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FULL PAPER

Characterization and epitope mapping of monoclonal antibodies to the nucleocapsid protein of severe acute respiratory syndrome coronavirus

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Abstract

The sudden emergence of severe acute respiratory syndrome (SARS) at the end of 2002 resulted in 774 reported deaths from more than 8000 cases worldwide. As no effective vaccines or antiviral agents are available, the most effective measure to prevent the expansion of a SARS epidemic is the rapid diagnosis and isolation of SARS patients. To establish specific diagnostic methods, we generated nine clones of monoclonal antibodies to nucleocapsid protein (NP) of SARS-coronavirus (SARS-CoV). On immunofluorescent antibody assay and Western blotting analysis, none of the monoclonal antibodies showed cross-reactivity to authentic and recombinant NPs of human coronavirus (HCoV) 229E strain. To determine the region on the NP molecule where the monoclonal antibodies bind, we generated four truncated recombinant NPs and analyzed the reactivity between monoclonal antibodies and truncated NPs. Two monoclonal antibodies reacted with a truncated NP covering from amino acid residues 111 to 230, and seven reacted with another truncated NP covering from amino acid residues 221 to 340. Epitope mapping analysis indicated that monoclonal antibody SN5-25 recognized the amino acid sequence Q^{245}TVTKK^{250} on SARS-NP. Within the epitope, Q245, T246, V247, K249, and K250 appeared to form an essential motif for monoclonal antibody SN5-25 to bind. The information about binding sites and epitopes of monoclonal antibodies may be useful for the development of new diagnostic methods for SARS and for analyzing the function of N protein of SARS-CoV.

Key Words: severe acute respiratory syndrome (SARS), coronavirus, nucleocapsid, epitope, monoclonal antibody

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Introduction

In late 2002, a severe respiratory illness of unknown etiology emerged in Guangdong Province, China, and spread to many parts of the world[18, 20, 28, 29]. In March 2003, the World Health Organization (WHO) issued global alerts and designated the disease severe acute respiratory syndrome (SARS). A global research network funneled information to WHO. In April 2003, a novel coronavirus, designated SARS-coronavirus (SARS-CoV), was isolated as the causative agent[1,10, 19]. By the time the epidemic was confined in July 2003, the number of probable cases had mounted to more than 8000, with 773 deaths in 32 countries or regions[26]. Several additional cases in Guangdong were later reported, as were laboratory-acquired infections in Singapore, Taipei, and Beijing.

Coronaviruses (*genus Coronavirus,* family *Coronaviridae*) generally target respiratory and intestinal tract organs. Although coronaviruses have traditionally been classified into three groups, based on their antigenic characterization, SARS-CoV appears to be distant from these groups, and was classified into a novel coronavirus group[16, 21]. Coronaviruses were thought to be species-specific, but SARS-CoV infected monkeys, cats, ferrets, mice, and rats, as well as humans. The virus was identified in masked palm civets and raccoon dogs in a Guangdong animal market[4], as well as dogs and cats during the 2003 Amoy Gardens epidemic in Hong Kong[17]. Recent studies suggested that horseshoe bats may carry SARS-CoV-related viruses[11, 13].

With a spherical virion 100-200 nm in diameter, a coronavirus has a positive sense, single-stranded genomic RNA, wrapped in an envelope. The three envelope proteins are spike (S) protein, responsible for large projections from the envelope; membrane (M) protein; and envelope (E) protein. S protein binds the virion to cells and is the target of neutralizing antibodies. Inside the virion, the viral genome is associated with nucleocapsid protein (NP). The functions of NP may be related to viral RNA replication, transcription, and RNA packaging[6,7,14, 23, 27]. As large amounts of NP are produced in infected cells[9,21], and anti-NP antibodies have been detected in more than 90% of patients[5,12, 22, 24, 25], NP may be an appropriate immune response target.

Kogaki *et al.*[8] expressed NP in *E. coli* and produced monoclonal antibodies to NP to establish a diagnostic method for SARS. Their findings suggested that monoclonal antibodies can detect SARS-CoV NP with high sensitivity. Here we characterized these monoclonal antibodies by analyzing the binding sites and epitopes of the monoclonal antibodies to SARS-CoV NP. This analysis may clarify the significance of the monoclonal antibodies as SARS-CoV-specific diagnostic reagents and tools for analyzing the function of SARS-CoV NP.

Materials and methods

Cells and viruses

Vero E6 cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 0.292 g/l L-glutamine and 5% heat-immobilized fetal bovine serum (FBS). The Hanoi strain of SARS-CoV was kindly provided by Dr. Koichi Morita, Nagasaki University, Japan, and propagated in Vero E6 cells. A monolayer of Vero E6 cells was infected with SARS-CoV and cultured for 2 days at 37℃ in a CO_2 incubator. The culture fluid of the infected cells was collected and centrifuged at 700×g for 5 min. The supernatant was stored at −80℃ as stock virus. The SARS-CoV-infected cells were transferred to sample tubes with a cell scraper. The cells were washed with phosphate-buffered saline (PBS), and the pellets were stored at −80℃ until use. L132 cells and the human coronavirus (HCoV) 229E strain were kindly provided by Dr. Shigeru Morikawa, National Institute of Infectious Diseases, Japan. The L132 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 0.292 g/l L-glutamine and 5% FBS. They were then infected with HCoV 229E and cultured for 3 days at 37℃ in

a CO_2 incubator. The culture fluid and HCoV 229E -infected cells were collected as described above.

Establishment of monoclonal antibodies to SARS-CoV NP

The cDNA of SARS-CoV Urbani strain NP was prepared from synthetic oligonucleotides and polymerase chain reaction (PCR), using Pfu Taq DNA polymerase[2]. The cDNA was cloned into the pW6A vector, and the plasmid containing the SARS-CoV NP gene was designated pWSN. The recombinant SARS-CoV NP was expressed in *E. coli* BL 21 cells and purified by DEAE-Sepharose (Whatman DE52) ion exchange chromatography and Phenyl-Sepharose Fast Flow (Pharmacia) chromatography[3]. We prepared mouse monoclonal antibodies to both the purified NP and a synthetic peptide (GQTVTKKSAAEASKKPR) that was equivalent to the amino acid sequence (244-260 aa) of the Urbani strain NP[8], thus establishing monoclonal antibodies rSN16a, rSN17, rSN18, rSN21, rSN21-2, rSN113, and rSN150 against the purified recombinant NP and monoclonal antibody SN5-25 against the synthetic peptide. The monoclonal antibodies used in this study are listed in Table 1.

Preparation of rabbit immune serum

A Japanese white rabbit (Japan SLC Inc.) was immunized with recombinant NP six times at 2-week intervals. At the first immunization, recombinant NP mixed with complete Freund's adjuvant was injected into the animal. In the latter five immunizations, recombinant NP mixed with incomplete Freund's adjuvant was injected. The rabbit was sacrificed by cardiac puncture under anesthesia, and the collected rabbit serum was heat-inactivated at 56℃ for 30 min and stored at −40℃ until use. All animal experiments were performed according to the guidelines of the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated with Isogen (Nippon Gene), according to the manufacturer's protocol. The reverse transcription mixture was prepared in a tube by adding 4 μl of 5× first-strand buffer, 2 ml 0.1M dithiothreitol, 1μl 10 mM dNTP mixture, 1 μl of 50 μM oligo dT_{20} (Invitrogen), 1 μl of sample RNA solution containing 1 μg of total RNA, and diethylpyrocarbonate-treated water, for a final volume of 19 μl. The mixture was heated at 70℃ for 10 min and kept at 42℃ for 2 min, after which 1 μl (200 units) of Superscript II (Invitrogen) was added. The tube was maintained at 42℃ for 50 min, heated at 70℃ for 15 min, and then used as cDNA.

Table 1. Monoclonal antibodies used in this study

Name	Immunogen	Source
rSN16a	Recombinant SARS-CoV NP[a]	This report
rSN17	Recombinant SARS-CoV NP	This report
rSN18	Recombinant SARS-CoV NP	Kogaki et al[8]
rSN21	Recombinant SARS-CoV NP	This report
rSN21-2	Recombinant SARS-CoV NP	Kogaki et al[8]
rSN113	Recombinant SARS-CoV NP	This report
rSN122	Recombinant SARS-CoV NP	Kogaki et al[8]
rSN150	Recombinant SARS-CoV NP	Kogaki et al[8]
SN5-25	Synthetic peptide[b]	Kogaki et al[8]

[a] Full length SARS-CoV NP expressed in *E. coli*.
[b] Synthetic peptide (GQTVTKKSAAEASKKPR) equivalent to amino acid residues 244-260 of SARS-CoV Urbani strain NP.

Amplification of full-length and truncated SARS-CoV N protein genes

The PCR mixture was prepared in 0.2-ml tubes, with addition of 0.5 μl of 5 U/μl Platinum Taq polymerase HiFi (Invitrogen), 5 μl of 10× HIFI buffer, 1 μl of 10 mM dNTP, 2 μl of 50 mM $MgSO_4$, 37.5 μl of water, 1μl of 10 mM primers, and 2 μl of cDNA. PCR was performed at 94℃ for 2 min, followed by 35 thermal cycles of 94℃ for 2 min, 60℃ for 30 s, and 68℃ for 30 s. The regions of amplified NP genes were nucleotide residues 1-1269 (NP Full), 1-360 (trNP1), 331-690 (trNP2), 661-1020 (trNP3), and 991-1269 (trNP4; Fig. 1).

Cloning of SARS-CoV NP genes

To add the one adenine base overhang at the 3' end of the amplified DNA fragments, 0.25 μl of Ampli Taq (Applied Biosystems) was added to the PCR mixture and kept at 70℃ for 10 min. The amplified DNA fragments were electrophoresed in SeaKem GTG agarose gels (Cambrex). DNA fragments of the correct molecular size were cut from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The purified DNA fragments were ligated with the pCR2.1 vector using a TA Cloning Kit (Invitrogen). Plasmids containing the NP gene were obtained from the cultures of transformed TOP10F' (Invitrogen) *E. coli* cells, and the NP gene inserts in the plasmids were sequenced. The nucleotide sequence of the Hanoi strain N gene perfectly matched that of the SARS-CoV Urbani strain [AY278741]. The plasmids with the NP genes and pET43.1a (Novagen) were digested with *Pst*I and *Eco*RI. The NP gene and pET43.1a were then ligated using a DNA Ligation Kit (Takara Bio Inc.).

Expression of recombinant NP

The pET43.1a plasmids containing NP genes were selected, multiplied, and purified as described above. *Escherichia coli* AD494pLys cells were transformed with the plasmids and cultured at 37℃ overnight in 2 ml of Luria-Bertani (LB) medium with 50 μg of ampicillin. The bacterial cultures (40 μl) were inoculated into 4 ml of fresh LB medium and cultured at 37℃ for 3 h. IPTG was added to the cultures, and the bacteria were

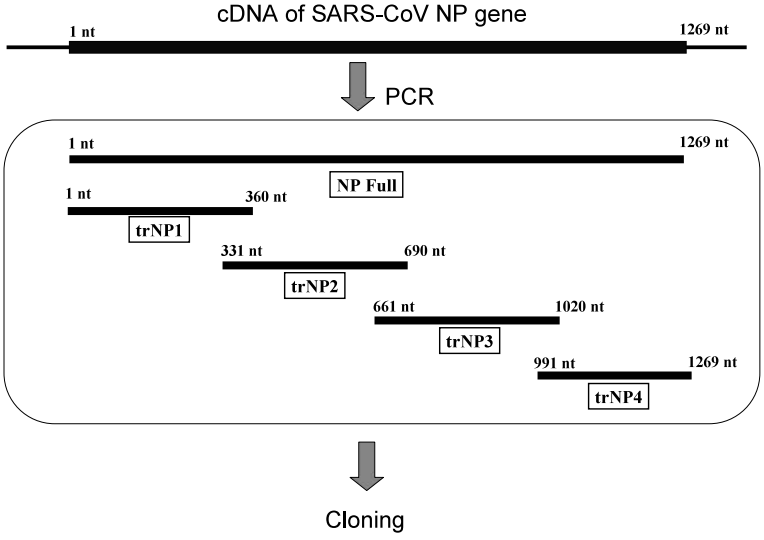


Fig. 1. Amplification of the nucleocapsid protein (NP) gene of SARS-CoV. Total RNA from infected Vero E6 cells was subjected to reverse transcription. The regions of NP genes amplified by PCR were nucleotide residues 1-1269 (NP Full), 1-360 (trNP1), 331-690 (trNP2), 661-1020 (trNP3), and 991-1269 (trNP4). The amplified DNA fragments were ligated into the pCR2.1 vector using a TA Cloning Kit (Invitrogen), and sequenced. The plasmids with the NP genes and pET43.1a (Novagen) were digested with *Pst*I and *Eco*RI. The digested NP genes were cloned into pET43.1a.

further cultured for 3 h. After culturing, the bacteria were collected by centrifugation at 5000 × g for 5 min and washed with 1 ml of 20 mM Tris-HCl (pH 8.0). The bacterial pellets were lysed with 400 µl of BugBuster reagent (Novagen) and 4 µl of Benzonuclease (Invitrogen) at room temperature for 20 min. After centrifugation at 16000 × g at 4℃ for 20 min, the supernatant was filtered through a 0.45-µm pore size membrane filter (Millipore), and used as the crude protein fraction. The recombinant NP was purified using ProBond resin (Invitrogen), according to the manufacturer's instructions. Briefly, aliquots of 300 µl of the resin were washed once in 2.0-ml tubes with 600 µl of water and equilibrated three times with 20 mM imidazole binding buffer. The soluble proteins were added to the resin and kept at 4℃ for 1 h. After centrifugation at 4℃ for 1 min, the resin was washed four times with 600 µl of 60 mM imidazole binding buffer. Finally, 1 ml of 300 mM imidazole binding buffer was added to the resin, mixed at 4℃ for 10 min, and the supernatant was collected as recombinant NP solution. The SARS-CoV NPs NP Full, trNP1, trNP2, trNP3, and trNP4 were expressed as fusion proteins with an NusA tag.

Western blotting analysis

Recombinant NPs were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (Immunobilon PVDF, Millipore). The membranes were blocked with BlockAce (Dainippon Pharmaceutical Co. Ltd.) at 4℃ overnight and washed with Tris-buffered saline (TBS: 25 mM Tris-HCl, pH7.4, and 150 mM NaCl). After washing three times with a mixture of TBS and 0.05% Tween 20 mixture (TBST), the membranes were immersed in 1:500-diluted mouse monoclonal antibody to SARS-CoV NP at 37℃ for 1 h. After washing with TBST, the membranes were immersed in 1:2000-diluted alkaline phosphatase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratory Inc.) at 37℃ for 1 h. The membranes were washed five times, and the bands were visualized using an AP Detection Reagent Kit (Novagen).

Enzyme-linked immunosorbent assay (ELISA)

Recombinant NP-NusA and NusA proteins were diluted with carbonate buffer (Sigma-Aldrich), and 50-µl aliquots of these solutions were added to 96-well assay plates at 4℃ overnight. The plates were washed five times with PBS containing 0.05% Tween 20 (PBST) and blocked with 200 µl of 1:4-diluted BlockAce (Dainippon Pharmaceutical Co. Ltd.) at 37℃ for 1 h. The plates were washed five times and reacted with 50 µl/well of monoclonal antibodies diluted 1:1000 with 0.5% bovine serum albumin (BSA) in PBST (BSA-PBST) at 37℃ for 1 h. After washing, 50 µl/well of peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratory Inc.), diluted at 1: 5000 with BSA-PBST, were applied to the plates, which were then incubated at 37℃ for 1 h. After washing, 100 µl o-phenylenediamine (Sigma-Aldrich) with H_2O_2 were added to the plates, which were then left at room temperature for 30 min. The optical densities at 450 nm (OD_{450}) and 620 nm (OD_{650}) were measured to calculate the ELISA value (OD_{450}-OD_{650}).

Indirect immunofluorescence assay (IFA)

Vero E6 cells grown on Lab-Tek eight-well chamber slides (Nalge Nunc) were infected with 3.52×10^6 tissue culture infective dose 50 ($TCID_{50}$) SARS-CoV at 37℃ for 1 h, after which 400 µl of minimal essential medium (MEM) were added to each well. The infected cells were cultured at 37℃ for 24 h in a CO_2 incubator, fixed with 800 µl of methanol per well, and irradiated with ultraviolet light for 30 min at room temperature. The fixed slides were stored at −40℃ until use. The monoclonal antibodies were serially diluted with PBS and applied to the slides (100 µl/well), which were then incubated at 37℃ for 1 h and washed three times with PBS. FITC-conjugated anti-mouse IgG antibody (MP Biomedicals) diluted with 1:400 PBS was added to the slides (100 µl/well). After washing, the slides were embedded with buffered glycerol (1 PBS:9 glycerol), covered with a cover glass, and observed with a confocal laser microscope.

Epitope mapping of monoclonal antibodies by the 9-fluorenylmethyloxycarbonyl (Fmoc) method

To determine the antibody epitopes on SARS-CoV NP, peptides consisting of ten NP amino acid residues were synthesized on membranes. All peptides were synthesized with an Autospot Peptide Synthesizer, ASP222 (AbiMED), using an Fmoc-protected system and Amino-Peg-membranes (INTAVIS Bioanalytical Instruments AG). The peptide-spotted membranes were rinsed once in methanol and twice in PBST. The membranes were blocked with BlockAce for 30 min at room temperature and washed three times with PBST. The membranes were soaked in 1:500-diluted monoclonal antibody, incubated at 37℃ for 60 min, and washed three times with PBST. The membranes were then soaked with 1:2000-diluted horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories), incubated at 37℃ for 60 min, and washed three times with PBST. To visualize peptide spots that reacted with the monoclonal antibodies, the membranes were stained with a 3-amino-9-ethylcarbazole (AEC) detection system (Sigma-Aldrich). After incubation with AEC reagent for a few minutes, the membranes were washed with distilled water and air-dried.

Results

Reactivity of monoclonal antibodies to authentic SARS-CoV and HCoV 229E NPs

To develop specific diagnostic methods for SARS, nine clones of mouse monoclonal antibodies to SARS-CoV NP were generated by immunizing a Japanese white rabbit with synthetic peptide or recombinant NP. To determine the reactivity of these monoclonal antibodies to authentic NP, Vero E6 cells infected with SARS-CoV were stained with monoclonal antibodies. Cells infected with SARS-CoV showed strong fluorescent signals with all of the antibodies, but neither cells infected with HCoV 229E strain nor normal cells showed a signal (Fig. 2). To determine the specificity of the antibodies in more detail, Western blotting analysis was performed. All antibodies showed a distinct signal at 46 KDa corresponding to NP in SARS-CoV-infected Vero E6 cells, but not in HcoV-229E-infected L132 cells or normal cells (Fig. 3). The NPs of SARS-CoV, HCoV 229E, and HCoV OC43 were expressed in bacterial cells, and the reactivities of these NPs to monoclonal antibodies were analyzed by Western blotting (Fig. 4). All nine monoclonal antibodies reacted only with SARS-CoV NP, and no cross-reactivity was observed with NPs of HCoV 229E or OC43 strains. In ELISA, all monoclonal antibodies reacted with full-length NP in a dose-dependent manner (Fig. 5).

Reactivity of monoclonal antibodies to truncated SARS-CoV NPs

To determine the binding regions of monoclonal antibodies on NP, four different truncated NPs (trNPs) were expressed as fusion proteins with an NusA tag in *E. coli,* and purified. On Western blots, two antibodies (rSN113 and rSN122) showed distinct signals with trNP2, covering NP amino acid residues 111-230 (Fig. 6). The remaining seven antibodies (SN5-25, rSN16a, rSN17, rSN18, rSN21, rSN21-2, and rSN150) showed a signal to trNP3 covering amino acid residues 221-340. In ELISA, rSN113 and rSN122 also showed signals to trNP2,and SN5-25 reacted with trNP3, but the remaining antibodies showed no reactivity to any of the trNPs (data not shown).

Epitope mapping by Fmoc solid-phase peptide synthesis

To determine the epitopes of the monoclonal antibodies, we performed epitope mapping using the Fmoc solid-phase peptide synthesis method. As SN5-25 was generated by immunization with the synthetic peptide GQTVTKKSAAEASKKPR, which was equivalent to amino acid residues 244-260 of NP, various ten-amino-acid peptides were synthesized from the 244-260 amino acid sequence of NP by spotting peptides of nine amino acids at various places on a membrane. Positive signals were observed at sites containing Q^{245}TVTKK^{250}

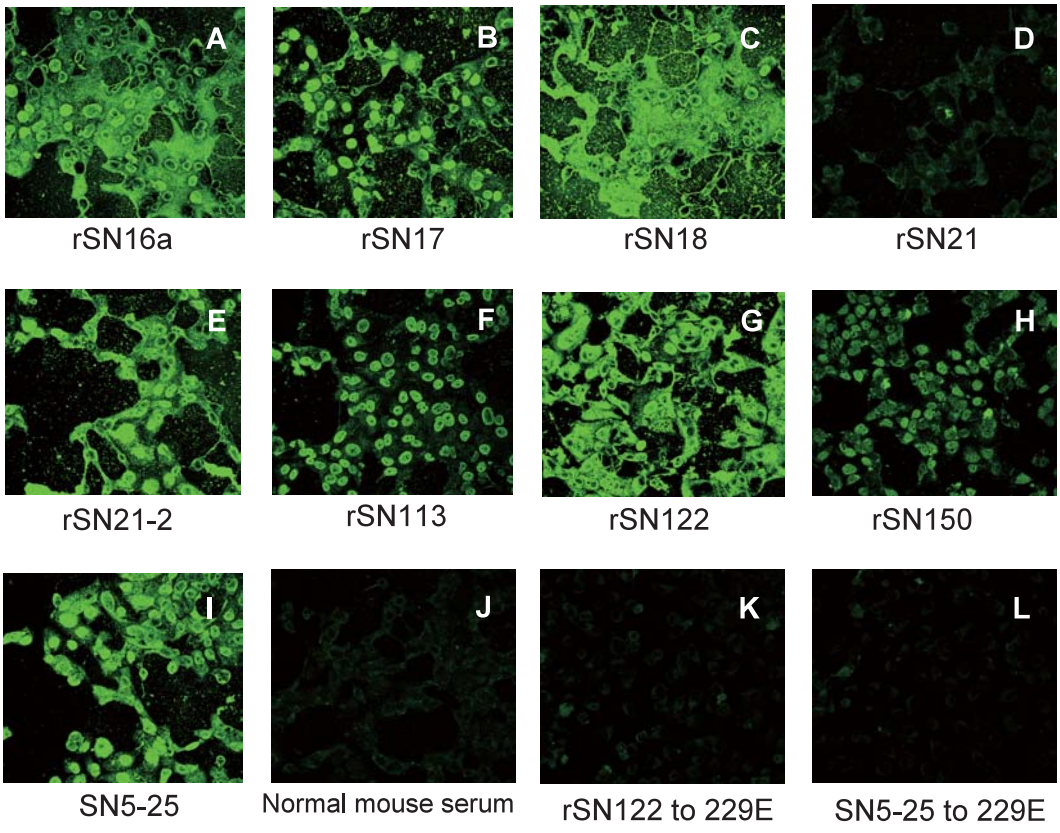
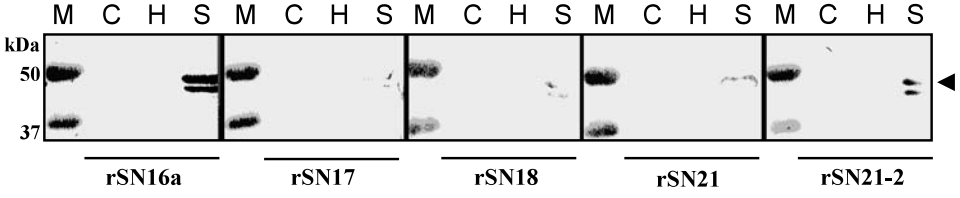


Fig. 2. Reactivity of monoclonal antibodies to SARS-CoV-infected Vero E6 cells. Vero E6 monolayers grown in an eight-chamber slide were infected with SARS-CoV Hanoi strain, and the cells were cultured at 37℃ for 1 day in a CO_2 incubator. The cells were fixed with methanol and reacted with the monoclonal antibodies. The reaction was visualized with FITC-conjugated anti-mouse IgG antibody and observed with a confocal laser microscope.



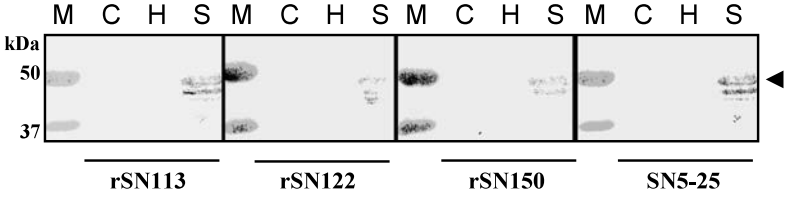


Fig. 3. Reactivity of monoclonal antibodies to authentic viral NPs on Western blotting analysis. Molecular marker (M), lysates of normal Vero E6 cells (C), human coronavirus 229E-infected L132 cells (H), and SARS-CoV-infected Vero E6 cells (S) were subjected to SDS-PAGE and transferred onto PVDF membranes. After reaction with the cell lysates, each monoclonal antibody was analyzed for binding specificity.

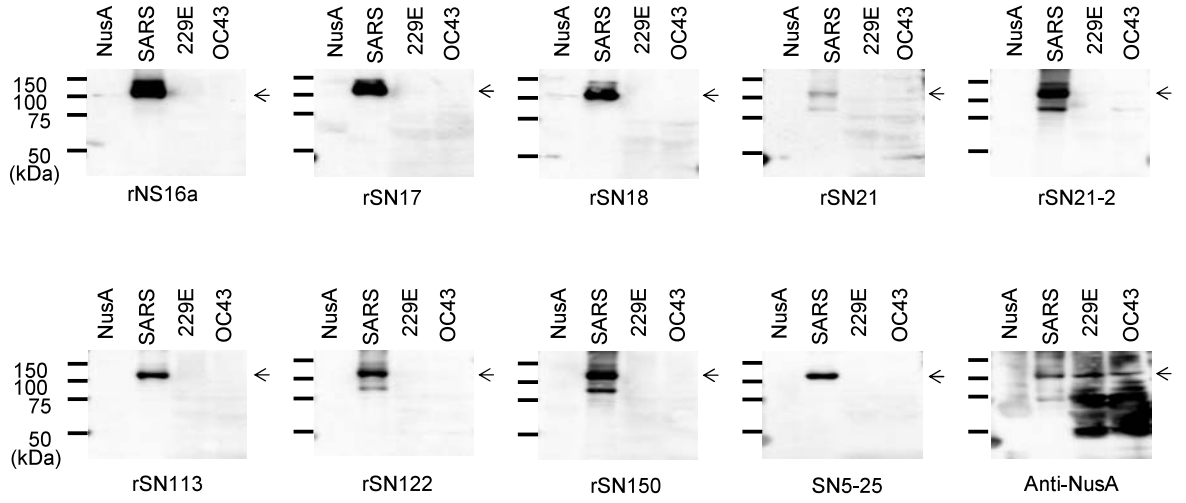


Fig. 4. Reactivity of monoclonal antibodies to recombinant NPs of SARS-CoV and human coronaviruses (HCoV). NPs of SARS-CoV, HCoV 229E, and HCoV OC43 were cloned into pET43.1a and expressed as fusion proteins with NusA protein in *E. coli*. The bacterial lysates were subjected to SDS-PAGE, transferred onto PVDF membranes, and analyzed by Western blotting.

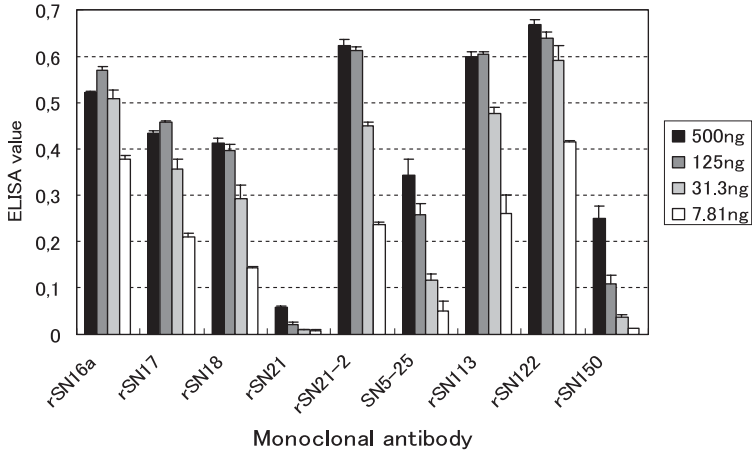


Fig. 5. Reactivity of monoclonal antibodies to SARS-CoV NP in ELISA. SARS-CoV NP was expressed as a fusion protein with NusA protein in *E. coli*. The bacterial lysate was purified and used as the antigen for ELISA. Full-length NP and NusA (2.5ng each) were used to coat plates and reacted with each monoclonal antibody in different amounts (7.82, 31.3, 125, and 500 ng/well). The ELISA values for each antibody were calculated as the differences in optical densities between wells with NP and NusA.

(Table 2), suggesting that this sequence is the epitope for monoclonal antibody SN5-25. To determine the essential amino acids in the epitope for monoclonal antibody SN5-25 binding, six amino acid peptides with substitutions of Alanine (A) were also analyzed for binding to SN5-25 (Table 3). Most of the substituted peptides lost reactivity, but QTVAKK reacted with SN5-25. Therefore, Q245, T246, V247, K249, and K250 appear to form an essential binding motif of SN5-25. We used the same strategy to search for the remaining eight monoclonal antibody epitopes, but found none. The NP gene amino acid sequence in the pET 43.1 vector was mutated by site-directed mutagenesis, yielding A^{245}TVTKK^{250} and Q^{245}TVTKA^{250}, which lost reactivity in the Fmoc method. The lysates of the mutated plasmid-transformed *E. coli* did not react with SN5-25 on Western blotting analysis (data not shown).

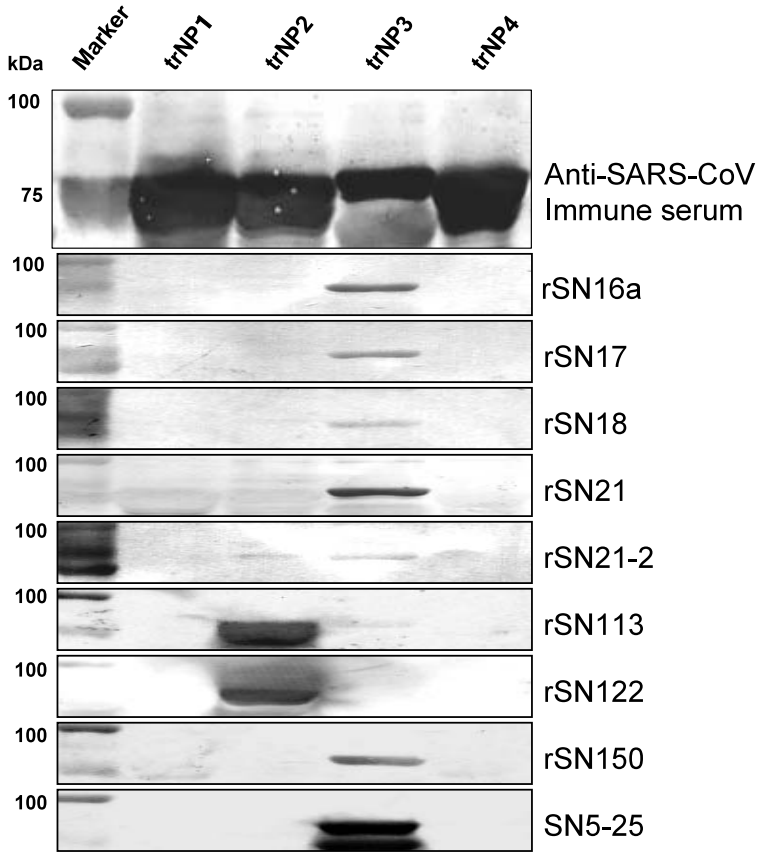


Fig. 6. Analysis of the regions on NP bound by the monoclonal antibodies. Four different truncated NPs were expressed as fusion proteins with NusA in *E. coli,* and the purified truncated NPs were subjected to SDS-PAGE. The truncated NPs were then transferred onto PVDF membranes, and the binding region of each monoclonal antibody was analyzed by Western blotting.

Table 2. Mapping of the epitope for SN5-25 monoclonal antibody on SARS-CoV NP[a)]

Amino acid sequence on SARS-CoV NP	Position of amino acid residue	Reaction with SN5-25
QQQQGQTVTK	240-249	−
QQQGQTVTKK	241-250	+
QQGQTVTKKS	242-251	+
QGQTVTKKSA	243-252	+
GQTVTKKSAA	244-253	+
QTVTKKSAAE	245-254	+
TVTKKSAAEA	246-255	−
VTKKSAAEAS	247-256	−

[a)] Peptides consisting of ten NP amino acid residues were synthesized on a membrane using an Fmoc-protected system. The peptide-spotted membrane was immunostained with an SN5-25, horseradish peroxidase-conjugated anti-mouse IgG, and 3-amino-9-ethylcarbazole detection system.

Table 3. Identification of the essential amino acid motif within the epitope for SN5-25[a)]

Amino acid sequence	Reaction with SN5－25
ATVTKK	－
QAVTKK	－
QTATKK	－
QTVAKK	＋
QTVTAK	－
QTVTKA	－
QTVTKK	＋

[a)] The epitope Q^{245}TVTKK^{250} for SN5-25 was synthesized with substitutions on a membrane using the Fmoc method. The binding ability of SN5-25 to the various peptides was visualized with the immunostaining protocol described in Table 2. Bold "A" represents the substituted alanine residue.

Discussion

The 2002-2003 global epidemic of SARS had social impacts around the world. Although the epidemic was confined, the re-emergence of SARS is possible. In fact, several laboratory infections occurred after the epidemic. Recent epidemiological studies have suggested that bat species in southern China are the reservoirs for SARS-CoV-related viruses[11,13)], and eradicating SARS-CoV in nature may prove impossible. Moreover, antiviral agents and vaccines have yet to be developed. Thus, reliable and sensitive diagnostic methods are critical for minimizing possible SARS epidemics. Detecting the infection in acute-phase patients is vital to guard against further infections, as the virus is shed in the acute phase in respiratory droplets. Although a variety of diagnostic methods have been developed, detecting SARS infection is still problematic. For example, antibodies are not detectable for $\geq$10 days after onset. The detection rates of RT-PCR and loop-mediated amplification (LAMP) 2-3 days after onset are only about 35-65%. Although an antigen-detection test by ELISA targeting NP has been developed, the assay takes several hours, which is not optimal for bedside diagnosis of SARS patients.

We established nine clones of mouse monoclonal antibodies to NP by immunization of mice with recombinant NP and a synthetic peptide. Kogaki *et al.*[8)] used two different monoclonal antibodies to develop a sensitive antigen-detection method targeting NP by immunochromatography, which can detect $\geq 1.99\times10^2$ $TCID_{50}$/ml virus within 15 min. This method may be suitable for the rapid diagnosis of SARS, but the rationale for the high sensitivity was not determined and specificity was not evaluated. Therefore, in the present study, we analyzed epitopes and recognition sites of the monoclonal antibodies, as well as their specificity. Although all of the antibodies reacted with native SARS-CoV NP (Figs. 2 and 3), none reacted with the NP of the HCoV 229E strain. SARS-CoV and 229E were cross-reactive, but the monoclonal antibodies were not cross-reactive with 229E. In immunochromatography, rSN122 and rSN21-2 showed the highest specificity[8)]. The binding sites of rSN122 and rSN21-2 are in the regions from amino acid residues 111-230 (trNP2 region) and 221-340 (trNP3 region), respectively. Therefore, two monoclonal antibodies recognizing the trNP2 and trNP3 regions may not inhibit their binding. On the other hand, when two monoclonal antibodies recognizing the same region were used, the immunochromatographic test showed less sensitivity. The 3D structures of NP in the N-terminal and C-terminal regions have been determined[7,27)], but the actual distance between the trNP2 and trNP3 regions is unclear, as the 3D structure of the whole NP has not yet been ascertained. However, the distance between trNP2 and trNP3 may be large enough for rSN122 and rSN21-2 to bind NP at the same time, without interference. Using two different antibodies that bind to the different regions of NP may be essential to detect the protein with high sensitivity. Two monoclonal antibodies and the remaining seven antibodies bound to trNP2 and trNP3, respectively. This information may be also useful for functional analysis of SARS-CoV NP.

We determined the minimum epitope of the monoclonal antibody SN5-25 on NP. As the amino acid sequence of the epitope (Q^{245}TVTKK^{250}) is

unique to SARS-CoV, and no such sequence is found among the causative agents of human respiratory illnesses, SN5-25 is quite useful for diagnosing SARS.

In conclusion, our analysis of monoclonal antibodies to NP revealed that antibodies are very useful in establishing sensitive diagnostic methods for SARS as well as for analyzing the function of SARS-CoV NP.

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