. Biol. 5 (25), 1–11. https://doi.org/10.1016/j.jsb.2017.05.008 (S1047847717300886).

Koenig, P.A., Das, H., Liu, H., et al., 2021. Structure-guided multivalent nanobodies block SARS-CoV-2 infection and suppress mutational escape. Science 371 (6530).

Kong, Q., Yao, Y., Chen, R., Lu, S., 2014. Progress in nanobody and its application in diagnosis. Chin. J. Biotechnol. 30, 1351.

Lu, Q., Zhang, Z., Li, H., et al., 2021. Development of multivalent nanobodies blocking SARS-CoV-2 infection by targeting RBD of spike protein. J. Nanobiotechnol. 19 (1), 33.

Mast, F.D., Fridy, P.C., Ketaren, N.E., et al., 2021. Nanobody repertoires for exposing vulnerabilities of SARS-CoV-2. bioRxiv 4 (10), 1–45. https://doi.org/10.1101/2021.04.08.438911.

Olival, K.J., Cryan, P.M., Amman, B.R., Baric, R.S., Blehert, D.S., Brook, C.E., Calisher, C. H., Castle, K.T., Coleman, J., Daszak, P., Epstein, J.H., Field, H., Frick, W.F., Gilbert, A.T., Hayman, D., Ip, H.S., Karesh, W.B., Johnson, C.K., Kading, R.C., Kingston, T., Lorch, J.M., Mendenhall, I.H., Peel, A.J., Phelps, K.L., Plowright, R.K., Reeder, D.M., Reichard, J.D., Sleeman, J.M., Streicker, D.G., Towner, J.S., Wang, L. F., 2020. Possibility for reverse zoonotic transmission of SARS-CoV-2 to free-ranging wildlife: a case study of bats. PLoS Pathog. 16, e1008758.

Pymm, P., Adair, A., Chan, L.J., et al., 2021. Nanobody cocktails potently neutralize SARS-CoV-2 D614G N501Y variant and protect mice. Proc. Natl. Acad. Sci. U. S. A. 118 (19).

Saied, A.A., Metwally, A.A., Mohamed, H., Haridy, M., 2021. The contribution of bovines to human health against viral infections. Environ. Sci. Pollut. Res. Int. 28, 46999–47023.

Schoof, M., Faust, B., Saunders, R.A., et al., 2020a. An ultra-high affinity synthetic nanobody blocks SARS-CoV-2 infection by locking spike into an inactive conformation. bioRxiv 8 (10), 1–48.

Schoof, M., Faust, B., Saunders, R.A., et al., 2020b. An ultrapotent synthetic nanobody neutralizes SARS-CoV-2 by stabilizing inactive spike. Science 370 (6523), 1473–1479.

Sun, Z., Chen, C., Li, W., et al., 2020. Potent neutralization of SARS-CoV-2 by human antibody heavy-chain variable domains isolated from a large library with a new stable scaffold. MAbs 12 (1), 1778435.

Valenzuela Nieto, G., Jara, R., Watterson, D., et al., 2021. Potent neutralization of clinical isolates of SARS-CoV-2 D614 and G614 variants by a monomeric, sub-nanomolar affinity nanobody. Sci. Rep. 11 (1), 3318.

Wrapp, D., De Vlieger, D., Corbett, K.S., et al., 2020. Structural basis for potent neutralization of betacoronaviruses by single-domain camelid antibodies. Cell 181 (5), 1004–1015 (e15).

Xu, J., Xu, K., Jung, S., et al., 2021. Nanobodies from camelid mice and llamas neutralize SARS-CoV-2 variants. Nature 595 (7866), 278–282. https://doi.org/10.1101/2021.03.04.433768.

Xu, J., Xu, K., Jung, S., et al., 2021. Multimeric nanobodies from camelid engineered mice and llamas potently neutralize SARS-CoV-2 variants. bioRxiv 4 (4), 1–5. https://doi.org/10.1101/2021.03.04.433768.

Ye, G., Gallant, J.P., Massey, C., et al., 2020. The development of a novel nanobody therapeutic for SARS-CoV-2. bioRxiv 11 (17), 1–48. https://doi.org/10.1101/2020.11.17.386532.