

Production of an Anti-Severe Acute Respiratory Syndrome (SARS) Coronavirus Human Monoclonal Antibody Fab Fragment by Using a Combinatorial Immunoglobulin Gene Library Derived from Patients Who Recovered from SARS

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A combinatorial human immunoglobulin gene library was constructed from the peripheral lymphocytes of two patients who recovered from severe acute respiratory syndrome (SARS). The library was screened for the production of Fab antibody fragments to a recombinant spike protein of SARS-associated coronavirus (SARS-CoV). One Fab clone, AS3-3, reacted with the spike protein in an enzyme-linked immunosorbent assay. The dissociation constant of AS3-3 was 1.98×10^{-8} M. Immunofluorescent microscopy revealed that it reacted with SARS-CoV-infected cells. The library seems to be a potent tool for the production of human antibodies to SARS-CoV.

Severe acute respiratory syndrome (SARS) is a life-threatening form of atypical pneumonia that is caused by a newly identified virus, the SARS-associated coronavirus (SARS-CoV) (3, 7). The disease emerged in southern China in late 2002 and spread worldwide. A total of 8,096 cases of SARS had been identified in 29 countries as of the end of 2003, and 774 patients had died; the fatality rate is 9.6% (World Health Organization [http://www.who.int/csr/sars/country/table_2004_04_21]). Despite the medical importance of this disease, an effective vaccine or therapy is not yet available. However, neutralizing antibody to SARS-CoV has been broadly elicited in SARS patients (5). It has also been reported that the transfer of mouse immune serum (passive immunity) has reduced pulmonary viral titers significantly in infected mice (8). SARS-CoV is a member of the family *Coronaviridae*. The spike glycoprotein is a highly antigenic envelope protein and is responsible for receptor binding and membrane fusion (1, 4). Therefore, human monoclonal antibodies to the spike protein may have potential as passive immunization reagents that can be used to reduce the rate of mortality from SARS-CoV infection. We report here on the generation of a human monoclonal antibody Fab fragment, AS3-3, to SARS-CoV spike protein from a combinatorial immunoglobulin gene library derived from two patients who recovered from SARS.

Five milliliters of peripheral blood was obtained from each of two patients who recovered from SARS at the Shanghai Hospital for Infectious Diseases. Both serum samples were positive for SARS-CoV by an enzyme-linked immunosorbent assay (ELISA), with titers greater than 1:2,000. Construction

of an immunoglobulin gene library from peripheral lymphocytes was performed as described previously (10). Briefly, total RNA was purified from lymphocytes and was subjected to reverse transcription-PCR. Genes coding the light (κ and λ) chain and the Fd region of the heavy (γ and μ) chain were amplified by 30 cycles of PCR. The light-chain genes were first ligated with an expression vector, pFab-His2, and introduced into *Escherichia coli* JM109 cells. The vector with inserts was then ligated with the Fd heavy-chain genes and introduced into *E. coli* cells.

For the preparation of the recombinant spike protein of SARS-CoV, the gene coding the protein (positions 21,477 to 25,244) of the Tor2 isolate was first synthesized by using overlapping 52 oligonucleotide primers, and then the partial gene corresponding to residues 258 to 573 was amplified by PCR with primers 5'-GCGCGGATCCAAGCCAACACTACATTTATG-3' and 5'-GGCCGAATTCCAATGTCTAATATTTTCAGATGT-3'. The amplified gene was ligated with the pET22a vector and was then introduced into *E. coli* BL21(DE3). The recombinant protein was obtained as an inclusion body and was then refolded. The first screening of positive clones producing anti-SARS-CoV antibodies was performed essentially as described previously (2). Briefly, approximately 5×10^3 *E. coli* colonies per 90-mm plate were grown on Luria broth agar containing 50 μ g/ml of ampicillin. Bacterial colonies were first transferred to nitrocellulose filters. The filters were replaced on the surface of fresh plates containing 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and ampicillin, and then they were incubated at 30°C for 6 h. The filters were treated with chloroform vapor and lysis buffer containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1.5% bovine serum albumin, 1 μ g of DNase per ml, and 40 μ g of lysozyme per ml overnight. After the filter was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), the filter was

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blocked with PBST containing 5% skim milk. Each filter was incubated with 125 μg of the recombinant spike protein of SARS-CoV and then with the sera from the patients who were the donors of lymphocytes. Positive signals on the filter were detected by horseradish peroxidase (HRP)-conjugated goat antibody to human immunoglobulin G (IgG) Fc (ICN Pharmaceuticals, Aurora, OH) and with an HRP-1000 immunostaining kit (Konica Co., Tokyo, Japan). Positive clones were identified in the original plates and were then cultured in 10 ml of super broth (30 g tryptone, 20 g yeast extract, 10 g 4-morpholinepropanesulfonic acid per liter, pH 7.0) containing ampicillin to an optical density (OD) at 600 nm of 0.8. Isopropyl- β -D-thiogalactopyranoside at a final concentration of 100 μM was added to the bacterial culture, which was then incubated overnight at 30°C for 12 h. The bacteria were pelleted by centrifugation, resuspended in 0.5 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The lysates were centrifuged at 10,000 $\times g$ for 10 min, and supernatants were subjected to a second screening by ELISA.

Each well of the ELISA plates was treated with the recombinant partial spike protein (0.1 $\mu\text{g}/\text{well}$) diluted in 50 mM sodium bicarbonate buffer. The plates were washed with PBST and then treated with PBS containing 1% skim milk for 1 h. One hundred microliters of the supernatant was added to the wells, and the plate was incubated for 1 h at room temperature. After the plate was washed, the wells were incubated with 100 μl of HRP-conjugated goat antibody to human IgG Fab (ICN Pharmaceuticals) for 1 h at room temperature and then treated with 200 μl of substrate (0.04% *o*-phenylenediamine in citric acid-phosphate buffer [pH 5.0] including 0.001% hydrogen peroxide). The reaction was stopped by the addition of 50 μl 2.5 N H_2SO_4 after 30 min, and the optical density at 490 nm was determined. The cutoff point for a positive result was defined as an OD value with 3 standard deviations above the mean for 10 healthy control serum samples diluted 1:400.

The positive clone was cultured in 1 liter of super broth medium, and 20 ml of the resultant supernatant was prepared by sonication as described above. Purification of Fab was performed by using His-Bind resin (Novagen, Madison, WI), according to the manufacturer's instructions. The reactivity of the purified Fab to the recombinant spike protein of SARS-CoV was examined by Western immunoblot analysis. The recombinant spike protein was electrophoresed under reducing conditions in a 10% acrylamide gel containing sodium dodecyl sulfate and was then transferred to a polyvinylidene difluoride membrane. The spike protein was blotted with the purified Fab, followed by HRP-conjugated goat antibody to human IgG Fab and a Konica HRP-1000 immunostaining kit. In both the ELISA and the Western immunoblotting assay, patients' sera and polyclonal rabbit antibody to the spike protein (Imgenex, San Diego, CA) were used as positive controls. Normal human Fab (OEM Concepts, Toms River, NJ), normal human serum, and normal rabbit IgG were used as negative controls.

SARS-CoV-infected Vero E6 cells were purchased from EUROIMMUN US LLC (Boonton Township, NJ) and used for the indirect immunofluorescence assay. After the cells were blocking with PBS containing 3% skim milk, the cells were incubated with 10 $\mu\text{g}/\text{ml}$ of the Fab or normal human Fab for 30 min. Fluorescein isothiocyanate-labeled goat antibody to

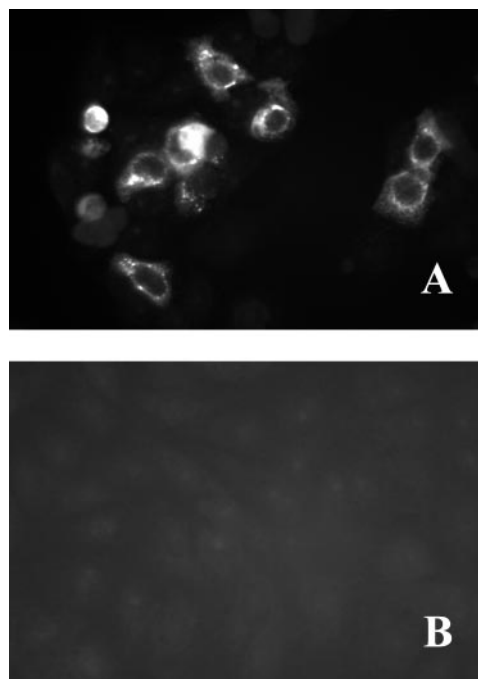


FIG. 1. Indirect immunofluorescent staining of SARS-CoV-infected cells (A) or noninfected cells (B) with the human monoclonal antibody Fab AS3-3.

human IgG Fab (ICN Pharmaceuticals) was used as the secondary antibody.

The affinity constant of Fab was assessed by determination of surface plasmon resonance with a BIAcore 3000 instrument (Biacore AB, Uppsala, Sweden). The recombinant spike protein was immobilized onto a CM5 chip (Biacore). Association and dissociation constants were determined by using BIAevaluation 3.1.

Clones (4.8×10^4) of the combinatorial immunoglobulin gene library were screened for the production of anti-SARS-CoV human Fab by incubation with recombinant SARS-CoV spike protein, followed by incubation with patients' sera and anti-human IgG Fc secondary antibody; three clones showed positive signals. In the second screening of these three Fab clones by ELISA, only one clone, designated AS3-3, reacted specifically with the recombinant spike protein. In Western blotting analysis, AS3-3 reacted with a 30-kDa protein, as did the patients' sera and commercial rabbit polyclonal antibody to the SARS-CoV spike protein (data not shown). AS3-3 was further tested for its ability to bind to native SARS-CoV in infected cells by an indirect immunofluorescence assay; the result showed that AS3-3 specifically recognized infected cells but not noninfected cells (Fig. 1). The affinity of AS3-3 to the recombinant protein was analyzed by a surface plasmon resonance assay; the association and dissociation constants were $5.05 \times 10^7 \text{ M}^{-1}$ and $1.98 \times 10^{-8} \text{ M}$, respectively. The heavy- and light-chain genes were sequenced, and the deduced amino acids in the variable regions are shown in Fig. 2. When the nucleotide sequences were analyzed with the IgBlast program, for the heavy-chain genes, the nearest germ line of the V segment was VH5-51 (93% homology). The closest D-segment germ line was D3-3, and the closest J-segment germ line was

H chain

FR1	CD1	FR2	CDR2
QVQLVQSGAEVKKPGESLKISCKGSGYSFA	NHWIA	WVRQMPGKGLEWMG	IFNPDNSDTTYSFSFQG
FR3	CDR3	FR4	
QVTFSAKKSISIAYLHWSSLKASDTAIYYCAR	LALSDGWLHDF	WGQGLTVTVSS	

L chain

FR1	CDR1	FR2	CDR2
EIVMTQSPFLSLPVTLGQPASISC	KSSQSLVDITNGNTYLI	WFHQRPQGSPRRLIY	QVSKRDS
FR3	CDR3	FR4	
GVPDRFSGSGSGTDFTLKISRVEAEDVGVYFC	MQGTYPPT	LGQGTKVEIKR	

FIG. 2. Deduced amino acid sequences of genes coding for the heavy (H) and light (L) chain variable regions of the human monoclonal antibody Fab, AS3-3, which is specific for the spike protein of SARS-CoV.

JH1. The identity of the heavy chain to the closest heavy chain (GenBank accession no. AAH89417) was 87% (E value, e^{-116}). The closest V-segment germ line of the light chain gene was A17, and the closest J-segment germ line was J κ 1. The identity of the light chain to the closest light chains (GenBank accession no. BAC01729, AAH22362, BAC01734, and BAC01687) was 92% (E values, e^{-116}).

Several methods for the preparation of human monoclonal antibody have recently been developed, and one of the effective methods is the phage display system. Indeed, it has been reported that recombinant neutralizing human antibodies to the SARS-CoV spike protein could be generated from large naïve human antibody phage display libraries (9, 12, 14).

By contrast, in the present study, a recombinant human monoclonal antibody Fab fragment to the SARS-CoV spike protein could be generated from a considerably smaller library derived from patients who had recovered from SARS. Moreover, for the screening of the library, antibodies were expressed as Fab fragments in *E. coli*, and the bacterial colonies were examined directly without the use of a phage display system.

In SARS patients, it has been reported that anti-SARS-CoV antibodies become detectable between days 10 and 15 after infection and that they correlate with a decline in viral load (6). It has also been demonstrated that 85.9% of serum samples obtained from 623 SARS patients contained neutralizing antibodies against SARS-CoV, and most of the neutralizing activities could be attributed to IgG (5). The high IgG titers to SARS-CoV shown by ELISA were also confirmed in the two patients in our study. Recently, we have generated neutralizing human monoclonal antibodies to a surface lectin of the protozoan parasite *Entamoeba histolytica* using combinatorial immunoglobulin gene libraries prepared from a symptomatic patient and an asymptomatic cyst passer (11). Although the antibody titer in the cyst passer was lower than that in the symptomatic patient, the proportion of antibodies recognizing the surface antigen seemed to be higher in the asymptomatic individual. The use of lymphocytes from patients who recovered from symptomatic conditions would be a useful way to

obtain neutralizing antibodies. The efficiency of using memory B cells from patients who recovered from SARS for the production of human monoclonal antibodies to SARS-CoV has also been demonstrated by the method of immortalizing B cells with Epstein-Barr virus (13).

It has been demonstrated that the natural receptor of SARS-CoV is angiotensin-converting enzyme 2 (4) and that the binding site for the receptor is between residues 318 and 510 of the spike protein (15). Although the neutralizing activity of AS3-3 toward SARS-CoV has not yet been evaluated, the recombinant spike protein used for the screening in the present study (residues 258 to 573) includes the receptor-binding site. Recently, it has been reported that one of the neutralizing human single-chain variable-region 80R fragments binds to a similar part (residues 261 to 672) of the spike protein with a dissociation constant of 3.23×10^{-8} M (9). Therefore, the affinity of AS3-3 to the spike protein demonstrated in this study seems to be 1.6-fold higher than that of the 80R single-chain variable-region fragment.

In conclusion, use of the immunoglobulin gene library prepared from patients who recovered from SARS would be a potent strategy for the production of human antibodies to SARS-CoV.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB245095 and AB245096.

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