Development of Human Single-Chain Antibodies against SARS-Associated Coronavirus

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Key Words
Antibody \cdot Severe acute respiratory syndrome \cdot Single-chain variable fragment \cdot Immunology

Abstract
The outbreak of severe acute respiratory syndrome (SARS), caused by a distinct coronavirus, in 2003 greatly threatened public health in China, Southeast Asia as well as North America. Over 1,000 patients died of the SARS virus, representing 10% of infected people. Like other coronaviruses, the SARS virus also utilizes a surface glycoprotein, namely the spike protein, to infect host cells. The spike protein of SARS virus consists of 1,255 amino acid residues and can be divided into two sub-domains, S1 and S2. The S1 domain mediates the binding of the virus to its receptor angiotensin-converting enzyme 2, which is abundantly distributed on the surface of human lung cells. The S2 domain mediates membrane fusion between the virus and the host cell. Hence two strategies can be used to block the infection of the SARS virus, either by interfering with the binding of the S1 domain to the receptor or by blocking the fusion of the virus with the cell membrane mediated by the S2 domain. Several antibodies against the S1 domain have been generated and all of them are able to neutralize the virus in vitro and in vivo using animal models. Unfortunately, point mutations have been identified in the S1 domain, so that the virus isolated in the future may not be recognized by these antibodies. As no mutation has been found in the S2 domain indicating that this region is more conserved than the S1 domain, it may be a better target for antibody binding. After predicting the immunogenicity of the epitopes of the S2 domain, we chemically synthesized two peptides and also expressed one of them using a recombinant DNA method. We screened a phage displaying library of human single-chain antibodies (ScFv) against the predicted epitopes and obtained a human ScFv which can recognize the SARS virus in vitro.

Introduction
Severe acute respiratory syndrome (SARS) is a life-threatening form of atypical pneumonia caused by a newly identified SARS-associated coronavirus (SARS-CoV) [1, 2]. It apparently began in Guangdong province of Chi-
served than the S1 domain and may be a better target for neutralization and antibody production [10, 13]. After using a computer program to predict the immunogenicity of the S2 domain, peptides were prepared by chemical synthesis and recombinant protein expression, and used to screen the phage library displaying human scFv antibodies.

One highly specific scFv, designated A11, was isolated, sequenced, expressed and purified. It showed specific reactivity with SARS-CoV-infected Vero cells in vitro. Although neutralization activity of this antibody is undetermined, the high specificity of this antibody to SARS-CoV makes it useful for diagnosis of SARS-CoV infection.

Materials and Methods

Peptide Synthesis and Carrier Protein Conjugation of S2a (5783–798)

A 16-amino acid peptide designated as S2a from amino acid 783 to 798 (NFSQILPDPLKPTKRS) and a 26-amino acid peptide designated as S2b from amino acid 803 to 828 (LLFNKVT-LADAGFMKQYGECLGDINA) of the spike protein, a 20-amino acid peptide (ERLFLSHPQTKYYPHFDLH) designated as ZETA (irrelevant control) from human ζ-globin protein were synthesized by the core facility of the University of California at San Francisco and were assessed to be >95% pure by HPLC. The peptides were conjugated to carrier protein bovine serum albumin (BSA; Pierce) or ovalbumin (OVA; Pierce) as described by the manufacturer (Imject Immunogen EDC Kit; Pierce) to facilitate the panning.

Expression and Purification of Recombinant S2b (S803–828)

The amino acid sequence of the SARS-CoV spike protein was used to design a codon-optimized version of the gene encoding the spike protein, as described elsewhere [14]. An oligonucleotide based on the DNA sequence encoding amino acids 803–828 (LLFNKVT-LADAGFMKQYGECLGDINA) of the spike protein was synthesized. The sequence of the oligonucleotide is 5’-AATTGCCT-GTTTTATATTTAATAGGTGACCCCTGGCCAGCGGGGTTCA-TGAAACAGTATGGTGAATGCTTGGGCGATATTAACGC-TGTTATTTAATAAGGTGACCCTGGCAGACGCGGGTTCA-.

The synthetic gene was cloned into the EcorI/NcoI sites of bacterial expression vector pET32b (Novagen) fused with the 3’-terminal of the thioredoxin (Trx) gene and the sequence was confirmed by DNA sequencing. The ligated vector was transformed into Escherichia coli BL21 (Novagen). The S2b Trx fusion protein and the Trx control protein from the blank pET32b vector were induced to express by isopropyl-b-D-thiogalactopyranoside (IPTG) and purified with Ni-nitrilotriacetic acid (Ni-NTA) resin (QIAGEN) according to the manufacturer’s protocols.

Phage Display Library Construction

A scFv phage antibody library was constructed in fd phage [15]. The fd phage display library was derived from a phagemid

na in November 2002 and spread to several neighboring areas, including Hong Kong [3]. Although the epidemic appears to have abated, it is believed that new outbreaks of SARS are possible in the future. Since there is no effective treatment currently available, the development of effective diagnostics and therapeutics to control the disease are of paramount importance [3].

During the last epidemic, SARS patients showed clinical improvement if they were given convalescent serum collected from recovered subjects [4]. However, the source of convalescent serum is extremely limited and the sera have the possibility of transmitting other blood-borne infections. Although animals can be used to develop antisera against this virus, these sera, being foreign proteins, will elicit immune responses and may cause anaphylactic reactions. Thus, it would be ideal to develop human antibodies against the SARS-CoV for prophylaxis or treatment [5]. This approach of immune therapy has been clinically successful when applied in malignant diseases and viral infections [5–8].

There are several ways of developing humanized antibodies. We chose to select antibodies from a phage library displaying human single-chain antibodies to facilitate the identification and development of specific affinity antibodies in a rapid and cost-effective manner. This phage library containing millions of independent clones of single-chain variable fragment (scFv) antibodies [9] was used to select specific antibodies against the spike protein, a major antigenic determinant of SARS-CoV [10]. In order to develop a potent neutralizing high-affinity antibody against SARS-CoV, the spike protein, responsible for receptor binding and membrane fusion [10], is targeted for generation of neutralizing human monoclonal antibodies.

The spike protein of SARS-CoV consists of 1,255 amino acid residues and can be divided into two sub-domains, S1 and S2. The S1 domain mediates the binding of the virus to its receptor, angiotensin-converting enzyme 2, that is richly distributed on the surface of human lung cells [11]. The S2 domain mediates membrane fusion between the virus and the host cell. Two strategies can be employed to block the infection of SARS-CoV, blocking either the binding of the S1 domain to the receptor or the fusion of the virus mediated by the S2 domain.

Several humanized antibodies against the S1 domain have been generated [12] and all of these antibodies can neutralize the virus in vitro and in vivo. However, point mutations have been identified in the S1 domain, so that the virus in future outbreaks may not be recognized by these antibodies. To our knowledge no mutation has been found in the S2 domain. Hence this region is more conserved than the S1 domain and may be a better target for neutralization and antibody production [10, 13]. After using a computer program to predict the immunogenicity of the S2 domain, peptides were prepared by chemical synthesis and recombinant protein expression, and used to screen the phage library displaying human scFv antibodies.

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library in pHEN1 vector [16] by subcloning the Sfil/NotI scFv inserts into Sfil/NotI sites of fd vector [17]. Ligated mixtures were used to transform E. coli TG1 and the transformation mixture plated on TYE plates [16] containing 15 μg/ml tetracycline. Library size was calculated by counting the number of tetracycline-resistant colonies. Library quality was verified by determining the percentage of clones with inserts of appropriate size for an scFv gene, performed by colony PCR screening using the primer fdseq and fdl2 [15]. Library diversity was confirmed by fingerprinting the amplified scFv genes after digestion with BstNI [16]. The library was stored in 2× YT medium containing 15 μg/ml tetracycline and 15% glycerol at −80°.

Phage Library Selection

Phage-displayed scFvs specific for S2a synthesized spike peptide or recombinant S2b peptide were affinity-selected using proteins absorbed to immunotubes (Nunc, Maxisorb). Immunotubes were coated with 10 μg/ml of BSA-conjugated S2a or recombinant S2b peptide overnight at 4°. The coated immunotubes were blocked with 2% (w/v) skimmed milk powder in phosphate-buffered saline (PBS) for 1 h at room temperature. To eliminate non-specific binding, the phage library was adsorbed in PBS containing 2% skimmed milk powder. Subsequently, plaque-forming units (9 × 1011) of phage-scFvs prepared from the phage library were mixed with blocking buffer and introduced into immunotubes for panning. Unbound phages were removed by 6 washes with PBS containing 0.02% Tween 20, followed by 10 washes with PBS. Bound phages were eluted with 1 ml of 100 mM triethylammonium (Sigma), neutralized with 0.5 ml of 1 M Tris-HCl (pH 7.4) and then used to infect 20 ml of exponentially growing E. coli TG1. E. coli was grown at 37° for 30 min and then the culture was plated on TYE plates containing 15 μg/ml tetracycline. After overnight growth, colonies were scraped from the plates and used to generate phage for a second round of selection as described [15].

For the subsequent rounds of panning, selection was alternated between OVA-conjugated and BSA-conjugated S2a protein to prevent selection against the carrier protein. For selection of scFvs to the recombinant peptide (S2b) fused with Trx protein, 1 ml of 10 μg/ml recombinant Trx control protein prepared from the pET32b blank vector without insert was incubated with the library for 60 min at room temperature for depletion.

Phage ELISA

Antigen-binding phage antibodies were identified by phage ELISA. Individual colonies were picked into 96-well microtiter plates containing 2× YT with 15 μg/ml tetracycline. Bacteria were grown overnight at 30°, and then the bacteria were pelleted, and the supernatant, containing phage particles, was used for ELISA. The spike protein fragments, 10 μg/ml of S2a conjugated with BSA or OVA or recombinant antigen S2b-Trx, were coated onto 96-well plates in 0.1 M sodium bicarbonate solution (pH 9.6) overnight at 4°. The next day, the wells were blocked for 1 h at room temperature with 2% skimmed milk powder in PBS. 100 μl of scFv phage supernatant were added to the wells and incubated for 1 h at room temperature. The plates were washed and phage binding was detected with anti-M13 antibody (Amersham Pharmacia) as described by the manufacturer. The absorbance was read at 405 nm by a plate reader (Molecular Device: Spectra Max 190).

DNA Fingerprinting and Sequencing

The number of unique phage antibodies was estimated by PCR fingerprinting of the scFv genes with the restriction enzyme BstNI (New England Biolab) and confirmed by DNA sequencing [5]. Sequencing runs were performed in both directions for each clone using the fd2 primer 5’-TTTTTTGAGATTTCAC-3’ and the fdl2 primer 5’-GAATTTCTGTATGAGG-3’.

Production of Single-Chain Variable Fragment (scFv)

The scFv gene fragments were subcloned into pSyn1 vector and transformed into E. coli BL21 (DE3; Novagen). The scFv expression was induced by growth in 2× YT medium supplemented with 100 μg/ml ampicillin and 1 mM isopropyl-d-thiogalactoside for 4 h at 30°. The scFv was collected from the periplasm. Soluble periplasmic extracts were obtained by osmotic shock at 4° using lysing buffer containing 20% sucrose, 1 mM EDTA, and 300 mM Tris-HCl (pH 8). All the scFvs contain a His-6 tag that allows purification by Ni-NTA agarose column (Qiagen). The scFvs purified from the periplasmic extracts were dialyzed with PBS and concentrated.

Western Blot Assay

The recombinant S2b protein was prepared with 2× SDS loading buffer under reducing conditions (60 mM Tris-HCl, pH 6.8), 1% SDS, 20 mM dithiothreitol, 10% glycerol, 0.02% bromophenol blue). Proteins were separated in 10% PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked in 5% nonfat milk in PBS with 0.05% Tween-20 and probed with mouse anti-His tagged antibody at a dilution of 1:10,000 at 4° overnight. The membranes were incubated with rabbit anti-mouse horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) at a dilution of 1:4,000 for 2 h at room temperature. The reaction was developed with enhanced chemiluminescence reagent (Pierce).

Immunofluorescence Staining of Vero Cells Infected with SARS-CoV

BIOCHIPS slides (Euroimmun, Lübeck, Germany), coated with SARS-CoV-infected cells and non-infected cells were utilized. 25 μl of diluted scFv or SARS patient serum was added to a well of the slide and incubated overnight at 4°. The slide was washed with PBST (PBS with 0.05% Tween-20), then 20 μl of the diluted fluorescein-labeled mouse anti-c-myc secondary antibodies (1:100) was added to the scFv well and 20 μl of the diluted fluorescein-labeled goat anti-human secondary antibodies (1:100) was added to the positive control well, and the slide was incubated at room temperature for 30 min. The slide was then washed with PBST, air dried and mounted. The mounted slide was observed under a fluorescence microscope (Leica DML).

Results (SARS-CoV)

Selection and Expression of Spike Protein Fragment for Phage Panning

Between the two domains the spike protein of the CoVs, the S2 domain at the carboxyl end is more conserved than the S1 subunit [10, 13]. The amino acid sequence of spike protein was analyzed to determine the
likelihood that a sequence may trigger an immune response in human. Based on the predicted antigenicity, hydrophilicity, accessibility, and secondary structures, two epitopes, S2a (S783–798) and S2b (S803–828), on the S2 domain of spike protein were determined to be antigenic and were used to select single-chain antibodies. Pure synthetic S2a peptide was conjugated to BSA or OVA carrier protein to increase the coating efficiency on immunotubes for screening. One problem with this method may arise in which antibodies recognizing BSA and OVA carrier protein can also be selected. These irrelevant bindings can be avoided by alternate use of BSA- or OVA-conjugated S2a peptide in selection rounds. For phage scFv antibody selection, chemically synthesized peptide S2b was tested, but it was found to be very hydrophobic and insoluble. S2b was later expressed as a fusion protein with Trx in *E. coli* to solve the solubility issue.

**Panning against Purified SARS-CoV Spike Protein Fragment**

A human non-immune phage display antibody library was then screened to select the scFv antibodies specific to the predicted antigenic determinant region (S2a or S2b) of the spike protein. Each round of panning comprised 4 steps in a cycle of scFv-phage binding to the immobilized antigen, washing away of unbound and nonspecifically bound scFv-phage, elution of the specifically bound scFv-phage, and propagation of enriched scFv-phage in bacteria before the output phage scFv goes into the next round of panning. The titer of the recovered phage was 7.3 × 10^8 and 3.6 × 10^6 cfu/ml after 4 rounds of selection on carrier protein-conjugated S2a and S2b-Trx fusion protein (Table 1). An increase in the number of eluted phages after several rounds of panning usually indicates specific bindings [18].

**Table 1.** Results obtained after panning a human phage displaying the scFv library (9 × 10^{11} cfu diversity) against spike protein fragments S2a and S2b

<table>
<thead>
<tr>
<th>Panning</th>
<th>S2a synthetic peptide</th>
<th>S2b fusion protein</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>bait</td>
<td>phage eluted cfu/ml</td>
</tr>
<tr>
<td>1st</td>
<td>S2a-BSA</td>
<td>1.7 × 10^4</td>
</tr>
<tr>
<td>2nd</td>
<td>S2a-OVA</td>
<td>7.8 × 10^5</td>
</tr>
<tr>
<td>3rd</td>
<td>S2a-BSA</td>
<td>3.0 × 10^5</td>
</tr>
<tr>
<td>4th</td>
<td>S2a-OVA</td>
<td>7.3 × 10^6</td>
</tr>
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**Fig. 1.** Polyclonal ELISA for S2a (A) and S2b (B). Polyclonal phages obtained from each round of panning were tested for binding to the target antigen and control antigen in ELISA. Binding phages were detected with an HRP-conjugated anti-M13 antibody. Positive clones specific to the target were enriched after four rounds of selection.
To determine the outcome of the selection strategy, polyclonal phages were prepared after the 4th round of selection and analyzed for their binding to two targets fragment (S2a and S2b) by ELISA. After each round of panning, the output phage was amplified and applied to microtiter wells coated with the respective antigens. Each microtiter well was coated with 10 μg/ml of the antigen. Bound phages were detected by horseradish peroxidase (HRP)-conjugated anti-M13 antibody. Phage ELISA shows the enrichment of S2a- and S2b-specific phages during panning cycles (fig. 1).

**Selection and Identification of scFvs to SARS-CoV Spike Protein**

To identify phages that specifically bind antigens, they were prepared from individual colonies from the 4th round of selection. The binding specificity of the scFv was determined by phage ELISA using the target antigen and control proteins as substrates. Forty-eight and 96 individual clones were picked for the ELISA analysis against S2a and S2b spike protein fragments, respectively (table 2).

**Selection on Conjugated Synthetic S2a Spike Protein Fragment**

Of 48 random clones analyzed, 16 clones specifically recognized S2a conjugated OVA and BSA proteins, but not a control ζ-globin peptide-conjugated OVA protein, OVA or BSA proteins (fig. 2). To determine the number of unique antibodies generated, the scFv gene was amplified by PCR and the PCR product was digested with the frequently cutting restriction enzyme BstNI (PCR fingerprinting). From the 16 S2a-binding scFvs, the same fingerprint was observed, indicating that they were derived from the same phage (fig. 4A). DNA sequences of representative scFv clones 6 and 13 were identical (fig. 5).

**Selection on Purified Recombinant S2b Spike Protein**

After four rounds of selection, a total of 96 clones were screened for S2b-specific binding by ELISA. Seventy clones specifically recognized S2b-Trx protein, but not
Fig. 3. The binding specificity of the selected phage antibodies to S2b. Phages were prepared from individual colonies from the 4th round of selection and analyzed for specific binding by ELISA. The specificity of anti-S2b scFvs was tested against carrier protein (BSA and OVA), cross-reactivity with conjugated S2a peptide, S2b-Trx fusion protein and control protein (Trx).

Fig. 4. DNA fingerprint analysis of the scFv genes of individual antibodies from the 4th round of selection. scFv DNA was amplified by PCR directly from colonies and digested with the frequently cutting restriction enzyme BstNI. A S2a. A unique banding pattern was observed, representing a unique antibody sequence. B S2b. A diverse banding pattern was observed, with each unique pattern representing a unique antibody sequence.

<table>
<thead>
<tr>
<th>Heavy Chain</th>
<th>Framework 1</th>
<th>CDR1</th>
<th>Framework 2</th>
<th>CDR2</th>
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</thead>
<tbody>
<tr>
<td>S2a (C6, 13)</td>
<td>QVQLVSEGGGLVQPGGLRSLCAAAGFTFS</td>
<td>SYAMS</td>
<td>WVRQAPKGLLEWS</td>
<td>GISGSSGSTYAADSVKG</td>
</tr>
<tr>
<td>S2b (A11)</td>
<td>QVELVESGGGLVKPGGLRSLCAAAGFTFS</td>
<td>AYAMS</td>
<td>WVRQAPKGLLEWS</td>
<td>AISGSSGSTYAADSVKG</td>
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<tr>
<th>Light Chain</th>
<th>Framework 1</th>
<th>CDR1</th>
<th>Framework 2</th>
<th>CDR2</th>
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<tbody>
<tr>
<td>S2a (C6, 13)</td>
<td>RFTISRDNKNLTLQMNLSLRAEDTVYYCAK</td>
<td>KQTSSGWYGKDY</td>
<td>WGGGLTVYSS</td>
<td>GVNQPS</td>
</tr>
<tr>
<td>S2b (A11)</td>
<td>RFTISRDNKNLTLQMNLSLRAEDTVYYCAK</td>
<td>GGYCTGGVCSLDY</td>
<td>RGQGLTVYSS</td>
<td>DVSNRPS</td>
</tr>
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</table>

Fig. 5. The sequences of the binding phage antibodies.
S2a and vector control protein (table 2). Eight representative clones showing the highest signals in ELISA were identified (fig. 3) and 24 S2b-specific clones were subjected to DNA fingerprint analysis and a diverse pattern was observed (fig. 4B). Figure 5 shows the DNA sequencing analysis of the clone A11 that has the highest specificity and affinity binding to S2b.

**Immunofluorescence Assay on SARS-CoV-Infected Cells**

Four scFvs with higher binding activity (C6, C13, A11 and G10) tagged with His-6 in pSyn1 vector were expressed in *E. coli* and purified by immobilized metal affinity chromatography. Reactivity with SARS-CoV-infected cells by scFvs was assessed by indirect immunofluorescence. This test utilized BIOCHIP containing SARS-CoV-infected cells and non-infected cells positioned side by side in each reaction field. Immunofluorescence analysis revealed that the scFv-A11 specifically recognized SARS-CoV infected (fig. 6A) but not uninfected Vero cells (fig. 6B). Although the other 3 ScFvs (C6, C13, G10) bound S2b fusion protein on ELISA, they did not bind the infected Vero cells. The negative binding of G10 is shown in figure 6E.

**Specificity of A11 to Recombinant Spike Protein**

A11 scFvs was demonstrated to show specific binding to the spike protein fragment peptide in ELISA assays and to SARS-CoV-infected cells. To further confirm the interaction between A11 scFv and recombinant spike protein, Western blot was performed. In figure 7, A11-scFv was found to have specific binding to recombinant S2b protein, but not to the vector control. Anti-His-tagged protein is shown as control.

**Discussion**

The sequence variation of infectious viruses limits the development of effective vaccines or neutralization antibodies against these viruses. Like other coronavirus, the SARS virus also uses its S1 domain of the spike protein to bind the receptor of the host cells. The best way to block the infection of the virus is to block the binding of the virus to the receptor. Although neutralization antibody against this domain has been developed, most mutations of the SARS virus have been found in this region, and hence some virus may escape recognition by the neutralization antibody. However, the S2 domain is very well conserved among all corona viruses and so far no mutation has been found in the S2 domain of SARS virus. Hence this region is a better target than the S1 domain for the development of vaccine and neutralization antibodies against the SARS virus. Furthermore, the combination of different neutralization antibodies against multiple epitopes may facilitate the clearance of virus from human blood.

The epitope Leu803-Ala828, S2b fragment, was identified previously as a peptide that had strong binding to convalescent sera from SARS patients [19]. It was also selected as a potential vaccine sequence for SARS virus. However, this peptide is hydrophobic and very hard to dissolve in water. Hence we synthesized the DNA fragment for this peptide and cloned the DNA fragment into the pET32b vector. The peptide was expressed as a soluble fusion protein in bacteria, after purification, the recombinant peptide was used to screen the human ScFv library for SARS antibody.

We obtained 8 unique clones for S2b recombinant peptide after 3 rounds of screening, but only one clone, A11 had specific binding activity for SARS virus. Perhaps the fusion of the S2b peptide changed the conformation of fusion protein and generated new epitopes in the fusion protein. Nevertheless, we were able to obtain one clone highly specific to the S2 domain of the spike protein. Hence this method may be useful for other hydrophobic peptide target. The library can be depleted by blank recombinant protein without the peptide and the recombinant peptide does not need conjugation with carrier proteins such as BSA or OVA.
The A11 scFv antibody may have possible therapeutic utility for neutralizing SARS-CoV infection. Thus, it is worthwhile to further investigate the A11-scFv interaction with native spike protein and to produce the whole humanized antibody for viral-neutralizing assays to provide a more precise assessment on its neutralizing effect and the potential for further in vivo and clinical studies.

In summary, a human scFv antibody A11 directed to the spike protein Leu803-Ala828 of SARS-CoV has been identified which can bind specifically to SARS-CoV protein and viral particles. The VH and VL of the A11 antibody has been sequenced, thus facilitating molecular approaches including site direct mutagenesis for affinity maturation and construction of a whole recombinant human antibody for studies of SARS-CoV neutralization.

Acknowledgement

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References
