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H. 知的財産権の出願・登録状況

現在出願予定はない。

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

1. Fuxun Yu, Mai Quynh Le, Shingo Inoue, Futoshi Hasebe, Maria del Carmen Parquet, Shigeru Morikawa, and Kouichi Morita: Recombinant Truncated Nucleocapsid Protein as Antigen in a Novel Immunoglobulin M Capture Enzyme- Linked Immunosorbent Assay for Diagnosis of Severe Acute Respiratory Syndrome Coronavirus Infection. Clin Vac Immunol, 14, 146-149, 2007
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III. 研究成果の刊行物・別刷

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Recombinant Truncated Nucleocapsid Protein as Antigen in a Novel Immunoglobulin M Capture Enzyme-Linked Immunosorbent Assay for Diagnosis of Severe Acute Respiratory Syndrome Coronavirus Infection▽

Fuxun Yu,[1] Mai Quynh Le,[2] Shingo Inoue,[1] Futoshi Hasebe,[1] Maria del Carmen Parquet,[1] Shigeru Morikawa,[3] and Kouichi Morita[1]*

Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan[1]; Department of Virology, National Institute of Hygiene & Epidemiology, (NIHE), Hanoi, Vietnam[2]; and Department of Virology, National Institute of Infectious Disease, Tokyo, Japan[3]

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We report the development of an immunoglobulin M (IgM) antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) for severe acute respiratory syndrome coronavirus (SARS-CoV) by using recombinant truncated SARS-CoV nucleocapsid protein as the antigen. The newly developed MAC-ELISA had a specificity and sensitivity of 100% as evaluated by using sera from healthy volunteers and patients with laboratory-confirmed SARS. Using serial serum samples collected from SARS patients, the times to seroconversion were determined by IgM antibody detection after SARS-CoV infection. The median time to seroconversion detection was 8 days (range, 5 to 17 days) after disease onset, and the seroconversion rates after the onset of illness were 33% by the first week, 97% by the second week, and 100% by the third week. Compared with the results of our previous report on the detection of IgG, the median seroconversion time by IgM detection was 3 days earlier and the seroconversion rate by the second week after the illness for IgM was significantly higher than by IgG assay. Our results indicating that the IgM response appears earlier than IgG after SARS-CoV infection in consistent with those for other pathogens. Our newly developed MAC-ELISA system offers a new alternative for the confirmation of SARS-CoV infection.

Severe acute respiratory syndrome (SARS) is a human disease associated with pneumonia that has emerged recently (5, 7, 10, 15). After the emergence of the SARS coronavirus (SARS-CoV), there were several reports dealing with the detection of specific immunoglobulin M (IgM) by indirect enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescent assay (IFA) (1, 3, 13). In these studies, IgM antibodies became detectable later than IgG antibodies, which is in contrast to the phenomena described for most known pathogens. The reason for an earlier IgG response is unclear. It may be due to the earlier development of IgG antibodies than IgM antibodies, or alternatively, the observation may just reflect the low sensitivity of the assays for the detection of IgM. It is known that the detection of IgM without the separation of IgG yields higher rates of false-positive and false-negative results (3). Therefore, the serological response of SARS-CoV-infected patients should be examined using more-sensitive methods.

Antibodies against SARS-CoV nucleocapsid protein are longer-lived and occur in greater abundance in SARS patients than antibodies against other viral components, such as the spike, membrane, and envelope proteins (2, 4, 12, 14, 16). In our previous study, we reported that an indirect IgG ELISA based on an N-terminally truncated nucleocapsid protein is a safe, specific, and sensitive test for the diagnosis of SARS-CoV infection (16). All these data indicated that the nucleocapsid protein of SARS-CoV is a good target antigen for diagnosis.

In this study, we developed a specific and sensitive IgM antibody capture ELISA (MAC-ELISA) for SARS CoV by using recombinant truncated SARS-CoV nucleocapsid protein as the antigen. This MAC-ELISA was designed to specifically detect the IgM-type antibody. The sensitivity and specificity of this MAC-ELISA were assessed. In addition, using serial serum samples collected from SARS patients, the times required for patients to exhibit IgM seroconversion after SARS-CoV infection were determined and compared to results from our previous study for IgG detection, and we found that in SARS-CoV infection, the IgM response appears earlier than the IgG response, resembling the host response to other known pathogens.

MATERIALS AND METHODS

Serum samples. One hundred seventy-five serum samples were collected from healthy volunteers from Hanoi, Vietnam, before the SARS outbreak, which were used as negative serum controls. Serial serum samples from 36 patients with laboratory-confirmed SARS at the French Hospital, Hanoi, Vietnam, were collected from 11 March to 3 April 2003. All sera were heat inactivated at 56°C for 30 min before use.

Protein preparation. SARS-CoV NΔ121 protein, encompassing amino acid residues 122 to 422 of the nucleocapsid protein (an N protein construct with 121 amino acids of the N terminus truncated), was expressed and purified as described previously (16). The NΔ121 protein was used for animal immunization as well as the antigen in the MAC-ELISA.

* Corresponding author. Mailing address: Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Phone: 81 95 849 7829. Fax: 81 95 849 7830. E-mail: moritak@net.nagasaki-u.ac.jp.

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TABLE 1. Seroconversion times detected by the SARS-CoV NΔ121 protein-based MAC-ELISA and indirect IgG ELISA

Ig	Time (days) to seroconversion after the onset of illness		
	Earliest	Latest	Median
IgM	5	17	8
IgG	6	21	11

Production of hyperimmune mouse ascites fluid. Three-week-old BALB/C mice were injected intraperitoneally with 100 μl (100 μg) of purified NΔ121 protein emulsified with an equal volume of Freund's complete adjuvant (MP Biomedicals). Two booster injections were given by the same dose of NΔ121 protein in Freund's incomplete adjuvant (MP Biomedicals) at 14-day intervals. One week after the final booster injection, 1×10^6 SP2/0 myeloma cells were injected intraperitoneally and ascites fluid collected.

Indirect IgG ELISA. The indirect IgG ELISA, based on the recombinant truncated SARS-CoV NΔ121 protein, was described previously (16).

IgM capture ELISA procedure. A SARS-CoV-specific IgM test was done by coating the wells of immunoplates with goat anti-human IgM antibody, adding patient or control sera to the anti-IgM-coated wells, and then allowing the captured IgM to react with the recombinant SARS-CoV NΔ121 protein. Captured SARS-CoV NΔ121 protein was detected with SARS-CoV virus hyperimmune mouse ascitic fluid. Bound anti-NΔ121 protein antibody was detected with horseradish peroxidase-conjugated anti-mouse IgG, followed by H_2O_2-ABTS (2,2′-azino-diethylbenzothiazolinesulfonic acid) substrate. Optimal dilutions of all reagents were determined by checkerboard titration with reference sera. The reference sera were selected according to our preliminary experimental results and serum availability. The reference sera were used for inner control of the assay. The actual procedures were as follows. Ninety-six-well Falcon immunoplates (Becton Dickinson) were coated with 100 μl of a 1:250 dilution of goat anti-human IgM (BioSource International) diluted in phosphate-buffered saline (PBS) (pH 7.4), and plate coating was conducted at 4°C overnight. The plates were then washed five times with PBS-T (PBS plus 0.1% Tween 20) before they were allowed to react with the patient serum. The patient serum samples were diluted at a 1:100 dilution in assay diluent (PBS-T plus 5% skim milk). Each patient serum was added to four wells of the plate. The patient samples were incubated on the plates for 1 h at 37°C, followed by washing. Antigen (purified recombinant truncated SARS-CoV NΔ121 protein) was then added at a concentration of 0.2 μg (in assay diluent) to the upper two wells, while assay diluent only was added to the lower two wells as a negative control, and the plate was incubated for 1 h at 37°C. The plates were washed, and a 1:4,000 dilution of an anti-SARS CoV NΔ121 protein hyperimmune mouse polyclonal ascitic fluid was added and incubated for 1 h at 37°C. The plates were washed, a 1:5,000 dilution of goat anti-mouse IgG-horseradish peroxidase conjugate (Biosource, CA) was added, and the mixture was incubated at 37°C for 1 h. The plates were washed, 100 μl of an ABTS-peroxidase substrate (Kirkegaard & Perry Laboratories) was added, and the plates were incubated in the dark for 30 min at 37°C. Optical densities (OD) were measured at 405 nm. The specific activity of each serum (net OD) was calculated by subtracting the nonspecific background OD in the wells without antigen from the specific OD in the wells with SARS-CoV NΔ121 protein antigen. The values reported represent the averages of results from duplicate wells for each sample. On all test plates, 1:100 dilutions of negative control, weakly positive control (the OD of the positive samples over the OD of the negative control [*P/N*] was approximately equal to 5), and strongly positive control ($P/N \cong 20$) serum samples were run simultaneously. The negative control was one of the blood samples from the 175 healthy subjects. The OD value for this control was established from the mean OD for the 175 SARS-negative subjects plus 2 standard deviations. Each sample was tested in duplicate, and the mean OD for each sample was calculated. The cutoff for the assay was twice the mean OD for the negative control serum sample ($P/N \geq 2$).

RESULTS

Specificity of MAC-ELISA of SARS-CoV using the recombinant truncated N protein. The specificity of the MAC-ELISA was estimated using 175 sera collected from healthy volunteers in Hanoi, Vietnam, before the SARS outbreak. All 175 serum samples were found to be nonreactive in our newly developed SARS-CoV MAC-ELISA; the specificity of the SARS-CoV MAC-ELISA was 100%.

TABLE 2. Rates of seroconversion arranged by length of time after the onset of illness as detected by the SARS-CoV NΔ121 protein-based MAC-ELISA and indirect IgG ELISA

Ig	% of patients who seroconverted during the indicated wk after the onset of illness[b]		
	First	Second[a]	Third
IgM	33 (12/36)	97 (35/36)	100 (36/36)
IgG	22 (8/36)	69 (25/36)	100 (36/36)

[a] A *P* value of <0.005 was determined by the χ^2 test.
[b] Values in parentheses are numbers of patients who seroconverted over the total number tested.

Sensitivity of the MAC-ELISA for SARS-CoV using the recombinant truncated N protein. To determine the sensitivity of the MAC-ELISA, serum samples serially collected from 36 patients with laboratory-confirmed SARS in Vietnam were investigated for their reactivity. All 36 patient sera were found to be reactive in the newly developed MAC-ELISA; the sensitivity of the SARS-CoV MAC-ELISA was 100%.

Reproducibility of MAC-ELISA results for SARS-CoV using the recombinant truncated N protein. The control serum OD were stable not only in different plates at the same test time but also between different test times; the negative control serum was always negative and the OD variations for the weakly and strongly positive control sera were less than 0.2 between different assay times.

Comparison of seroconversion times and seroconversion rates by time to detection by the NΔ121 protein-based IgG ELISA and MAC-ELISA. The times of seroconversion for the 36 patients from whom serial serum samples were collected are shown in Table 1. For IgM detection, the median time to seroconversion was 8 days (range, 5 to 17 days) after disease onset. For IgG detection, the median time to seroconversion was 11 days (range, 6 to 21 days) after disease onset. The seroconversion rates by time after onset of illness as detected by the NΔ121 protein-based IgG ELISA and MAC-ELISA for the 36 patients from whom serial serum samples were collected are shown in Table 2. For IgG detection, the anti-NΔ121 protein IgG seroconversion rates after the onset of illness were 22% by the first week, 69% by the second week, and 100% by the third week. For IgM detection, the anti-NΔ121 protein IgM seroconversion rates after the onset of illness were 33% by the first week, 97% by the second week, and 100% by the third week. The IgM seroconversion rate by the second week after the illness was significantly higher than that observed for IgG ($P < 0.005$).

DISCUSSION

IgM antibody is produced early in the immune response and rises rapidly during the disease course, and hence the detection of IgM antibody is a valuable tool for the rapid diagnosis of acute viral infections (6). The MAC-ELISA was designed specifically for IgM. The capture format of the MAC-ELISA eliminates any potential background caused by extraneous antibody, which in turn results in less frequent nonspecific reactions as well as in the absence of false-positive results

caused by rheumatoid factor. Competition between IgM and IgG for antigen binding is minimized, reducing the occurrence of false-negative results, thus allowing more-reliable and -sensitive detection of IgM antibody against the antigen (8, 9, 11).

After the emergence of SARS-CoV, several studies dealing with the detection of specific IgM antibody by using indirect ELISA or the IFA method have been reported. Woo et al. reported that IgM antibodies became detectable later than IgG antibodies in both the indirect ELISA and the IFA (13). Hsueh et al. reported that IgG seroconversion occurred simultaneously, or 1 day earlier, than IgM seroconversion by IFA. They absorbed IgG before the detection of IgM to avoid the interference of IgG antibody (3). Chang et al. also reported a similar IgG IgM subclass response after SARS-CoV infection (1). In all these studies, IgM antibodies became detectable later than or simultaneously with IgG antibodies, which is in contrast to the phenomena described for most of the known pathogens, against which IgM antibodies often appear a few days earlier than IgG antibodies. It is known that the detection of IgM antibodies without separating IgG antibodies yields higher rates of false-positive and false-negative results. Hence, the reason for the earlier IgG response observed in the studies mentioned above may be because of the low sensitivity of their assay systems for the detection of IgM, rather than the biology of the host immune response.

Using recombinant truncated SARS-CoV nucleocapsid protein as the antigen, we developed an IgM capture ELISA system for SARS-CoV. The newly developed MAC-ELISA had a specificity and sensitivity of 100%, evaluated by using sera from healthy volunteers and patients with laboratory-confirmed SARS, indicating that the assay system is sensitive and reliable. Our newly developed MAC-ELISA system is the first reported IgM capture assay for SARS-CoV using a recombinant protein. It offers one more choice for the serological diagnosis of SARS.

Using serial serum samples collected from 36 patients with laboratory-confirmed SARS, we compared the IgM and IgG antibody seroconversion times after SARS-CoV infection. For IgM antibody, the median time of seroconversion detected was 8 days after disease onset and the seroconversion rates after the onset of illness were 33% by the first week, 97% by the second week, and 100% by the third week. For IgG antibody, the median time of seroconversion detected was 11 days after disease onset and IgG seroconversion rates after the onset of illness were 22% by the first week, 69% by the second week, and 100% by the third week. The mean seroconversion time for IgM was 3 days earlier than that for IgG, in addition to which the rate of positivity for IgM was significantly higher than that for IgG by the second week after the onset of illness. Our results indicate that the IgM response appears earlier than the IgG response after SARS-CoV infection. This is consistent with the phenomena observed for most known pathogens, against which IgM antibodies often appear a few days earlier than IgG antibodies. Our results suggest that the earlier IgG than IgM responses previously reported by other researchers might be due to the low sensitivity of the test systems employed rather than representing the actual timing of the IgM-to-IgG switch.

The results presented here in combination with our former report (16) clearly indicate that the recombinant truncated SARS-CoV nucleocapsid protein is a good target antigen for SARS diagnosis. The results were highly reproducible. In our newly developed system, the use of infectious virus for antigen production, which requires a high level of microbiological security and a proper way to inactivate and to monitor the inactivation process of the virus, is not required. Hence, it is a safer method for diagnosis. The advantages of using a prokaryotic host to produce recombinant protein would be considerable due to the ease of scale-up and the low costs involved in growing bacteria. The recombinant product can be obtained within a relatively short time (within 1 week after cloning), and the expression and purification procedures are simple and easy to perform. From 1 liter of cultured bacterial medium, we obtained more than 10 mg of purified NΔ121 protein. Our method would be much less expensive for the preparation of antigen than using virus-infected cell culture or the eukaryotic expression systems. It would be especially useful in developing countries as well as in cases of large-scale epidemiological investigations.

ACKNOWLEDGMENTS

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Rapid Genome Sequencing of RNA Viruses

Tetsuya Mizutani,* Daiji Endoh,† Michiko Okamoto,‡ Kazuya Shirato,* Hiroyuki Shimizu,* Minetaro Arita,* Shuetsu Fukushi,* Masayuki Saijo,* Kouji, Sakai,* Chang Kweng Lim,* Mikako Ito,* Reiko Nerome,* Tomohiko Takasaki,* Koji Ishii,* Tetsuro Suzuki,* Ichiro Kurane,* Shigeru Morikawa,* and Hidekazu Nishimura‡

We developed a system for rapid determination of viral RNA sequences whereby genomic sequence is obtained from cultured virus isolates without subcloning into plasmid vectors. This method affords new opportunities to address the challenges of unknown or untypeable emerging viruses.

Over the past few years, global migration has led to emerging infectious diseases that pose substantial risks to public health. To prevent potential outbreaks, early detection of infectious pathogens is necessary. In particular, the recent outbreak of severe acute respiratory syndrome (SARS) provided important lessons on how unknown viruses should be detected rapidly. Thus, a standardized and qualified system is required for rapid nucleic acid sequence determination for newly emerging viruses.

Recently, we developed a new method for detecting RNA viruses. This method, based on cDNA representational difference analysis (cDNA RDA), uses 96 hexanucleotides that are not suitable for priming ribosomal RNAs but that normally prime most of the genome of an RNA virus as primers for reverse transcription in cDNA RDA (*1*). However, the RDA method with a cloning step requires at least 1 week for the determination of the nucleic acid sequence.

The Method

Our new system for rapid determination of viral RNA sequence (RDV) uses whole-genome amplification and direct sequencing techniques (Figure 1). The RDV method comprises 6 procedures: 1) effective destruction of cellular RNA and DNA for semipurification of viral particles, 2) effective elimination of DNA fragments by using a prefiltration column system and elution of small amounts of RNA, 3) effective synthesis of first- and second-strand cDNAs, 4) construction and amplification of a cDNA library, 5) construction of a second cDNA library, and 6) direct sequencing using optimized primers. The RDV method enables a broad range of partial nucleotide sequences within the entire viral RNA genome to be obtained within 2 days without cloning into plasmids.

To eliminate contaminating cellular RNA and DNA from the samples, 0.001 μg of RNase A (Qiagen, Hilden, Germany) and 1 μL (2 U) of Turbo DNA-free DNase I (Ambion, Austin, TX, USA) with 1× Turbo DNA-free buffer were incubated at 37°C for 30 min under conditions that prevented destruction of viral RNA in the viral particles. The RNA in the viral particles was then extracted within 30 min by using a total RNA isolation mini kit (Agilent Technologies Inc., Palo Alto, CA, USA). We confirmed that DNA was effectively eliminated by this RNA extraction kit.

In accordance with the Invitrogen manual, cDNA was synthesized, by using random hexamers (Takara Bio Inc., Kyoto, Japan) and Superscript III (Invitrogen, Carlsbad, CA, USA) lacking RNase H activity, at 50°C for 1 h. Then 60 U of RNase H (Takara Bio Inc.) added before synthesis of second-strand cDNA at 50°C for 1 h. In accordance with the manual, a whole genome amplification system (WGA; Sigma-Aldrich, Saint Louis, MO, USA), which was developed for amplification of genomic DNA, was used to amplify viral double-stranded cDNA. This process was

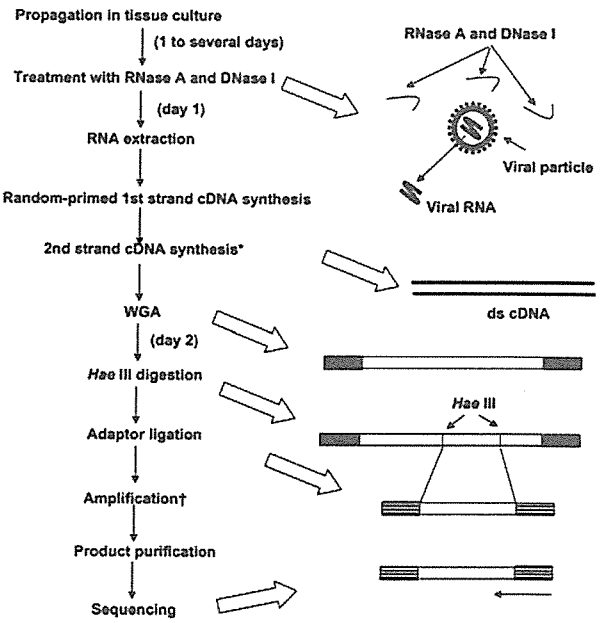

Figure 1. Overall scheme of the rapid determination of viral RNA sequence method. *By adding RNase H; WGA, whole genome amplification; †With specially designed primer sets as shown in Figure 2.

*National Institute of Infectious Diseases, Tokyo, Japan; †Rakuno Gakuen University, Ebetsu, Japan; and ‡Sendai Medical Center, Sendai, Japan

performed within 90 min. Instead of the Taq polymerase recommended in the kit, we used 1.25 U of AmpliTaq Gold LD (Applied Biosystems, Foster City, CA, USA) to obtain a high yield of the PCR products. Primers were provided in the WGA kit, but no information regarding their sequences was obtained. The reaction mixture was heated at 95°C for 9 min (for activation of AmpliTaq Gold), followed by 70 cycles of amplification using Mastercycler (Eppendorf AG, Hamburg, Germany). Each PCR cycle consisted of annealing at 68°C for 1 min, primer extension at 72°C for 5 min, and denaturation at 94°C for 1 min.

The 1st cDNA library was digested with 40 U of *Hae*III (Takara Bio Inc.) at 37°C for 30 min. DNA was purified by using the MonoFas DNA isolation system (GL Science, Tokyo, Japan), and a blunt *Eco*RI-*Not*I-*Bam*HI adaptor (10 pmol; Takara Bio Inc.) was ligated at 16°C for 30 min by using DNA Ligation Kit, Mighty Mix (Takara Bio Inc.). The second cDNA library was amplified by PCR with specially designed primer sets in which 6 nucleotides composed of CC (*Hae*III-digested sequence) and 4 variable nucleotides were added to the 3′ end of the adaptor sequence (Figure 2). For example, 1 primer set was as follows: forward primer, H1–1: 5′-AATTCGGCGGCCGCG GATCCCCGGGG-3′; reverse primer H9–3: 5′-AATT CGGCGGCCGCGGATCCCCAGGA-3′ (the adaptor sequence is underlined, and the *Hae*III-digested sequence is shown in italics) (Figure 2).

We always used >12 primer sets and 0.83 μmol of each primer per cDNA library. PCR was performed with AmpliTaq Gold Master Mix (Applied Biosystems). The reaction mixture was heated at 95°C for 12 min, followed by 70 cycles of amplification. Each PCR cycle consisted of annealing and primer extension at 72°C for 30 s and denaturation at 94°C for 30 s. A single band was consistently obtained in ≈50% of the reactions. DNA was purified from the PCR by using MonoFas. Occasionally, we purified DNA fragments from the gels when >2 bands were detected. Direct sequencing was performed with the forward primer, reverse primer, or both.

When the number of viral particles in the sample was high, we omitted the RNase A and DNase I treatments and used the RNeasy Mini Kit (Qiagen) for RNA extraction. We occasionally used a whole transcriptome amplification kit (Rubicon Genomics Inc, Ann Arbor, MI, USA) instead of the WGA kit because both kits yielded similar amplification results.

In preliminary studies that used referential RNA viruses, we attempted to determine the nucleic acid sequences of SARS coronavirus, mouse hepatitis virus, West Nile virus, Japanese encephalitis virus, and dengue virus type 2 in culture supernatants (10–100 μL) by using the RDV method. The percentages of positive fragments (number of fragments containing viral nucleic acid/total number of

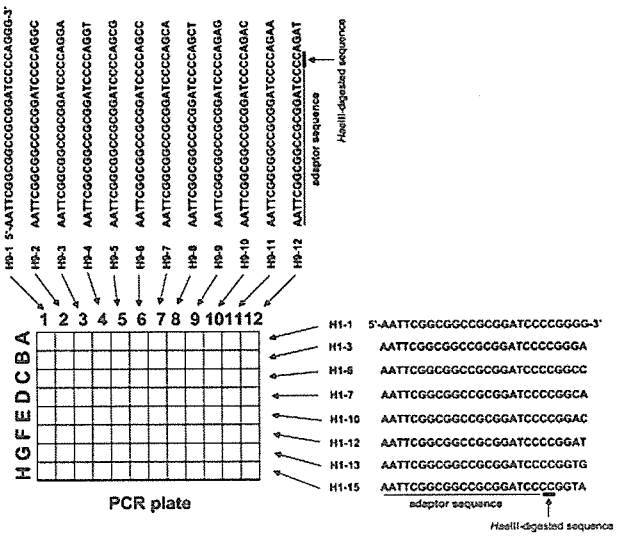

Figure 2. Primers used in rapid determination of viral RNA sequence method.

sequenced fragments) in the reactions for detection of these 5 viruses were 60% (3/5), 45% (5/11), 100% (12/12), 50% (5/10), and 40% (4/10), respectively. As a clinical application, a throat swab specimen from a patient with fever and upper respiratory infection was characterized. Although the specimen exhibited enterovirus-like cytopathic effect by inoculation into HEF and GMK cells when cell culture system for virus isolation was used (*2*), extracted RNA from the supernatant of the cells showed no amplification by reverse transcription–PCR (RT-PCR) when 1 of the conventional primer sets for human enteroviruses was used (*3,4*). In the cell culture supernatant analysis by the RDV method, the specimen exhibited amplification of the partial nucleotide sequences of coxsackie A14 virus (nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession nos. AB275848–AB275853). Thus, the RDV method could detect unidentified cytopathic-effect agents such as enterovirus that could not be detected by RT-PCR when the conventional primer set for enteroviruses was used.

Conclusions

The RDV method is a rapid method for the direct determination of viral RNA sequences without using the cDNA cloning procedure. The limitations of the RDV method are the requirement for cell culture isolate and the large number of steps. However, RDV would be useful for species-independent detection of RNA viruses including unknown or untypeable emerging RNA viruses. Furthermore, with minor modifications, this method would also be applicable to the detection of DNA viruses and bacteria.

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Dr Mizutani is a senior researcher at the National Institute of Infectious Diseases, Tokyo, Japan. His current research focus is infectious disease surveillance by using new technologies.

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Address for correspondence: Tetsuya Mizutani, Department of Virology 1, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama City, Tokyo 208-0011, Japan; email: tmizutan@nih.go.jp

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Development of vaccines and passive immunotherapy against SARS corona virus using SCID-PBL/hu mouse models

Masaji Okada [a,*], Yoshinobu Okuno [b], Satomi Hashimoto [a], Yoko Kita [a], Noriko Kanamaru [a], Yasuko Nishida [a], Yoshie Tsunai [a], Ruriko Inoue [a], Hitoshi Nakatani [a], Reiko Fukamizu [a], Yumi Namie [a], Junko Yamada [a], Kyoko Takao [a], Ritsuko Asai [a], Ryoko Asaki [a], Tetsuo Kase [b], Yuji Takemoto [a], Shigeto Yoshida [c], J.S.M. Peiris [d], Pei-Jer Chen [e], Naoki Yamamoto [f], Tatsuji Nomura [g], Isao Ishida [h], Shigeru Morikawa [i], Masato Tashiro [i], Mitsunori Sakatani [a]

[a] *Clinical Research Center, National Hospital Organization Kinki-Chuo Chest Medical Center, 1180 Nagasone, Kitaku, Sakai, Osaka 591-8555, Japan*
[b] *Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, 3-69 Nakamichi 1-chome Higashinari-ku, Osaka 537-0025, Japan*
[c] *Department of Infection and Immunity, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Tochigi 329-0498, Japan*
[d] *Department of Microbiology, The University of Hong Kong, Prokfulam Road, Hong Kong*
[e] *Hapatitis Research Center, National Taiwan University College of Medicine, Room 328, 3F, No. 1, Sec. 1, Ren-ai Rd., Jhongjheng District 100, Taipei, Taiwan*
[f] *AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjyuku, Tokyo 162-8640, Japan*
[g] *Central Institute for Experimental Animals, 1430 Nogawa, Miyamae, Kawasaki, Kanagawa 216-0001, Japan*
[h] *Pharmaceutical Frontier Laboratory, Pharmaceutical Division, Kirin Brewery Co., 3 Miyahara, Takasaki, Gunma 370-1295, Japan*
[i] *National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*

Abstract

We have investigated novel vaccine strategies against severe acute respiratory syndrome (SARS) CoV using cDNA constructs encoding the structural antigens: (S), (M), (E), or (N) protein, derived from SARS CoV. PBL from healthy human volunteers were administered i.p. into IL-2 receptor γ-chain disrupted SCID mice, and SCID-PBL/hu mice were constructed. These mice can be used to analyze the human immune response in vivo. SARS M DNA vaccine and N DNA vaccine induced human CTL specific for SARS CoV antigens. Alternatively, SARS M DNA vaccines inducing human neutralizing antibodies and human monoclonal antibodies against SARS CoV are now being developed. These results show that these vaccines can induce virus-specific immune responses and should provide a useful tool for development of protective and therapeutic vaccines.

Keywords: SARS DNA vaccine; SCID-PBL/hu; Human neutralizing antibody against M

1. Introduction

The causative agent of severe acute respiratory syndrome (SARS) has been identified as a new type of corona virus, SARS corona virus (SARS CoV) [1–3]. SARS has infected more than 8400 patients in about 7 months in over 30 countries and caused more than 800 deaths. However, no SARS vaccine is currently available for clinical use. Therefore, we have developed novel vaccine candidates against SARS CoV using cDNA constructs encoding the structural antigens; S, M, E, or N protein. In immunized mice, neutralizing antibodies against the virus and T cell immunity against virus-infected-cells were studied, since these immunities play important roles in protection against SARS CoV and many virus infections. In particular, $CD8^+$ CTL plays an important role in T cell immunity dependent protection against virus infections and the eradication of murine and human cancers [4,5]. In the present study, the SCID-PBL/hu model, which is capable of analyzing in vivo human immune response, was

* Corresponding author. Tel.: +81 72 252 302; fax: +81 72 21 2153.
E-mail address: okm@kch.hosp.go.jp (M. Okada).

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used because it is a more relevant translational model for human cases [4]. These vaccines induce neutralizing antibody and CTL. This is the first report inducing antibody against SARS M. Theses vaccines should provide useful tool for development of protective vaccines in human.

2. Materials and methods

Three kinds of SARS CoV strains: HKU39849 [1], TW-1 and FFM-1 [2] and their cDNAs were used. S, M, N or E cDNA was transferred into pcDNA 3.1(+) vector [4]. PBL from healthy human volunteers were administered i.p. into IL-2 receptor γ-chain disrupted NOD SCID mice [IL-2R(−/−) NOD-SCID], and SCID-PBL/hu mice were constructed [4]. pcDNA 3.1(+) vector, 50 μg each, containing SARS S, M, N, or E DNA was injected i.m. into SCID-PBL/hu three times, at an interval of 7 days. Neutralizing antibodies against SARS CoV in the serum from the mice were assayed by use of Vero-E6 cell. CTL activity against SARS CoV was studied using human cells, expressing SARS antigens. CTL activity of human CD8-positive lymphocytes in the spleen from SCID-PBL/hu was assessed using 51Cr-release assay [5,6]. Human monoclonal antibodies were produced from B cell hybridoma using P3U1 myeloma cell and spleen cells from human immunoglobulin transchromosomic mice (KM mice) [7].

3. Results

3.1. Induction of human immune responses against SARS CoV using SCID-PBL/hu

The production of neutralizing antibodies against SARS CoV was observed in the serum from mice immunized with S DNA vaccine SARS (M) DNA vaccine and N DNA vaccine induced murine T cell responses against SARS [4]. To analyze the human immune responses, SCID-PBL/hu were constructed and human CD3-positive T lymphocytes and human B cells and macrophages were replaced in the spleen cells and PEC from these SCID-PBL/hu mice, as shown in Fig. 1.

3.2. SARS M DNA vaccine induced the production of human neutralizing antibodies against SARS CoV in SCID-PBL/hu model

Human neutralizing antibodies were induced from SCID-PBL/hu mice vaccines with SARSS [6] and M DNA vaccines (Fig. 2). Titer of neutralizing antibody in the serum from SCID-PBL/hu mice immunized with SARS (M) DNA vaccine was 1:10. In contrast, inhibition of cytopathic effect was observed even in 100-fold dilution of the same serum. Furthermore, B cell hybridoma producing human monoclonal antibodies were established by the fusion of P3U1 cells and

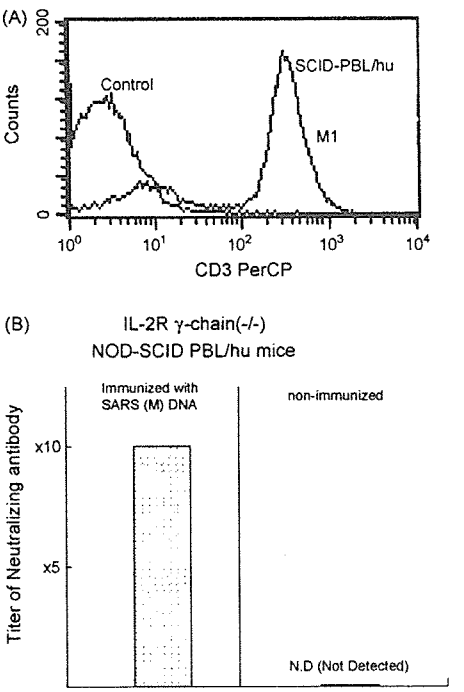

Fig. 1. (A) Human CD3-positive T cells in the spleen from SCID-PBL/hu mice. 1×10^7 PBL from healthy human volunteers were administered i.p. into IL-2R(−/−) NOD-SCID. The number of human CD3-positive T cells were assessed by using anti-human CD3 antibody and FACS. (B) Induction of human neutralizing antibody against SARS coronavirus M protein in SCID PBL/hu mice by SARS (M) DNA vaccination. Titration of neutralizing antibody against SARS CoV in the serum from these mice was assessed by Vero-E6 cells.

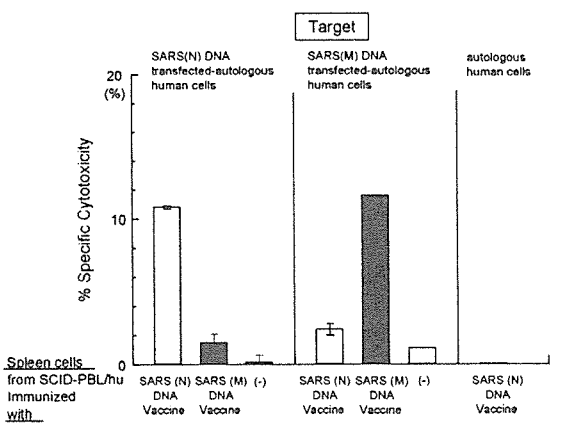

Fig. 2. SARS (N) DNA vaccine and SARS (M) DNA vaccine induces in vivo human CTL against SARS CoV in the SCID-PBL/hu human immune systems. Autologous B blastoid cells transfected with SARS (N) DNA or SARS (M) DNA were used as target cells using ^{51}Cr release assay. E/T ration was 10:1.

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Table 1
Method for establishment of hybridoma producing human monoclonal antibody against SARS CoV

Humanized monoclonal antibody against SARS-S protein
SARS TW1 strain
S protein (S431-447-KLH)
↓
Human immunoglobulin gene transchromosomic mice (KM mouse)
↓
Spleen + P3U1
↓
Hybridoma (Screening)
Humanized monoclonal antibody against SARS S (431–447) peptide: clones (21 clones)

SARS S peptide (S431-447 KLH) was immunized to KM mouse. Hybrid clones producing human monoclonal antibody against SARS S peptide were selected.

spleen cells from human immunoglobulin transchromosomic mice immunized with SARS antigens (Table 1). Specificity is now being studied.

3.3. SARS M DNA and N DNA vaccines induced human T cell immune responses (CTL and proliferation) in SCID-PBL/hu model

The M DNA vaccine enhanced the CTL activity against antologous B blast cells transfected with SARS M DNA but not with SARS N DNA in SCID-PBL/hu mice (Fig. 2). On the other hand, SARS N DNA vaccine augmented the CTL activity specific for autologous B blast cells transfected with SARS N DNA.

4. Discussion

We have demonstrated that SARS (M) DNA and (N) DNA vaccines induce virus-specific immune responses (CTL and T cell proliferation) in the mouse systems using type II lung alveolar T cell lines in clone target models [4]. Gao et al. developed adenovirus based a SARS DNA vaccine encoding S1 polypeptide was capable of inducing neutralizing antibody, while another SARS DNA vaccine encoding N protein generated IFN-γ producing T cells in rhesus monkeys [8]. SARS S DNA vaccine which elicits effective neutralizing antibody responses that generate protective immunity in a mouse model [9]. However its immunogenicity in humans has yet to be established. Therefore, it is very important to evaluate the efficacy of SARS DNA vaccine in a SCID-PBL/hu mice, which is a highly relevant translational model for demonstrating human immune responsiveness.

In the present study, SARS M DNA vaccine and N DNA vaccine induced human CTL specific for SARS M antigen and SARS N antigen, respectively. Furthermore, SARS M DNA as well as SARSS DNA vaccine induce human neutralizing antibodies against SARS CoV by the SCID-PBL/hu model. Antibody against SARS M antigen exerted high inhibitory activity against cytopathic effect by SARS CoV. It was reported that monoclonal antibodies against external domain of M neutralize murine hepatitis corona virus, but only in the presence of complement, and both E and M proteins are required for budding of virions [10]. Neutralizing antibody activity against M protein was not at all eliminated by the inactivation of complement. Antibody against M protein might inhibit the growth of SARS CoV in the cells. Therefore, the effect of combination immunization with such SARS vaccines (M vaccine and S vaccine) and the specificity of human monoclonal neutralizing antibodies are now being studied. These vaccines are expected to provide useful tool for development of therapeutic vaccines.

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Participation of both Host and Virus Factors in Induction of Severe Acute Respiratory Syndrome (SARS) in F344 Rats Infected with SARS Coronavirus▽

Noriyo Nagata,[1]* Naoko Iwata,[1] Hideki Hasegawa,[1] Shuetsu Fukushi,[2] Masaru Yokoyama,[3] Ayako Harashima,[1] Yuko Sato,[1] Masayuki Saijo,[2] Shigeru Morikawa,[2] and Tetsutaro Sata[1]

Departments of Pathology[1] and Virology I,[2] and Center for Pathogen Genomics,[3] National Institute of Infectious Diseases, Tokyo, Japan

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To understand the pathogenesis and develop an animal model of severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV), the Frankfurt 1 SARS-CoV isolate was passaged serially in young F344 rats. Young rats were susceptible to SARS-CoV but cleared the virus rapidly within 3 to 5 days of intranasal inoculation. After 10 serial passages, replication and virulence of SARS-CoV were increased in the respiratory tract of young rats without clinical signs. By contrast, adult rats infected with the passaged virus showed respiratory symptoms and severe pathological lesions in the lung. Levels of inflammatory cytokines in sera and lung tissues were significantly higher in adult F344 rats than in young rats. During in vivo passage of SARS-CoV, a single amino acid substitution was introduced within the binding domain of the viral spike protein recognizing angiotensin-converting enzyme 2 (ACE2), which is known as a SARS-CoV receptor. The rat-passaged virus more efficiently infected CHO cells expressing rat ACE2 than did the original isolate. These results strongly indicate that host and virus factors such as advanced age and virus adaptation are critical for the development of SARS in rats.

The epidemic of severe acute respiratory syndrome (SARS) spread rapidly worldwide during the winter of 2003 to 2004 (16; http://www.who.int/csr/sars/country/en/). SARS-associated coronavirus (SARS-CoV) has been identified as the etiological agent of SARS (4, 5, 12, 14, 28). SARS-CoV has caused progressive respiratory failure and death of approximately 800 individuals, approximately 10% of over 8,000 patients (http://www.who.int/csr/sars/country/en/). Common symptoms of SARS are fever, nonproductive cough, myalgia, and dyspnea. An age of 60 years or older, comorbid disease, male sex, high neutrophil counts, and several biochemical abnormalities are associated with poor outcomes (1, 3, 16, 27, 38). Advanced age in particular is recognized as an independent correlate of adverse outcomes and a predictor of mortality.

Experimental animals, particularly monkeys, have been infected with SARS-CoV to analyze various pathogenic aspects of SARS according to Koch's postulates and to develop animal models to evaluate potential vaccines and antiviral agents (2, 5, 6, 7, 14, 17, 24, 30, 31, 34). Cats, ferrets, mice, pigs, guinea pigs, hamsters, chickens, and rats have also been investigated for SARS-CoV susceptibility (22, 23, 32, 33, 39). All these animals are susceptible to SARS-CoV after intrarespiratory inoculation and exhibit virus excretion in pharyngeal or nasal swabs, histopathological pulmonary lesions, and seroconversion. In monkeys, aged mice, and Syrian hamsters, infection is not lethal but results in consolidative pneumonitis that resolves within 1 week (7, 24, 30, 32, 33, 34). Thus, existing animal models are useful to analyze the pathology associated with early phases of SARS-CoV infection and to provide insights into early events in SARS-CoV infection.

The SARS-CoV spike (S) protein mediates the infection of receptor-bearing cells. In the case of several avian and mammalian coronaviruses, the S protein is cleaved by furin or a related protease into S1 and S2 proteins. The S1 protein bears the receptor attachment site, and the S2 protein mediates fusion activity (15). Angiotensin-converting enzyme 2 (ACE2) is a functional receptor for SARS-CoV that binds SARS-CoV S protein with a high affinity (18, 19, 20). Several reports suggest that ACE2 is a physiologically relevant receptor during infection. Its protein expression pattern corresponds to the localization of virus infection in humans and animals (10, 35). Also, the efficiency of infection in humans and other species correlates with the ability of ACE2 in that species to support viral replication (18, 21). Structural analysis of the peptidase domain of human, palm civet, mouse, and rat ACE2 with the SARS-CoV receptor-binding domain of S1 has identified aspects of that interface that enable efficient cross-species infection and human-to-human transmission (18). Interestingly, rat ACE2 does not support infection by SARS-CoV.

The objectives of this study were to understand the pathogenesis of and develop an animal model for SARS. We found that the Frankfurt 1 isolate of SARS-CoV replicated in the respiratory tracts of F344 rats without associated clinical symptoms. We passaged the Frankfurt 1 isolate serially in young F344 rats and found that by the 10th passage, the virus was altered such that it replicated more efficiently in rats than did the original virus. Furthermore, adult rats showed more severe acute lung injury than did young rats after infection with the passaged virus. Higher levels of cytokines were seen in adult

* Corresponding author. Mailing address: Department of Pathology, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771. Fax: 81-42-561-6572. E-mail: nnagata@nih.go.jp.

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rats than in young rats after infection. Analysis of the nucleotide sequence of passaged virus encoding relevant S1 domains identified a missense mutation in the receptor binding domain. We found that this mutation is responsible for more efficient viral replication in rats. Comparative analysis of immune responses including an elevation in cytokine levels and histopathological findings in young and adult animals is crucial for understanding SARS pathogenesis.

MATERIALS AND METHODS

Viruses and cells. The SARS-CoV Frankfurt 1 isolate used here was kindly supplied by John Ziebuhr, Institute of Virology and Immunology, University of Würzburg, Würzburg, Germany. Virus was propagated twice in Vero E6 cells purchased from the American Type Culture Collection (Manassas, VA). Vero E6 cells were cultured in Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum, 50 IU of penicillin G, and 50 μg of streptomycin per ml. Titers of this stock were expressed as 50% of the tissue culture infectious dose ($TCID_{50}$), which was calculated according to the Behrens-Kärber method using Vero E6 cells. Work with infectious SARS-CoV was performed under biosafety level 3 conditions.

Experimental infection of rats with SARS-CoV. F344 rats (4-week-old females purchased from Japan SLC, Inc.) were inoculated intranasally with SARS-CoV in a volume of 100 μl into the left nostril under anesthesia using an intraperitoneal injection of 0.1 ml/10 g body weight of 1.00 mg of ketamine (Ketalar) plus 0.02 mg of xylazine. Each animal was bled under ether anesthesia and sacrificed on days 3, 5, 7, and 21 postinoculation (p.i.). Animals were housed in biosafety level 3 animal facilities. Protocols for animal experiments were approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases, Tokyo, Japan.

Serial in vivo passage of SARS-CoV in rats. The Frankfurt 1 isolate was serially passaged 10 times in 4-week-old female F344 rats. After intranasal inoculation, three rats were sacrificed on day 3 p.i. to collect bronchoalveolar wash fluids. Lungs were removed under sterile conditions, washed three times, and homogenized in 2 ml phosphate buffer containing 0.1% bovine serum albumin, 20 IU of penicillin G, 20 μl of streptomycin, and 1 μg of amphotericin B per ml. The wash fluid was then serially inoculated into F344 rats 10 times. At the 5th and 10th passage, wash fluids (F-ratV and F-ratX, respectively) were checked for virulence in rats. After 10 passages, lung homogenates were centrifuged at 2,000 rpm for 20 min, and the supernatant was used to infect Vero E6 cells. Cells were infected with 1 ml of the homogenates in 10 ml of MEM containing 2% fetal bovine serum. After 1 h of absorption, the inoculum was removed, and MEM containing 2% fetal bovine serum was added. Infected cell cultures were continuously incubated at 37°C with 5% CO_2. Cells were harvested 2 days after infection and treated once by freeze-thawing. After centrifugation at 2,000 rpm for 20 min, the supernatant was used as the virus inoculum (F-ratX-VeroE6).

Frankfurt 1, F-ratV, F-ratX, and F-ratX-VeroE6 were intranasally inoculated into 4-week-old female F344 rats. While still under ether anesthesia, the rats were bled and sacrificed by exsanguination on days 3, 5, 7, and 21 p.i., respectively. Three of the six rats were analyzed for virus replication and cytokine responses, and the other three were investigated histopathologically on each day. F-ratX-VeroE6 was similarly inoculated into adult F344 rats (7- to 8-month-old males purchased from Charles River, Inc., Japan). After intranasal inoculation with 100 μl of the virus, three adult rats were bled under ether anesthesia and killed by exsanguination on days 3, 5, and 7 p.i. to analyze virus replication and cytokine responses. Adult rats were also used for pathological examination on days 3, 5, 7, 14, and 21 p.i. Follow-up experiments were performed using 200 μl of strain F-ratX-VeroE6 using adult rats for pathological examination on days 3, 5, 7, and 14 p.i.

Virus isolation and titration. Tissue homogenates (20% [wt/vol]) from lung or maxilla including nasal cavity were prepared in MEM containing 2% fetal bovine serum, 50 IU of penicillin G, 50 μg of streptomycin, and 2.5 μg of amphotericin B per ml. Samples were clarified by centrifugation at 2,000 rpm for 20 min, and supernatants were inoculated onto VeroE6 cell cultures for virus isolation and titration.

Neutralizing antibody. Plasma samples were diluted twofold in a range from 1:10 to 1:320 with MEM containing 2% fetal bovine serum, 50 IU of penicillin G, 50 μg of streptomycin, and 2.5 μg of amphotericin B per ml. Each sample was mixed with the same volume of MEM containing SARS-CoV at an infectious dose of 100 $TCID_{50}$ per 100 μl, and the mixture was incubated for 1 h at 37°C for neutralization. After incubation, 100 μl of each sample was inoculated onto monolayers of Vero E6 cells in 96-well culture plates, which were incubated at 37°C with 5% CO_2. After 48 h, cells were examined for cytopathic effects (CPEs). The neutralizing antibody was determined as a reciprocal of the highest dilution at which a CPE was not observed.

Histopathology and immunohistochemistry. Animals were anesthetized and perfused with 10 ml of 10% phosphate-buffered formalin. Fixed tissues of lung, heart, kidney, liver, spleen, small and large intestine, brain, spinal cord, and maxilla including nasal cavity were routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Maxilla samples were decalcified in phosphate-buffered saline (PBS) (pH 7.4) plus 10% EDTA before embedding. Immunohistochemical detection of the SARS-CoV and ACE2 antigens was performed on paraffin-embedded sections. Rabbit antibodies against SARS-CoV and recombinant human ACE2 (R&D Systems, MN) were used as first antibodies. After deparaffining with xylene, sections were hydrated in ethanol and immersed in PBS. Antigens were retrieved by hydrolytic autoclaving for 20 min at 121°C in 10 mM/liter sodium citrate-sodium chloride buffer (pH 6.0). After cooling, sections were immersed in PBS. Endogenous peroxidase was blocked by 1% hydrogen peroxide in methanol for 30 min. After washing in PBS, the sections were treated with normal rabbit serum for 5 min and then incubated with antibodies against SARS-CoV or ACE2 overnight at 4°C. After three washes in PBS, the sections were incubated with biotin-conjugated anti-rabbit immunoglobulin G for 30 min at 37°C, followed by streptavidin-peroxidase for 30 min at room temperature. Peroxidase activity was developed in diaminobenzidine with hydrogen peroxide. Nuclei were counterstained by hematoxylin.

RNA extraction, RT-PCR, and sequencing. One hundred microliters of wash fluids and lung homogenates was treated with TRIzol (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions and then treated with DNase I (Promega, Madison, WI). RNA was dissolved in 20 μl RNase-free water. RNA extracted from the wash fluids and lung homogenates was used to generate cDNA. One microgram of eluted RNA samples was reverse transcribed using the ImProm-II reverse transcription (RT) system (Promega, Madison, WI) in a 20-μl reaction mixture containing 0.5 μg of random primers, 0.5 mM of deoxynucleoside triphosphates, 30 units of rRNasin RNase inhibitor, and 4 mM of $MgCl_2$. Mixtures were annealed at 25°C for 5 min and then incubated at 42°C for 60 min for extension, followed by heat inactivation at 70°C for 15 min. Reverse-transcribed products were stored at −20°C. A primer pair targeting the ORF7b region of SARS-CoV sequences (27415P [5′-CTCTTGCTGACAATAAAT-3′] and 27790N [5′-GAGAAGTTTCATGTTCGT-3′]) was used to detect deletion mutants. For PCR, 2.0 μl of cDNA was amplified in a 50-μl reaction mixture containing 0.2 μM each of forward and reverse primers and a high-fidelity PCR Master kit (Roche Diagnostics, Indianapolis, IN). PCR was performed as follows: (i) 4 min at 94°C and then (ii) 35 cycles, with 1 cycle consisting of 1 min at 94°C, 90 s at 55°C, and 2 min at 72°C. Products were analyzed by agarose gel electrophoresis. Water controls were included in each assay, and no false positives were observed in negative-control reactions.

In subsequent experiments, four pairs of primers targeted to spike coding region sequences (nucleotides 20751 to 26610) were used to analyze the virus genome from lung homogenates. Regions and primers were as follows: PCR15-2 (5′-AATACACCTACTTTAGCTGTACCCTACAAC-3′) and Sr10 (5′-ATCAC CGACTGTGACTTG-3′) for region 1, Seq51 (5′-TTGTCCGTGGTTGGGTT TTTGG-3′) and PCR16R (5′-GTAATAAAGAAACTGTATGGTAACTAGC AC-3′) for region 2, PCR17 (5′-CAGCTTGGCGCATATATTCTACTGGAAA C-3′) and Sr4 (5′-CCATTGAACTTCTGCGCA-3′) for region 3, and Sf7 (5′-C CTGACCCTCTAAAGCCA-3′) and PCR18R (5′-TCTGTAGACAACAGCAA GCACAAAACAAGC-3′) for region 4. For PCR, 1.0 μl of cDNA was amplified in a 50-μl reaction mixture containing 0.15 μM each of forward and reverse primers using the Expand Long PCR system (Roche Diagnostics, Indianapolis, IN). PCR was performed as follows: (i) 2 min at 94°C and (ii) 40 cycles, with 1 cycle consisting of 10 s at 94°C, 30 s at 55°C, and 8 min at 68°C. Correctly sized products were purified by using a QIAquick gel extraction kit (QIAGEN GmbH, Germany) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequencing products were analyzed by using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA). Both sense and antisense sequences of PCR products were sequenced at least once.

Cytokine multiplex analysis. Samples of sera and supernatants of 20% homogenates of lungs were analyzed for 10 cytokines (interleukin-1α [IL-1α], IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, tumor necrosis factor alpha [TNF-α], gamma interferon [IFN-γ], and granulocyte-macrophage colony-stimulating factor) with Luminex 200 (Luminex Co, Austin, TX) using a Rat Cytokine 10-plex antibody bead kit (BioSource International, Inc., Camarillo, CA) according to the manufacturer's instructions. Both samples were subjected to UV irradiation for 10 min and stored at −80°C. Homogenized lung tissue samples were diluted

1:1 in cell extraction buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, and 0.5% deoxycholate; BioSource International, Inc., Camarillo, CA) for 30 min on ice with vortexing at 10-min intervals and centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were diluted 1:5 in assay diluent and assayed. Multiplex beads were vortexed and sonicated for 30 s, and 25 μl was added to each well of a 96-well filter plate and washed twice with wash buffer. Samples were diluted 1:2 with assay diluent and loaded onto a Millipore Multiscreen BV 96-well filter plate to which 50 μl of incubation buffer had been added to each well. Serial dilutions of cytokine standards were prepared in parallel and added to the plate. Samples were incubated on a plate shaker in the dark at room temperature for 2 h. The plate was applied to a Millipore Multiscreen vacuum manifold and washed twice with 200 μl wash buffer, and 100 μl of biotinylated anti-rat multicytokine detector antibody was added to each well. The plate was shaken again as described above for 1 h, applied to a Millipore Multiscreen vacuum manifold, and washed twice with 200 μl wash buffer. One hundred microliters of streptavidin R-phycoerythrin was added directly to each well, and the plate was shaken again as described above for 30 min, applied to the vacuum manifold, and washed twice. One hundred microliters of wash buffer was added to each well, and the plate was shaken for 3 min. The assay plate was analyzed using the Bio-Plex Luminex 100 XYP instrument. Cytokine concentrations were calculated using Bio-Plex Manager 3.0 software with a five-parameter curve-fitting algorithm applied for standard curve calculations.

Infection of rat ACE2-expressing CHO cells with in vivo-passaged SARS-CoV. Rat ACE2 cDNA was amplified by PCR from reverse-transcribed rat kidney RNA using primers mACE2f2 (5′-TTGCTCAGTGGATGGGATCTTGGC-3′) and ratACE2r1 (5′-GCATACAGTAAAATGACGACGAGTG-3′) and cloned into a pcDNA 3.1(+) vector (Invitrogen, Grand Island, NY). A variant rat ACE2 gene with amino acid residues 82 to 84 (NYS) altered to residues corresponding to human ACE2 (MYP) was generated as described previously by Li et al. (20) and cloned into pcDNA 3.1(+). CHO cells were transfected with plasmids encoding either form. Cells were infected with the Frankfurt 1 isolate or F-ratX-VeroE6 at a multiplicity of infection of 0.002, and culture supernatants were harvested 72 h p.i. for virus titration.

Molecular modeling of a complex of rat-passaged SARS-CoV spike protein and rat ACE2. To predict the three-dimensional (3-D) structure of the receptor binding domain of rat-passaged SARS-CoV spike protein complexed with rat ACE2, we used the crystal structure of the receptor binding domain of SARS-CoV spike protein/human ACE2 complex at a 2.9-Å resolution (Protein Data Bank accession number 2AJF) (18) as a template for homology modeling. 3-D models were constructed independently by a homology modeling technique using MOE-Align and MOE-Homology in the Molecular Operating Environment (MOE) (Chemical Computing Group Inc., Canada) as described previously (11). 3-D structures were thermodynamically optimized by energy minimization using MOE and an AMBER99 force field (29). Physically unacceptable local structures of optimized 3-D models were further refined using the Ramachandran plot program packaged in MOE.

Statistical analysis. All data were analyzed by Student's *t* test.

RESULTS

Enhanced virulence of SARS-CoV after serial in vivo passage in rats. Three days after intranasal inoculation with the Frankfurt 1 isolate of SARS-CoV, histopathological lesions in lungs of young F344 rats (4-week-old females, three rats per group) were mild, and virus antigen-positive cells were rarely seen (Fig. 1A). During 10 serial passages of the Frankfurt 1 isolate, the virus was consistently identified in the maxillar tissue, including the nasal cavity, lung tissue, and lung fluids (Fig. 1B). In nasal wash fluids, infectious virus was not detectable after the fourth passage.

It has been reported that a variant of the Frankfurt 1 isolate emerged in which 45 nucleotides (nucleotides 27670 to 27714) are deleted within ORF7b upon replication in cell culture (36). Thus, the Frankfurt 1 isolate used in the present study was a mixture of the original virus without the deletion and a variant carrying the deletion (Fig. 1C). A variant with the deletion in the ORF7b region was detected in nasal and lung washes of

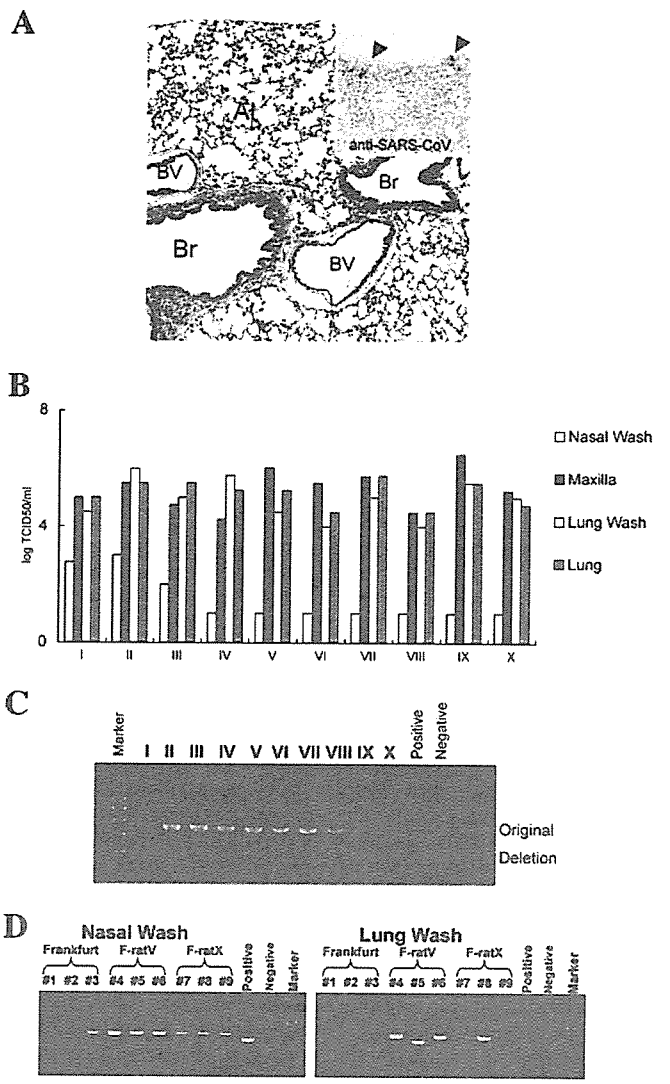

FIG. 1. Experimental infection and serial in vivo passages of SARS-CoV in young F344 rats (4-week-old females; $n = 3$). (A) Hematoxylin- and eosin-stained tissue section of lung. After intranasal inoculation with the Frankfurt 1 isolate, no inflammatory reaction in bronchi was observed. A few virus-positive cells in bronchi were seen by immunohistochemistry using SARS-CoV-specific antibody (inset, arrowheads). Br, bronchi; Al, alveoli; BV, blood vessel (magnification, ×20; inset magnification, ×40). (B) Virus titers were detected in the maxilla including nasal cavity and lung tissue homogenates and lung wash fluids following 10 serial passages. The detection limit was $10^{1.5}$ $TCID_{50}$/g of tissue. (C) DNA of serially passaged virus was amplified by RT-PCR of lung wash fluids using primers specific for ORF7b-encoding cDNA. No ORF7b deletion mutant was replicated during the serial passage. Positive indicates that the Frankfurt 1 isolate was amplified by RT-PCR; negative indicates that distilled water was used as a negative control. (D) Viral DNA was amplified by RT-PCR of nasal and lung wash fluids on day 3 p.i. using ORF7b primers. A 45-nucleotide in-frame deletion in ORF7b was detected in nasal and lung wash fluid from one animal (animal 3) and two animals (animals 1 and 2) after infection with the Frankfurt 1 isolate, respectively. F-ratV and F-ratX virus were passaged 5 and 10 times serially, respectively. The same positive and negative controls were used in C.

Frankfurt 1-infected rats; however, during serial passage in vivo, that variant was not detected (Fig. 1C and D). Replication and pathogenicity of the 5th and 10th serially passaged viruses (referred to as F-ratV and F-ratX, respectively) in the