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Recombinant scFv Antibodies against E Protein and N Protein of Severe Acute Respiratory Syndrome Virus

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Abstract Three single chain antibodies (scFv) against the proteins of severe acute respiratory syndrome coronavirus (SARS-CoV) were isolated by phage display from an scFv antibody library. Bio-panning was carried out against immobilized purified envelope (E) and nucleocapsid (N) proteins of SARS-CoV. Their binding activity and specificity to E or N protein of SARS-CoV were characterized by phage-ELISA. Two of them, B10 and C20, could recognize non-overlapping epitopes of the E protein according to the two-site binding test result. Clone A17 could recognize N protein. The sequence of the epitope or overlapping epitope of scFv antibody A17 was PTDSTDNNQNGGRNGARPKQRRPQ. The affinity (equilibrium dissociation constant, K_d) of SARS-CoV E protein was 5.7×10^{-8} M for B10 and 8.9×10^{-8} M for C20. The affinity of A17 for N protein was 2.1×10^{-6} M. All three scFv antibodies were purified with affinity chromatography and determined by Western blot.

Key words SARS-CoV; antibody; scFv; phage display; affinity chromatography

Severe acute respiratory syndrome (SARS) brought a global outbreak in spring of 2003 [1–3], and more and more attention has been paid on it when a new case resurfaced in Singapore last September [4]. By the end of May in 2003, WHO reported a cumulative total of 8202 infected cases with 725 deaths from 28 countries. Because of the high transmission and morality rate of SARS, scientists in many countries have made their efforts in studying SARS coronavirus (SARS-CoV) [5, 6]. Several genomes of SARS-CoV subgroup have been mapped and the functions of some proteins have been determined [7, 8]. On the basis of structural information of SARS-CoV proteins, three-dimensional model of several SARS proteins have been designed, including the S1 and S2 subunit of SARS CoV spike glycoprotein [9], small envelope (E) protein [10] and 3CL proteinase of SARS-CoV [11, 12]. The epitope of SARS-CoV nuleocapsid (N) protein has been identified, which might be helpful in designing specific antiviral drugs [13]. Wang *et al.* [14] also found that some peptides from SARS structural proteins appeared to be highly immunogenic and might be useful for serologic assays.

The SARS-CoV genome is 29,727 nucleotide in length with organization similar to that of other coronaviruses. There are eleven open reading frames corresponding to known coronavirus proteins including the polymerase protein, spike protein (S), small membrane protein (E), membrane protein (M) and N protein [15]. Researches have revealed that E and N protein play important multi-functional roles in coronavirus virion life cycle based on available information of other coronaviruses [16,17]. Such property makes it necessary to develop antibodies against theses proteins, which can provide basis for further study on SARS or candidates for specific antiviral drugs.

Phage display of antibody libraries provided a powerful tool for the isolation of recombinant antibodies against important viral pathogens [18–20], and in some cases needn't immunize animals [21–23]. This technique is based on recombinant DNA methods to allow co-selection of recombinant antibodies and their respective genes [24].

In this paper, with bio-panning technique, we selected three recombinant scFv antibodies against E and N protein of SARS-CoV respectively from a phage display

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scFv antibody library derived from murine. The primary properties of the three scFv antibodies were reported here. These scFv antibodies showed specific binding activity and relatively high affinity for E and N protein, which provided basis for further study on SARS virus.

Materials and Methods

Materials

The purified epitope and nuclear protein of SARS-CoV were kindly gifted by Prof. Hua-Liang JIANG and Prof. Xu SHEN (Shanghai Institute of Materia Medica, SIBS, CAS); the proteins were expressed in *E. coli* and purified by affinity chromatography. The N1-BSA and N2-BSA were kindly provided by Prof. Bing SUN (Institute of Biochemistry and Cell Biology, SIBS, CAS). The helper phage Vcsm13, *E. coli* XL1-Blue and BL21(DE3) were stored in our lab. BSA was purchased from Shenergy Biological Science & Technology Company (Shenergy, Shanghai). The purified BlyS (B lymphocyte stimulator) and BCMA (receptor-I of BlyS) protein were stored in our lab.

Phage display library

The mouse semisynthetic V_H+V_L scFv phage display library was prepared previously in our lab. The library was highly variable and contained approximately 10^6 different clones of scFv antibody. The library-derived phages were large-scale amplified as described previously [25] and used for bio-panning.

Bio-panning

For bio-panning, immunotubes (Nunc) were coated with 5 μg/ml N protein and E protein of SARS-CoV in 100 mM PBS buffer, pH 7.4, at 4 °C overnight. After blocking with 4% skimmed milk dissolved in PBS for 1 h at 37 °C, about 10^{12} pfu phage particles were added into the immunotube for binding at 37 °C. Two hours later, non-specific binders were washed away. The bound phages were eluted with 0.1 M HCl (adjusted to pH 2.2 with solid glycine) supplemented with 2% BSA (*W*/*V*), and neutralized with total 600 μl Tris-HCl (1 M, pH 7.5). The eluted phages were re-amplified by infecting freshly prepared *E. coli* XLI-Blue, and then plated onto SB agar plates. The clones were incubated at 37 °C overnight and scrapped into SB medium with shaking for 1 h at 37 °C, then cultured with 10^{12} pfu Vcsm13 helper phages (Strategene) for another hour at 37 °C in shaking. After adding 70 μg/ml kanamycin, the mixtures were further cultured at 37 °C overnight in shaking. The supernatant of the culture was collected by centrifugation and transferred to a fresh tube. Antibody phages in the supernatants were precipitated by 4% PEG8000 and 3% NaCl, followed by incubation for 30 min on ice, re-suspended in PBS buffer (pH 7.4), and used for analysis next round of panning. At the end of each round in bio-panning, single colony was taken from the titration plate for phage-ELISA detection and sequence analysis.

Phage ELISA

The 96-well plate was coated with 5 μg/ml E or N protein in PBS at 4 °C overnight, and blocked with 4% skimmed milk. The phage supernatant was added and incubated with the coated protein at 37 °C for 2 h. The bound phage clones were detected with HRP conjugated anti-M13 mouse antibody (Amersham Pharmacia) and the substrate TMB/H_2O_2 (Shenergy) for horseradish peroxide. Color development was stopped with 2 M H_2SO_4, and absorbance value at 450 nm was taken.

ELISA was performed similarly to phage-ELISA, except that the proteins were incubated with purified scFv antibodies at 37 °C for one hour instead of phage supernatant. Then ELISA was developed with HRP-conjugated anti-flag M2 mouse antibody (Sigma) and substrate TMB (Shenergy). The absorbance value at 450 nm was obtained.

Cross-reactivity with unrelated proteins

The antigen specificity of the scFv antibodies was evaluated by analyzing the reactivity to BlyS, BCMA and BSA. These antigens (1–5 μg/ml) were coated directly onto 96-well plates, and the phage-ELISA was conducted as described above.

Expression and purification of soluble recombinant anti-SARS scFv antibodies

The positive phage clones were digested with *Sfi*I and cloned into the expression vector pET15b digested with the same restriction enzyme. Then recombinants were transformed into *E. coli* BL21(DE3) and induced during log phase growth with 0.5 mM IPTG. After incubation at 37 °C overnight, the proteins in the periplasm were collected by solution I (20 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 20% sucrose) and solution II (20 mM Tris-HCl, pH 8.0, 2.5 mM EDTA) respectively. The anti-flag M2 agarose (Sigma) was used to incubate with the proteins from periplasm, the bound scFv antibodies were purified following the instructions of Sigma Company. The

purified proteins were then detected by SDS-PAGE and Western blot.

Western blot

The purified scFv antibodies were separated by 12% SDS-PAGE and transferred to PVDF membrane. Then the membrane was blocked with 5% skimmed milk, and incubated with HRP-conjugated mouse anti-flag M2 antibody. After washing with PBS, the substrate DNB (Shenergy) was added to develop color.

Determination of affinity constant

The affinity constant of selected scFv antibodies against SARS-CoV was determined following the protocol described by Friguet *et al.* [26]. Firstly, the 96-well plate was coated with 5 μg/ml E or N protein, then serially diluted purified scFv protein in 2% skimmed milk was added at 37 °C for 1 h, the bound antibodies were detected with normal ELISA. The concentration of scFv antibodies showing 50% of maximal antigen binding activity would be used in competitive ELISA.

Competitive ELISA was performed on immobilized E or N protein as described above except that the E or N protein at various concentration was mixed with a constant amount of antibody in 100 mM PBS (pH 7.4) supplemented with 10 mg/ml BSA. The antibody concentration used was deduced by ELISA as mentioned above. After incubation overnight at room temperature, 50 μl of each mixture was transferred into the well coated with E or N protein and incubated for 1 h at 37 °C. After washing, the bound scFv antibodies were detected as above in triplicate. Then the affinity of scFv antibodies was calculated by Scatchard Analysis equation [26].

Two-site binding test

Microtiter plates were coated with purified scFv B10 or C20 at 10 μg/ml, and kept at 4 °C overnight. Serially diluted E protein was added with PBS (without E protein) as control, then 5 μg/ml purified C20 or B10 scFv antibody was added subsequently, followed by the HRP-conjugated anti-flag M2 mouse antibody and substrate TMB/H_2O_2, and the color reaction was terminated by 2 M H_2SO_4 and the absorbance was detected at 450 nm.

Results

Screening of scFv antibodies against SARS proteins

After three rounds of bio-panning against the antigens of SARS-CoV, 100 clones were picked up randomly and their binding to E or N protein of SARS-CoV by phage-ELISA were assessed respectively. 34/100 clones were positive to E protein, and 11/100 to N protein. The cross-reactivity with three unrelated proteins BCMA, BSA and BlyS showed that only clone B10 and C20 were specific to the E protein, and clone A17 specific to N protein (data shown below).

Conforming the specificity of selected scFv clones

Binding specificity of scFv antibody B10 and C20 against E protein The binding specificity of two positive scFv clones B10 and C20 against E protein were determined by phage-ELISA with helper phage as a negative control. The results were shown in Fig. 1 and Fig. 2.

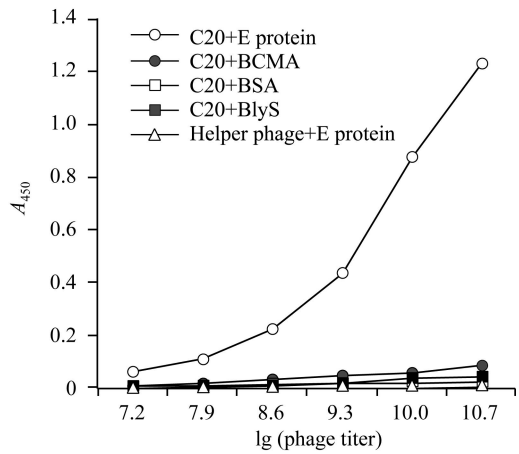


Fig. 1 Binding specificity of scFv antibody C20 to purified E protein of SARS-CoV with phage-ELISA

Helper phage with E protein of SARS-CoV was a negative control.

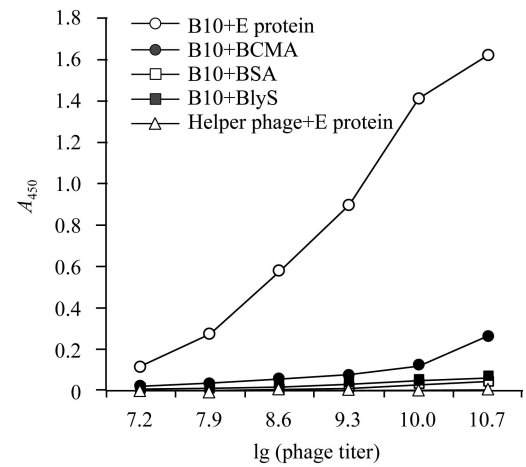


Fig. 2 Binding specificity of scFv antibody B10 to purified E protein of SARS-CoV with phage-ELISA

Helper phage with E protein of SARS-CoV was a negative control.

The cross-reactivity of the two clones with three unrelated proteins, BlyS, BCMA and BSA, were also detected, and the data revealed that there were almost no cross-reactivity between the selected clones with these unrelated proteins, except that clone B10 showed comparatively weak binding signal with purified BCMA protein, and the absorbance at 450 nm was 0.266 (Fig. 2).

Binding specificity of scFv antibody A17 against N protein As mentioned above, the binding specificity of clone A17 was determined by phage-ELISA with purified N protein of SARS-CoV coated on the 96-well plate. The results were shown in Fig. 3, in which clone A17 also didn't react with the unrelated proteins, BlyS, BCMA and BSA.

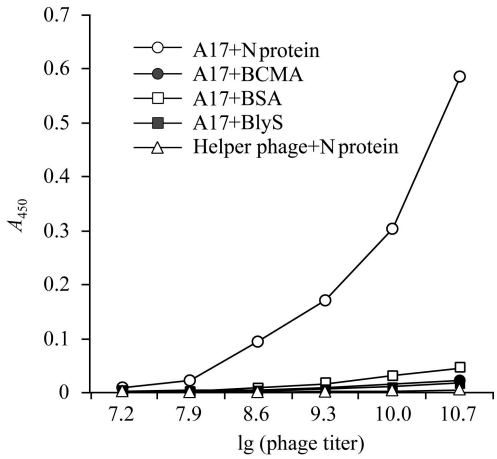


Fig. 3 Binding specificity of scFv antibody A17 to purified N protein of SARS-CoV with phage-ELISA

Helper phage with N protein of SARS-CoV was a negative control.

Determination of the epitope of scFv antibody A17 on N protein of SARS-CoV To investigate the binding sites of the scFv antibody A17 against N protein, two peptides N1 (PTDSTDNNQNGGRNGARPKQRRPQ), N2 (GALNTPKDHIGTRNPNNNAATVL) derived from the sequence of SARS putative nucleotide protein (GenBank No. NC 004718) conjugated with BSA were used to be immobilized in the 96-well plate respectively. The binding activity of scFv A10 with N1-BSA or N2-BSA was determined by phage-ELISA. Compared with N2-BSA and BSA protein, the binding signal of scFv antibody A17 with N1-BSA was much stronger (Fig. 4), which implied that A17 could bind N1 peptide specifically. The results also revealed that N1 peptide was one of the epitope or overlapping epitope of scFv antibody A17, and this epitope had been verified by Lin *et al.* [13] before.

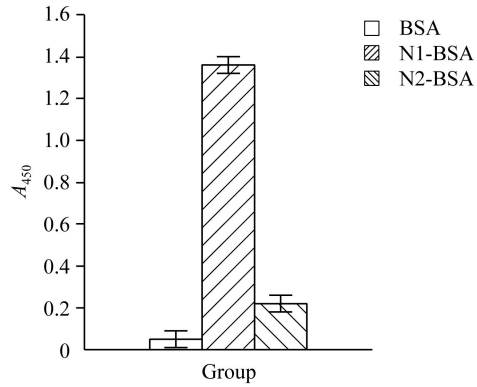


Fig. 4 Binding reactivity of scFv antibody A17 with N1 and N2 peptides

Phage-ELISA was performed with N1-BSA, N2-BSA and BSA proteins as described in materials and methods, phage supernatant of clone A17 was incubated with three coated proteins. The color was developed with TMB.

Purification of the scFv antibodies and Western blot

The scFv antibody genes were cut from the phagemid and inserted into the expression vector pET15b. The expressed scFv antibodies were collected from the periplasm of the bacteria and purified with anti-flag affinity chromatography. The purified scFv antibodies were detected by using HRP conjugated mouse anti-flag antibody. Western blot results were shown in Fig. 5. All three scFv antibodies showed a single band at about 30 kD.

Determination of the affinity constant of the scFv antibodies

According to the Scatchard analysis equation, the affinity of the scFv antibodies for the SARS proteins were

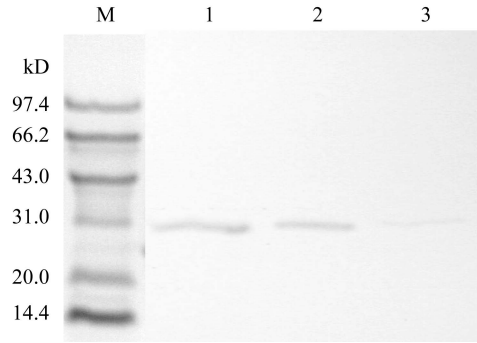


Fig. 5 Western blot of the purified scFv antibodies

Target antibody band was detected with HRP-conjugated anti-flag M2 mouse antibody and developed with DAB. M, molecular marker; 1, B10; 2, C20; 3, A17.

shown in Table 1. The affinities of scFv antibodies for E protein were much higher, with K_d being 5.7×10^{-8} M and 8.9×10^{-8} M for B10 and C20 respectively. The K_d of antibody A17 for N protein was 2.1×10^{-6} M.

Table 1 Affinity determination of the scFv antibodies against the proteins of SARS-CoV

scFv antibody	K_d (M)	
	E protein	N protein
B10	5.7×10^{-8}	–
C20	8.9×10^{-8}	–
A17	–	2.1×10^{-6}

Two-site binding assay of scFv antibodies and E protein

All possible pairs of scFv antibodies for E protein were tested for applicability by sandwich ELISA. The scFv antibody C20 with B10 as detector rendered the highest optical density. The purified scFv C20 was immobilized to trap E protein, and serially diluted E protein was added (PBS without E protein as control), the second purified scFv B10 was chosen as detector. Fig. 6 showed that scFv B10 and C20 were two-site binding antibodies against E protein, and they might recognize non-overlapping epitopes of E protein with relatively high affinity, which would be useful in further study of SARS-CoV.

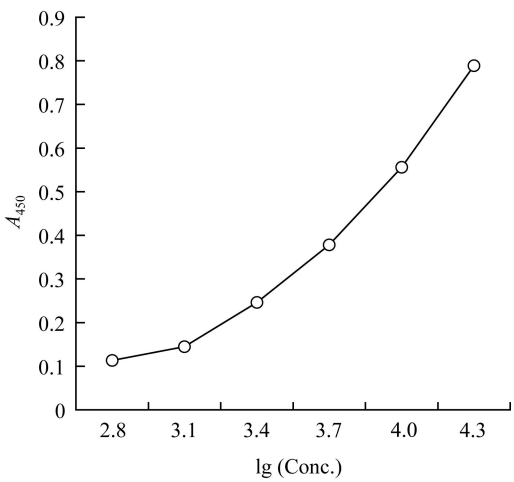


Fig. 6 Dose-response curve in two-site binding assay of scFv antibody C20 and B10 with E protein

Microtiter plates were coated with 10 μg/ml purified C20 scFv antibody, serially diluted E protein was added, followed by 5 μg/ml B10 scFv antibody as a detector.

Discussion

SARS brought a worldwide outbreak of lethal contagion in the spring in 2003. It is significant to develop antibodies against SARS-CoV, which may be helpful in figuring out the infection pathway of SARS virus and efficiently preventing it from infecting. Some clinical trials verified that the anti-SARS serum from the recovered patients could cure SARS patients [27], which indicated that the antibodies against SARS virus could efficiently block SARS virus. Theoretically, all proteins of SARS-CoV can be used as targets to screen anti-SARS antibodies. Research has revealed that E and N protein play important multifunctional roles in coronavirus virion life cycle. In this study, we selected specific recombinant scFv antibodies against E and N protein of SARS-CoV from the phage display library, which would provide basis for further study on the pathogenesis or early diagnosis of SARS virus. Three recombinant scFv antibodies were isolated herein with no detective cross-reactivity with other antigens. However, during the panning procedure, we also found lots of scFv antibodies could bind the proteins of SARS-CoV with significantly high affinity and weak cross-reactivity. But we didn't further study other clones. It was reported that the children patients of SARS presented mild clinical symptom compared to adults or older patients, and no case fatality in infected children has been found [28,29]. Maybe it was because the children have been immunized with multiple kinds of vaccines. However, it was unknown which kind of vaccine immunized before played the role in protecting the children against the SARS virus.

Single-chain fragment variable (scFv) antibody libraries displayed on filamentous phage can be derived from non-immunized or immunized animals [30]. Naïve scFv antibody libraries do not use animals, and a new library needs not to be constructed for each new antigen. A number of recombinant antibodies against a variety of antigens have been reported [31–33]. Due to these advantages, we tried to select scFv antibodies for SARS-CoV from a mouse phage display library directly. Herein, the affinities of scFv antibodies against E protein were relatively high compared with that of the clones for N protein of SARS-CoV. With the development of antibody engineering technology, the affinity and stability can be improved significantly by several kinds of approaches. So the engineered antibodies

can be further modified as a SARS targeting drug, with potential application in the diagnosis and treatment of SARS disease. Moreover, we also found that the scFv antibodies B10 and C20 could recognize the non-overlapping epitopes of E protein of SARS-CoV. The scFv antibody C20 with B10 as detector rendered the highest optical densities, which provided useful tool to study the pathogenesis of SARS. In another aspect, these three scFv antibodies could be produced in large-scale in *E. coli*. Such antibodies, with similar binding properties to polyclonal or monoclonal antibodies and decreased immunogenicity compared with the parental mouse antibodies, have the potential to simplify the production of specific reagents in large scale. Furthermore, our study provides basis for further research.

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