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Original article

Isolation and identification of an scFv antibody against nucleocapsid protein of SARS-CoV

Aizhi Zhao ^{a,*}, Weijun Qin ^{b,d,1}, Yueheng Han ^c, Weihong Wen ^c, Wenhong Zhang ^c,
Zhonghui Lian ^a, Gang Chen ^c, Zhuoli Zhang ^c, Jianqiang Peng ^a, He Wang ^b, Yinglu Guo ^d

^a Vector Gene Technology Company Ltd., Beijing 100176, People's Republic of China

^b Department of Urology, Xijing Hospital, The Fourth Military Medical University, Xi'an 710032, People's Republic of China

^c Department of Biochemistry and Biology, The Fourth Military Medical University, Xi'an 710032, People's Republic of China

^d Institute of Urology, The First Hospital, Peking University, Beijing 100034, People's Republic of China

Received 11 January 2007; accepted 14 April 2007

Available online 21 April 2007

Abstract

To develop reagents for early diagnosis and therapeutic drugs against SARS-associated coronavirus (SARS-CoV), a large (3×10^9) immunized human antibody library was constructed from peripheral blood mononuclear cells from six SARS convalescent patients. A single chain variable fragment antibody (N18) with high affinity against N protein of SARS-CoV was isolated. Sequence analysis revealed that the VL gene was composed of VL3 h (V lambda subgroup) and JL2 regions and the VH gene was composed of VH1-69 (VH1 subgroup), D2-15, D3-22 and JH6 regions. Soluble N18 antibody was expressed in *Escherichia coli* HB2151, purified by Ni-NTA affinity chromatography and verified by SDS-PAGE and Western blot. The potential application for early diagnosis was evaluated using N protein capture ELISA in which N18 antibody demonstrated high sensitive activity in detecting N protein of SARS-CoV. Finally, the potential usefulness of the N18 antibody in prophylaxis, vaccine design and therapy of SARS is discussed.

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Keywords: SARS-CoV; scFv; Nucleocapsid; Phage display

1. Introduction

Severe acute respiratory syndrome (SARS), an infectious disease, caused outbreaks in 29 countries in spring of 2003 with a reported 8098 clinical cases and 774 deaths, resulting in an overall mortality rate as high as 9.6% [1].

Of the initial clinical SARS cases, 11 occurred independently in geographically distinct areas of Guangdong, which, with the findings that the determined viral sequences grouped into different subtypes [2], suggests that the patients were infected by SARS-CoV from multiple sources rather than

a single source. Therefore, the possibility that SARS may be recurrent in human beings highlights the urgent need of accurate and timely diagnosis of SARS-CoV infection for the prevention of another global outbreak.

The three major diagnostic methods currently available are (i) viral RNA detection using real-time reverse transcription PCR (RT-PCR) [3], (ii) antibody detection using ELISA [4,5], and (iii) N protein detection by capture ELISA [6,7]. Although RT-PCR is a useful assay, it sometimes provides false-positive results due to contamination. Serological tests, however, do not provide early diagnosis [8]. Since SARS-CoV appears in the blood much earlier than the antibodies [9], the identification of SARS-CoV antigens in sera provides a possible method for early diagnosis. Several studies have found that N protein is highly immunogenic, and antibody response in SARS patients is directed most frequently and predominantly to the nucleocapsid [10,11]. Based on these findings, we

* Corresponding author. Laboratory of Cardiovascular Science, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA. Tel.: +1 410 558 8476.

E-mail address: zhaoaizhi@gmail.com (A. Zhao).

¹ Co-first author.

constructed a single chain antibody library to isolate anti-N protein antibodies for early diagnosis and possible therapeutic use.

2. Materials and methods

2.1. Peripheral blood mononuclear cells (PBMC) isolation and cDNA synthesis

PBMC were purified by layering 80 ml peripheral blood (donated by six convalescent SARS patients) on a Ficoll-Hypaque gradient (Sigma-Aldrich, Louis, MO). Total RNA was prepared using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. mRNA was purified from the total RNA using the Oligotex mRNA purification Kit (Qiagen, Valencia, CA) and used for the first strand cDNA synthesis using random hexamers (Promega, Pittsburgh, PA) following standard reverse transcription protocols.

2.2. Variable regions amplification and the assembly of single chain fragments of variable region (scFv)

The primers (30 oligos in total) used for amplifying the variable regions of the heavy (VH) and light (VL) chains were previously described [12]. All the oligos were added a conserved region at the 5' end to introduce restriction sites and tails that served as the annealing sequences for the second PCR (tails of the VL forward and VH reverse primers) or overlap extension PCR (tails of the VL reverse and VH forward primers served as the overlap sequences with the linker).

The tails are shown below (sequences in the same font and underline are annealing matching sequences).

VL Forward: 5'-agcaagcggcgcttggcgcgcatgcc-3' (annealing with the forward primer of the second PCR).

VL Reverse: 5'-GTTATGGTCGACCCTCCGGA-3' (overlap with the 5' end of the linker).

VH Forward: 5'-GTTATCCTCGAGCGGTACC-3' (overlap with the 3' end of the linker).

VH Reverse: 5'-gattggttgccttagctagc-3' (annealing with the reverse primer of the second PCR).

linker: 5'-TCCGGAGGGTCGACCATAACTTCGTATAATG TATACTATACGAAGTTATCCTCGAGCGGTACC-3'.

PCR was performed using each individual forward primer with the mixed reverse primers. After gel purification, 0.1 µg of VL, VH and equimole linker were mixed in 20 µl and assembled by overlap extension PCR using the conditions of 94 °C 1 min, 50 °C 1 min, 72 °C 1 min for 15 cycles. After assembly, the scFv fragments (VL + linker + VH) were further amplified and, concurrently, longer tails were added in order to facilitate the subsequent digestion by restriction enzymes. The primers for the second PCR are shown below:

Second VL Forward: 5'-tcaggctactgcagactgattacggt caatccgccgttagcaagcggcgcttggcgcg-3' (*Bss*HII).

Second VH Reverse: 5'-gctacagctcagctactgtggagaatcc gacgggtgttagattggttgccttagctagc-3' (*Nhe*I).

2.3. scFv library construction

Twenty-five microgram scFv fragments digested by *Nhe*I/*Bss*HII were ligated with 40 µg pDAN5 (a gift from Dr. Bradbury A.) [13]. The ligated DNA was electroporated into *Escherichia coli* TG1 cells. T4 DNA ligase was inactivated at 70 °C for 30 min before electroporation, which had been previously described as generating higher transformation efficiency [14]. *E. coli* TG1 competent cells, which have a transformation efficiency of 1.5×10^9 transformants/µg DNA, were prepared as previously described [15]. The library was aliquoted and stored at -80 °C. Stored bacteria library was inoculated into fresh 2YT medium, shaken to OD₆₀₀ = 0.5, then phagemid library was rescued by helper phage M13K07 (Amersham Pharmacia, Piscataway, NJ).

2.4. Vector construction for the prokaryotic expression of nucleocapsid of SARS-CoV

Viral RNA was extracted with the Trizol Reagent (Invitrogen, Carlsbad, CA) from SARS-CoV in a biosafety level III laboratory. The SARS genomic RNA was converted to cDNA by reverse transcription using random hexamers (Promega, Pittsburgh, PA). The coding region for SARS-CoV nucleocapsid was amplified by PCR using the resulting cDNA and pyrobest polymerase (Takara, Dalian, Liaoning). The sequence specific primers were designed according to the published cDNA sequences for SARS coronavirus strain BJ01 (GeneBank accession NO. AY278488). The primers were the following: Forward: 5'-cgcggatccatgtctgataatg gacccc-3' and Reverse: 5'-acgcgtcactatgctgagttgaaatcgc-3'. The amplified DNA was cloned into pBV220 by *Bam*HI/*Sal*I. The recombinant plasmid pBV220-N was identified by sequencing. The non-fusion protein expression vector pBV220 (a gift from Institute of Virology, Chinese Academy of Preventive Medicine) contains P_RP_L tandem promoters of λ bacteriophage for the high level expression and cIts857 restraining gene of λ bacteriophage adapted to heat-induced expression [16].

2.5. Expression and purification of the nucleocapsid protein of SARS-CoV

The recombinant plasmid pBV220-N was transformed into *E. coli* DH5α, and the expression of nucleocapsid protein was induced when the OD₆₀₀ reached 0.5 by the addition of 0.1 mM iso-propyl-1-thio-D-galactopyranoside (IPTG) in 1 L LB broth at 42 °C for 5 h. The cells were harvested by centrifugation and the pellet was resuspended in lysis buffer (pH 9.0, 100 mM Tris). Cells were ultrasonically lysed and the recombinant nucleocapsid protein was purified in two steps employing gel filtration chromatography (Sephacryl S-200 high resolution) and ion-exchange chromatography (SP Sepharose Fast Flow) (Amersham Pharmacia, Piscataway, NJ). Briefly, the supernatant solution was applied to Sephacryl S-200 high resolution column equilibrated with buffer A (pH 9.0, 100 mM Tris) and the eluted nucleocapsid protein peak was

collected. The pH was adjusted to 7.5, then the solution was loaded onto an SP Sepharose Fast Flow column equilibrated with buffer B (pH 7.5, 100 mM Tris). Nucleocapsid protein was eluted with buffer C (pH 7.5, 100 mM Tris, 1 M NaCl) and then the buffer was changed into PBS using Sephacryl S-200 high resolution column.

2.6. Panning

In order to obtain as more diverse scFv as possible, we employed first loose and then stringent panning conditions to enrich scFv-phage for N protein of SARS-CoV. In brief, the panning procedure was performed in 25 ml plastic tissue culture flasks (Nunc, Roskilde, Denmark) coated with purified nucleocapsid protein of SARS-CoV (5 ml, 50 µg/ml for the first round panning and 10 µg/ml for the following panning) in carbonate–bicarbonate buffer (0.2 M, pH 9.6). Five milliliter of the phage library (10^{13} phages) was added into the flask blocked with MPBS (PBS containing 4% non-fat dry milk). After incubation at room temperature for 2 h, the unbound phages were removed by 10 washes with PBST (PBS containing 0.05% Tween 20), followed by 10 washes with PBS (20 washes with PBST and 20 washes with PBS for the second to fourth panning). Ten milliliter log phase ($OD_{600} = 0.5$) TG1 *E. coli* bacteria were added into the flask to allow the bound phages to infect at 37 °C for 30 min without shaking. The infected bacteria were then spread on 2 × YT plates (containing 100 mg/ml ampicillin and 1% glucose) and incubated at 30 °C overnight. Phagemids were rescued from the bacteria by M13K07 helper phage and used for the next panning round. Phages at a colony forming units (cfu) of 1000 times of the elution titer were used for the next round panning, for one scFv-phage could be isolated in the panning only when its copies reached 100–1000. Four rounds of selections were performed.

2.7. Preparation of monoclonal scFv-phage and isolation of N protein binding monoclonal scFv-phage by phage ELISA

Following the last selection round, 285 individual colonies were randomly picked from plates for phagemid rescue and identification of N protein binding property by phage ELISA. In brief, four 96-well ELISA plates were coated with purified SARS-CoV nucleocapsid protein (1 µg/well), blocked and washed. Then each 100 ml monoclonal scFv-phage supernatant was added into 100 ml MPBS and incubated for 30 min to reduce nonspecific binding. The absorbed supernatant was added into each well of the plates. After incubation for 1 h at room temperature, plates were washed and the bound antibodies were detected by HRP-conjugated anti-M13 antibody (Amersham Pharmacia, Piscataway, NJ), followed by incubation with the substrate of TMB (3,3',5,5'-Tetramethylbenzidine). Absorbance was measured at 450 nm and evaluated by S/N ratio (sample/negative). The cut off value was $S/N > 2.1$. Strong positive colonies in the monoclonal phage ELISA were sequenced.

2.8. Soluble scFv expression, identification by Western blot analysis and purification

The selected scFv-phage was introduced into *E. coli* HB2151 (Amersham Pharmacia, Piscataway, NJ) to express a soluble scFv antibody in the presence of 1 mM IPTG. The soluble scFv was fused to a SV5 tag at the C-terminus to facilitate identification by Western blot using HRP-conjugated anti-SV5 antibody (Invitrogen, Carlsbad, CA). For purification, the supernatant was applied to a nickel–nitrilotriacetic acid (Ni–NTA) agarose column (Qiagen, Valencia, CA) equilibrated with PBS. After extensive wash with 50 mM imidazole, the soluble scFv was eluted with 500 mM imidazole, followed by buffer exchange in PBS using gel filtration chromatography.

2.9. Affinity measurement

The affinity of N18 was measured using the non-competitive ELISA described previously by the Beatty lab [17]. In brief, plates were coated with N protein at two concentrations, 5 µg/ml and 2.5 µg/ml in carbonate–bicarbonate buffer, blocked with MPBS. Then four-fold serial dilution N18 was added to the wells at a starting concentration of 0.125 nM. Wells were incubated with HRP-conjugated mouse anti-SV5 monoclonal antibody followed by an incubation with TMB substrate solution. The N18 concentration at OD_{50} absorbance for each N protein coating concentration was measured, and the K_{aff} was calculated by the equation $K_{aff} = 1/2(2[Ab']_t - [Ab]_t)$, where $[Ab']_t$ refers to the N18 concentration at OD_{50} for the N protein coating concentration at 2.5 µg/ml and $[Ab]_t$ refers to the N18 concentration at OD_{50} for the N protein coating concentration at 5 µg/ml.

2.10. Capture ELISA for detecting the nucleocapsid protein of SARS-CoV

Microtiter plates were coated with 100 µl/well of an anti-N protein monoclonal antibody (prepared by Vector Gene Technology Company), each at a concentration of 10 µg/ml in carbonate–bicarbonate buffer. After the plates were washed and blocked, 100 µl/well of a four-fold serial dilution (starting at 10 µg/ml) nucleocapsid protein was added. The dilution was performed in normal human serum. Each concentration was run in duplicate. After 1 h incubation at 37 °C and following six washes with PBST, the wells were incubated with 100 µl/well of 10 µg/ml soluble N18 scFv. Concurrently, a 1:100 diluted SARS convalescent serum that was previously identified as having positive reaction with nucleocapsid protein was used as a control. After six washes with PBST, the nucleocapsid protein was detected by the HRP-conjugated mouse anti-SV5 monoclonal antibody or HRP-conjugated goat anti human Ig (BD Biosciences, San Diego, CA), followed by incubation with the substrate of TMB. The absorbance was measured as described above with a cut off value of $S/N > 2.1$.

3. Results

3.1. scFv library construction

Two-hundred microgram total RNA was obtained from the PBMC, from which 2.8 μg mRNA was isolated. The variable

regions were successfully amplified and assembled into VL–linker–VH scFv format. The recombinant phagemid pDAN5 was transformed into *E. coli* TG1 to yield 3×10^9 individual clones. Thirty clones were picked randomly to check cloning efficiency of the library by PCR. Result showed that more than 96% (29/30) clones contained full-length scFv

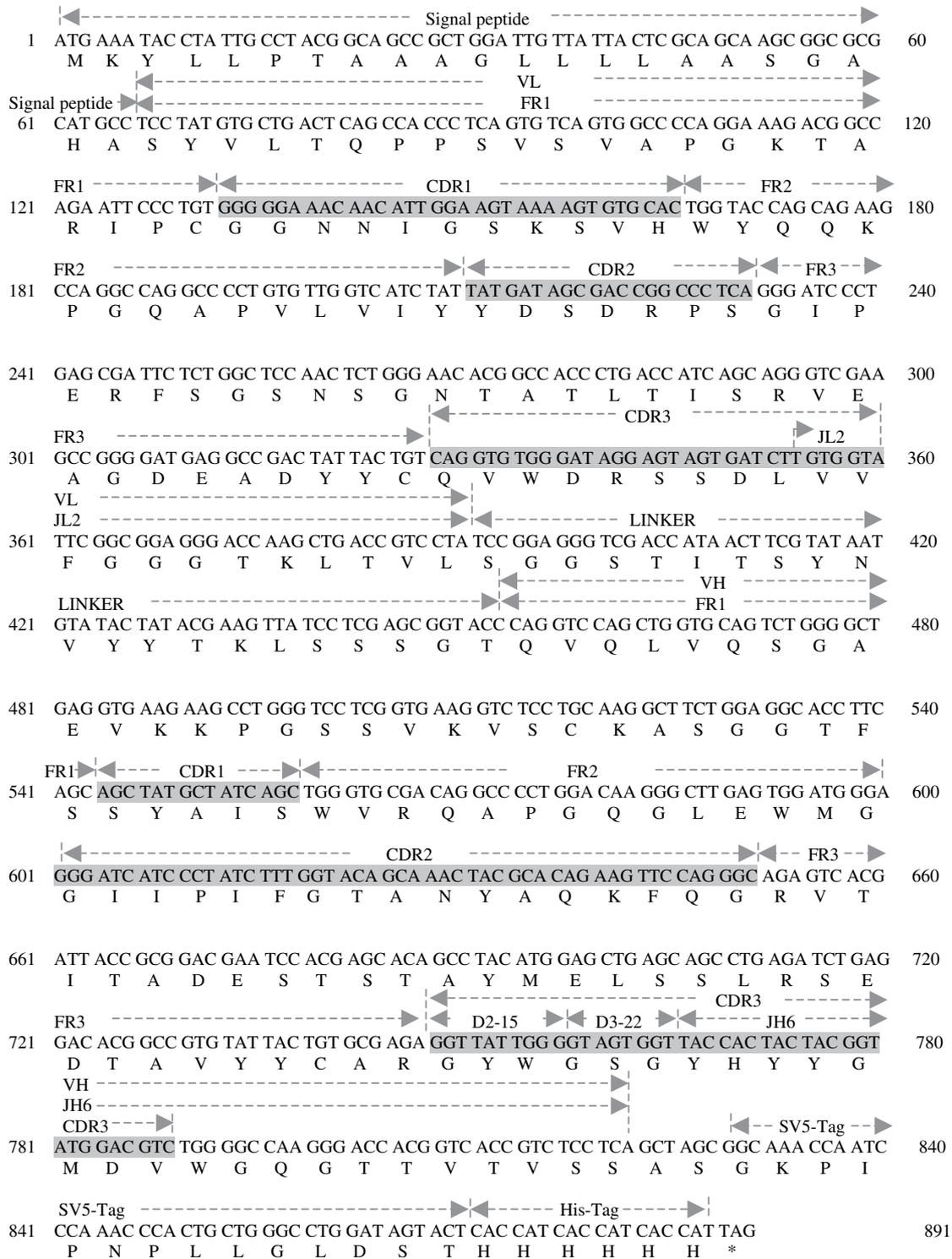


Fig. 1. Complete nucleotide sequence of N18 scFv antibody gene and derived amino acid sequence of the protein. The deduced amino acid sequence is listed starting with the initiation methionine and shown below the nucleotide sequence. All the annotations are displayed above the nucleotide and amino acid sequence. The gray highlighted areas depict complementary determinant regions (CDRs) of VH and VL.

Table 1
Amino acid sequence alignment of CDR3 of VL

AA NO.	89	90	91	92	93	94	95	95a	95b	96	97
VL3h	Q	V	W	D	S	S	S	D	H		
JL2										V	V
N18	*	*	*	*	R	*	*	*	L	*	*

“AA NO.” is the Kabat numbering of the amino acid; “*” denotes homology.

inserts. Ten randomly selected clones were sequenced and the variable regions of the heavy and light chains fell into different families without bias according to the V-Base (<http://vbase.mrc-cpe.cam.ac.uk>).

3.2. Expression and purification of nucleocapsid protein of SARS-CoV

The cloned cDNA fragment encoding N protein of SARS-CoV was identified by sequencing to be identical to the published sequence (GeneBank accession NO. AY278488). From 1 L culture, 160 mg purified nucleocapsid protein was obtained. As expected, the purified nucleocapsid protein was estimated to be approximately 50 kDa and the purity above 90% by SDS-PAGE.

3.3. Selection of scFv-phage to nucleocapsid of SARS-CoV

After the first round panning, 1.0×10^5 cfu scFv-phages were eluted. The titer increased with the growth of each panning round, 3.2×10^5 cfu after the second and 8.3×10^5 cfu after the third panning, and finally reached 1.0×10^6 cfu after the fourth panning. From the final panning round, 285 scFv-phage clones were randomly selected for ELISA to evaluate their binding activity to SARS-CoV nucleocapsid protein. The results revealed that all of the clones gave very strong positive signals with the OD₄₅₀ value around 2.0. Ten clones giving highest signals were picked for sequencing. The results indicated that all of the 10 clones shared the identical sequence, which is composed of 324 bp VL gene and 375 bp VH gene (Fig. 1). The scFv was designated as N18.

3.4. Sequence analysis

The VL and VH sequences of N18 were analyzed by comparing them to the V base database (human antibody gene database) (<http://vbase.mrc-cpe.cam.ac.uk>) and immunoglobulin BLAST (<http://www.ncbi.nlm.nih.gov/igblast/>). Results revealed that the VL gene was composed of VL3 h (V lambda subgroup) and JL2 regions and the VH gene was composed

Table 2
Amino acid sequence alignment of CDR3 of VH

AA NO.	94a	94b	94c	94d	94e	94f	95	96	97	98	99	100	101	102
JH6							Y	Y	Y	Y	G	M	D	V
N18	G	Y	W	G	S	G	*	H	*	*	*	*	*	*

“AA NO.” is the Kabat numbering of the amino acid; “*” denotes homology.

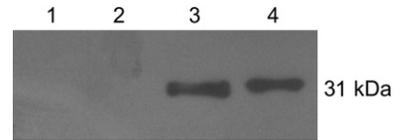


Fig. 2. Western blot analysis of the N18 soluble scFv antibody. Lane 1: culture supernatant of the *E. coli* HB2151; Lane 2: whole cell lysate of the *E. coli* HB2151; Lane 3: culture supernatant of the *E. coli* HB2151 infected by N18-phage; and Lane 4: whole cell lysate of the *E. coli* HB2151 infected by N18-phage.

of VH1-69 (VH1 subgroup), D2-15, D3-22 and JH6 regions (Fig. 1). There were no significant mutations in the frame regions, CDR1 (complementarity determining region) and CDR2 of the VH and VL gene except the mutation from T to P at the site of L22 (Kabat Numbering) in the FR1 of VL. However, a high degree of mutation appeared in the CDR3 regions. The sequence homology between VL CDR3 and the closest germline match (VL3 h) was 88.5% (23/26) with two amino acids mutation in the total of 11 (Table 1). While the sequence homologies between VH CDR3 and the closest germline matches were 71.4% (20/28) with D2-15, 88.2% (15/17) with D3-22 and 95.6% (23/24) with JH6. The long sequence GYWGSYH (from H94a-f to H96) (Table 2) was unique, which was a result of the mutation and rearrangement between the regions of D2-15, D3-22 and JH6.

3.5. Expression and identification of the soluble scFv

N18 soluble scFv antibody was expressed in *E. coli* HB2151 in the presence of 1 mM IPTG at 30 °C. After overnight expression, both the supernatant and the whole bacteria protein contained high concentrated soluble scFv antibody. The molecular weight was about 31 kDa as expected. Western blot analysis further confirmed the successful expression of the soluble N18 scFv antibody (Fig. 2).

3.6. Purification of soluble scFv antibody

The purification results of N18 scFv antibody are shown in Fig. 3. There is very little scFv appearance in the unbound solution or 50 mM imidazole wash. With the 500 mM imidazole elution, the scFv antibody was clearly eluted which migrated as a single protein band on SDS-PAGE. 4.9 mg purified N18 was obtained from 2 L culture.

3.7. Affinity measurement

The absorbance curves for the N18 at different N protein coating concentration are shown in Fig. 4. The $[Ab]_t$ was

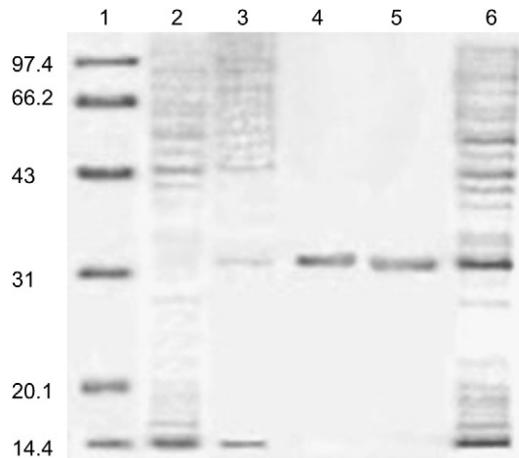


Fig. 3. Purification of N18 scFv antibody by Ni–NTA agarose column. The numbers on the left refers to the molecular masses of the standards. Lane 1: marker; Lane 2: unbound solution; Lane 3: 50 mM imidazole elution; Lane 4: 500 mM imidazole elution; Lane 5: purified scFv after the buffer exchange (imidazole was replaced by PBS); and Lane 6: unpurified culture supernatant of the *E. coli* HB2151 infected by N18-phage.

14.26 nM and $[Ab]_t$ 1.79 nM calculated by the software CURVE. The K_{aff} was therefore calculated at $1.87 \times 10^7 M^{-1}$.

3.8. Capture ELISA for detecting the nucleocapsid protein of SARS-CoV

For being lack of the serum from the SARS patients, we used the serially diluted nucleocapsid protein in normal human serum to check whether the soluble N18 scFv antibody could be used for detecting the nucleocapsid protein of SARS-CoV by capture ELISA. The results indicated that the soluble N18 scFv antibody could be detected as low as 10 ng/ml (1:1024 dilution) nucleocapsid protein in the capture ELISA.

4. Discussion

Phage display antibody library has been used as a powerful tool for generating monoclonal antibodies against viral pathogens [18–20] including SARS-CoV [21,22]. ScFv antibody libraries displayed on filamentous phage can be synthetic or derived from non-immunized or immunized animals or human. Among them, only the immunized human library is the resource to obtain high affinity human antibodies. Until now, no human anti-N protein antibodies isolated from the SARS patients have been reported. In this study, we constructed a large human scFv library from the peripheral blood mononuclear cells of six convalescent SARS patients and isolated one scFv antibody with high affinity against nucleocapsid protein of SARS-CoV.

It is very interesting that only one scFv was isolated from a large library (3×10^9 members) with good diversity. A similar result was reported in the isolation of scFv antibody against SARS-CoV from a semi-synthesized scFv library [22]. The reason behind our result may be that the isolated scFv antibody N18 has the competitive affinity advantage in

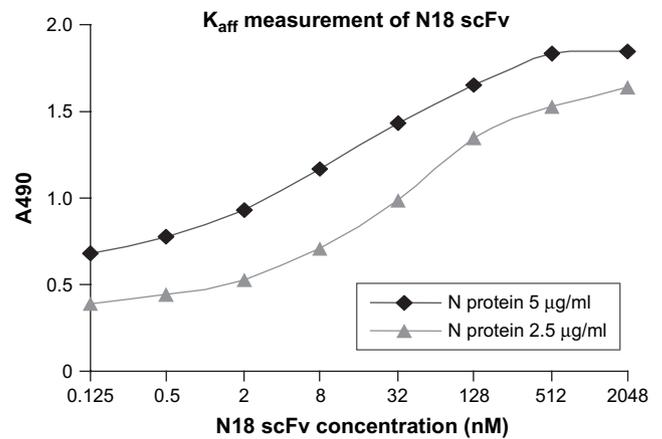


Fig. 4. The absorbance curves of the N18 at different N protein coating concentrations (the X-axis plotted on a log scale). The concentrations of N protein in the coating solutions were 5 µg/ml and 2.5 µg/ml. The calculated N18 concentrations at OD_{50} were 1.79 nM and 14.26 nM, respectively.

the panning and rapidly wiped out others in further stringent pannings. Therefore, fewer rounds of panning would help to get more diverse antibodies. More rounds of stringent panning may help to enrich the high affinity antibodies, but will reduce the diversity at the same time. In the present study, only four rounds of standard panning were performed, but the population was already overwhelmed by N18. It would be advisable to isolate scFv-phages after the second or third round of panning if one is interested in obtaining more diverse antibodies instead of the antibody with the highest affinity.

Analysis of the sequence of the N18 scFv antibody revealed that there were no mutations in the CDR1 and CDR2 in either the light or the heavy chains. Therefore, it is reasonable to believe that the two mutations in the CDR3 of the light chain and especially the long unique sequence of GYWGSYH (from H94a-f to H96) in the CDR3 of the heavy chain due to the rearrangement between the regions of D2-15, D3-22 and JH6 are necessary for recognition of the N protein epitope. The standard genetic mechanism for the VDJ rearrangement in the formation of immunoglobulins was that only one V, one D and one J gene segment join together. Two D segments appearing in one CDR3 is a rare phenomenon. Dr. Baskin reported that D–D fusions in one CDR3 could be observed in all the fetal livers as well as in the adult PBMC, as previously reported [23–25]. The D–D fusion could have resulted from an error in VDJ rearrangement, and may lead to increased antibody's diversity.

Several studies have found that N protein is highly immunogenic, thus antibody response in SARS patients is directed most frequently and predominantly to the nucleocapsid [10,11]. So far, there have been no reports about a human antibody sequence against N protein of the SARS-CoV developed in the SARS patients. This study may provide some useful information for studies in the human humoral immunization against SARS-CoV or the vaccine development.

Several studies confirmed that nucleocapsid protein capture ELISA was a valuable method for the early diagnosis of SARS-CoV infection [1,6,7]. The property of N18 scFv

antibody that could detect N protein in a very low concentration in the capture ELISA makes it a potential useful reagent in the SARS prophylaxis and treatment. Furthermore, due to its easy, rapid and cheap production, it may be a good substitute for the monoclonal antibodies and anti-N serum [6,7].

It is unlikely that the antibodies against N protein play a neutralizing role due to its internal position in the virion. However, some researchers reported that monoclonal antibodies (MAb) to N protein of murine hepatitis virus (MHV, an animal coronavirus) [26] and rotavirus [27] had protective effect on the virus infection even though the antibodies lacking of neutralizing activity. The latter phenomenon may be due to the ability of the IgA antibodies to enter the infected cells by the IgA-transcytosis pathway, thereby interfering with viral transcription [28]. So whether the N18 scFv antibody itself or a modified IgA format by being fused to an IgA Fc fragment has the similar protective effect in vivo needs to be further investigated.

In summary, we described here the isolation and characterization of the N18 scFv antibody which demonstrated a high affinity with the N protein of SARS-CoV. This scFv antibody may allow better understanding in the immunogenic characteristics of N protein, the SARS vaccine design, the studies of viral replication and antiviral activity, as well as early diagnosis and potential therapy of SARS.

Acknowledgements

This work was supported by National Natural Science Foundation of China (NO. 30340028) and CX01F002 Grant. We greatly appreciate Dr. Andrew Bradbury for providing us the gift of phagemid pDAN5 and his helpful discussions. Finally, we thank the NIH Fellows Editorial Board (FEB) for their critical review of this manuscript.

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