JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2005, p. 3718–3726 Vol. 43, No. 8
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.8.3718–3726.2005

Identification of Immunodominant Epitopes on the Membrane Protein of the Severe Acute Respiratory Syndrome-Associated Coronavirus

Yuxian He,[1] Yusen Zhou,[2] Pamela Siddiqui,[1] Jinkui Niu,[1] and Shibo Jiang[1]*

Viral Immunology Laboratory, Lindsley F. Kimball Research Institute, New York Blood Center, New York, New York 10021,[1] and Department of Molecular Biology, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, Peoples Republic of China[2]

Received 29 December 2004/Returned for modification 20 February 2005/Accepted 25 April 2005

Similar to other coronaviruses, the membrane (M) protein of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is a major transmembrane glycoprotein with multiple biological functions. To date, limited information is available about its antigenic properties. In this study, we identified two major immunodominant epitopes on the M protein located in the extreme N-terminal region (residues 1 to 31) and the interior C-terminal region (residues 132 to 161), respectively, by Pepscan analyses against convalescent-phase sera from SARS patients and antisera from virus-immunized mice and rabbits. Synthetic peptides M1-31 derived from the N-terminal epitope and M132-161 derived from the C-terminal epitope were highly reactive with all of the convalescent-phase sera from 40 SARS patients but not with 30 control serum samples from healthy blood donors, suggesting their potential application for serologic diagnosis of SARS. We showed that both peptides (M1-31 and M132-161) were able to induce high titers of antibody responses in the immunized rabbits, highlighting their antigenicity and immunogenicity. These findings provide important information for developing SARS diagnostics and vaccines.

In November 2002, a new infectious pneumonia, now known as severe acute respiratory syndrome (SARS), emerged in China and rapidly spread to 29 countries (19, 25, 34). More than 8,000 people were infected, and ca. 900 died during the outbreak in 2003 (www.who.int). A novel coronavirus (SARS-CoV) was identified as the etiological agent of SARS, and its genome was subsequently characterized (6, 17, 22, 28). Although the global outbreak of SARS was contained, serious concerns remain over its reemergence in the future (9). Development of reliable diagnostics and vaccines is still a priority to control a new SARS epidemic.

Phylogenetic analyses demonstrate that SARS-CoV is distinct from the three known antigenic groups of coronaviruses (22, 28). With a genomic organization similar to those of other coronaviruses, the large, positive-stranded RNA genome of SARS-CoV encodes four major viral structural proteins: the spike (S), membrane (M), envelope (E), nucleocapsid (N) proteins. The M gene, together with the genes for the other structural proteins, is located in the 3′ one-third of the viral genome, downstream from the S gene and upstream from the N gene, and encodes a glycoprotein with a predicted length of 221 amino acids. Recently, a number of studies suggest that the S protein of SARS-CoV is a promising antigen for developing SARS vaccines since it can mediate protective immunity (1–3, 12, 42) and that the N protein may serve as an ideal antigen for SARS diagnosis since it can induce an appreciable antibody response in infected SARS patients (14, 16, 20, 30). However, little information is available on the immune response to the M protein of SARS-CoV.

The M protein of coronavirus is the most abundant glycoprotein in the virus particle (29). The interaction between the M protein and the S protein, as well as the N protein, is essential for viral assembly and budding (29). The structure of M protein is characterized as having three domains: a short N-terminal ectodomain, a triple-spanning transmembrane domain, and a large interior C-terminal domain. It was previously demonstrated that the M proteins of coronaviruses were able to induce antibody responses in hosts infected by coronavirus or immunized by attenuated recombinant virus expressing the M protein (8, 18, 26, 35, 39). Monoclonal antibodies to the M protein of mouse hepatitis virus, a mouse coronavirus, could neutralize virus infectivity in vitro (5, 10) and protect animals against lethal virus challenge in vivo (10). These features make the M protein an attractive target for developing diagnostic tests and subunit vaccines (7, 8, 11, 18, 24, 26, 35, 37, 39), although its antigenic determinants remain to be defined. Computer-aided analyses suggest that the M protein of SARS-CoV has a structure similar to the M proteins of other coronaviruses (22, 28). It contains a short N terminus in the exterior of the virion (residues 1 to 14), three transmembrane helices (residues 15 to 37, 50 to 72, and 77 to 99), and a 121-amino-acid C-terminal region inside the virus particle (Fig. 1). In the present study, we sought to identify the immunodominant epitopes on the M protein of SARS-CoV.

MATERIALS AND METHODS

Synthetic peptides. A set of 30 overlapping peptides spanning the entire sequence of the M protein was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health. These overlapping peptides range from 15 to 20 amino acids in length. A set of peptides in Table 1 was synthesized by a standard solid-phase Fmoc (9-fluorenylmethoxy carbonyl) method in the MicroChemistry Laboratory of the New York Blood Center. Peptides were purified to homogeneity (purity of >95%) by high-performance liquid chromatography and identified by laser desorption mass spectrometry.

* Corresponding author. Mailing address: Lindsley F. Kimball Research Institute, New York Blood Center, 310 East 67th St., New York, NY 10021. Phone: (212) 570-3058. Fax: (212) 570-3099. E-mail: SJiang@NYBloodcenter.org.

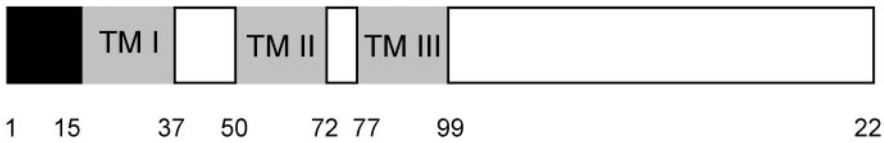

FIG. 1. Schematic diagram of SARS-CoV M protein. The M protein contains a short N terminus in the exterior of the virion (residues 1 to 14), three transmembrane helices (residues 15 to 37, 50 to 72 and 77 to 99), and a 121-amino-acid C-terminal region inside the virus particle.

Serum specimens from SARS patients. Serum samples were collected from 40 convalescent-phase SARS patients 30 to 60 days after onset of illness during the SARS outbreak in Beijing in 2003. The diagnostic criteria for SARS-CoV infection followed the clinical description of SARS released by the World Health Organization. All of the sera were verified to be positive for SARS-CoV by immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA) using commercially available diagnostic kits (Beijing Genomics Institute, Beijing, Peoples Republic of China). Sera from 30 healthy blood donors were used as controls.

Preparation of inactivated SARS-CoV. SARS coronavirus strain BJ01 (accession number AY278488) was used as viral source and propagated in Vero E6 cells (27). The infected cells were harvested and completely lysed by three cycles of freeze-thaw. β-Propiolactone was then added to the lysates at 1:2,000 ratio, followed by incubation at 37°C for 2 h. The inactivated virus was centrifuged at 10,000 rpm for 20 min. After removal of the cell debris, the supernatants were desalted with Sephadex G-50, concentrated with PEG-8000, and filtrated with Sepharose CL-2B sequentially. The inactivated SARS-CoV in the final prepara-

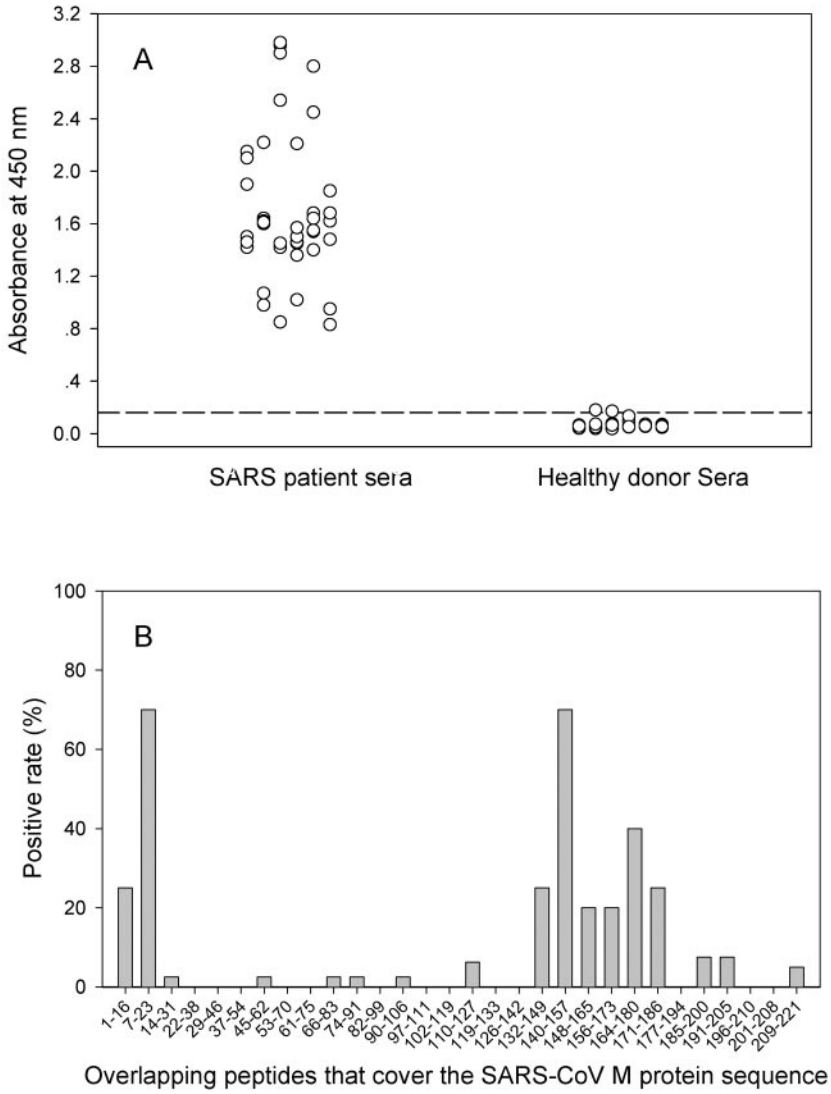

FIG. 2. Mapping of immunodominant epitopes on the M protein of SARS-CoV by ELISA. (A) Antibodies specific for SARS-CoV in the convalescent-phase sera from 40 SARS patients were measured by commercial diagnostic kit, in which the mixture of proteins purified from viral lysates of SARS-CoV was used as coating antigen. (B) Pepscan analysis against the convalescent-phase sera from 40 SARS patients with a set of 30 overlapping peptides that span the entire sequence of the M protein as coating antigens. Sera, tested at 1:50 dilution, were considered positive when the A_{450} values were above the cutoff value (mean A_{450} value of sera from health blood donors plus three standard deviations).

TABLE 1. Peptides overlapping the immunodominant epitopes of M protein

Peptide	Sequence
N-terminal peptides	
M1-16	MADNGTITVEELKQLL
M7-23	ITVEELKQLLEQWNLVI
M1-20	MADNGTITVEELKQLLEQWN
M1-23	MADNGTITVEELKQLLEQWNLVI
M4-26	NGTITVEELKQLLEQWNLVIGFL
M1-31	MADNGTITVEELKQLLEQWNLVIGFLFLAWI
C-terminal peptides	
M140-157	GAVIIRGHLRMAGHPLGR
M132-161	LMESELVIGAVIIRGHLRMAGHPLGRCDIK
M164-180	PKEITVATSRTLSYYKL
M158-185	CDIKDLPKEITVATSRTLSYYKLGASQR

tion, with >95% purity analyzed by high-pressure liquid chromatography, was confirmed by observing the coronavirus-like particles under an electron microscope and by determining the reactivity with convalescent-phase sera of SARS patients in Western blots (33).

Immunizations. BALB/c mice and New Zealand White rabbits were immunized intradermally with 10 and 30 μg, respectively, of purified β-propiolactone-inactivated viral particles as immunogen in the presence of Freund complete adjuvant and boosted with freshly prepared emulsion of the immunogen and Freund incomplete adjuvant at 2-wk intervals. Preimmune sera (preimmune) were collected before the primary immunization and antisera were collected 5 days after the third boost immunization. Rabbit antisera directed against peptides M1-31 and M132-161 were produced at Covance Research Products, Inc. (Denver, PA), by using their standard protocols. Briefly, New Zealand White rabbits were immunized intradermally with 250 μg of purified peptides resuspended in phosphate-buffered solution (pH 7.2) in the presence of Freund complete adjuvant and boosted with freshly prepared emulsion of the immunogen and Freund incomplete adjuvant at 3-week intervals. Rabbit antisera were collected 10 days after each boost. Sera were kept at 4°C before use.

ELISA. The reactivities of the M-protein-derived peptides with the convalescent-phase sera from SARS patients and the serum samples from mice and rabbits immunized with the inactivated viral particles or synthetic peptides were determined, respectively, by ELISA. Briefly, each peptide (10 μg/ml) was coated onto wells of a 96-well microtiter plate (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 2% nonfat milk, the plate was incubated with corresponding antisera at indicated dilution at 37°C for 1 h and then washed three times with PBS containing 0.1% Tween 20. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) or anti-mouse IgG or anti-rabbit IgG (Zymed, South San Francisco, CA), accordingly, at 37°C for 1 h, followed by washing. The reaction was visualized by addition of the substrate 3,3′,5,5′-tetramethylbenzidine, and the absorbance at 450 nm (A_{450}) was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC).

RESULTS

Mapping of immunodominant epitopes on the M protein in SARS patients. A total of 40 serum samples from convalescent

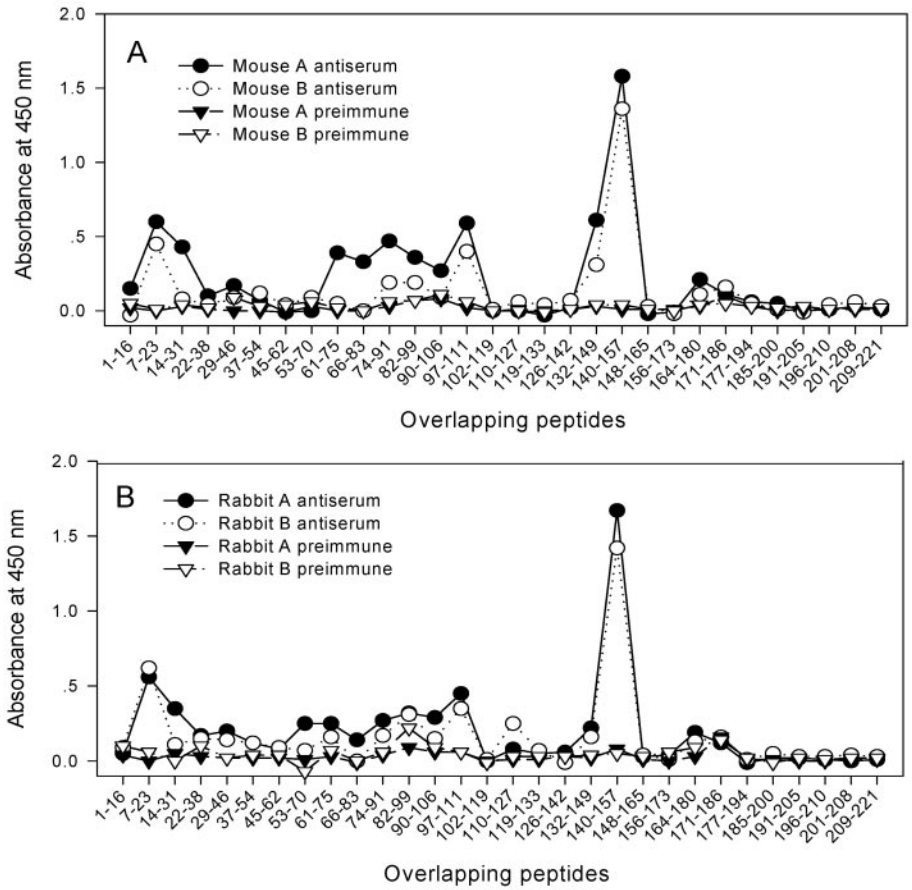

FIG. 3. Mapping of immunodominant epitopes on the M protein of SARS-CoV by Pepscan analysis against antisera from mice (A) and rabbits (B) immunized with inactivated SARS-CoV. A set of 30 overlapping peptides that span the entire sequence of the M protein were used as coating antigens in ELISA. Antisera were tested at 1:100 dilutions.

TABLE 2. Reactivity of convalescent sera from SARS patients with N-terminal or C-terminal peptides[a]

Serum	Viral proteins	Reactivity (A_{450}) with:								
		N-terminal peptides						C-terminal peptides		
		M1-16	M7-23	M1-20	M1-23	M4-26	M1-31	M140-157	M132-161	M158-185
SARS sera										
No. 1	**2.54**	0.09	**0.57**	**0.68**	**1.19**	**2.19**	**2.56**	**0.60**	**0.69**	**0.31**
No. 2	**0.85**	0.03	**0.94**	**1.55**	**2.51**	**2.12**	**1.99**	0.01	**0.67**	0.07
No. 3	**1.90**	**0.44**	**0.56**	**0.69**	**1.37**	**1.11**	**2.58**	**0.95**	**1.13**	**0.41**
No. 4	**1.62**	0.03	**0.21**	0.06	**0.46**	**0.62**	**2.36**	**0.54**	**0.76**	0.15
No. 5	**1.68**	**0.58**	**0.32**	**0.42**	**0.69**	**0.47**	**2.48**	**1.10**	**1.66**	**0.21**
No. 6	**1.57**	0.12	**0.88**	**0.58**	**1.10**	**0.77**	**2.44**	0.01	**0.90**	0.02
No. 7	**2.22**	0.08	0.04	0.14	**0.32**	**0.35**	**2.71**	**0.48**	**0.76**	**0.28**
No. 8	**1.50**	0.07	**0.25**	0.10	**0.27**	**0.34**	**1.44**	**0.47**	**0.73**	**0.20**
No. 9	**1.07**	0.11	0.10	0.18	**0.34**	0.06	**2.12**	0.07	**1.81**	0.10
No. 10	**2.21**	**0.26**	**0.30**	**0.28**	**0.27**	**0.25**	**0.85**	**0.68**	**1.16**	**0.29**
No. 11	**2.98**	0.13	**0.86**	0.09	**0.32**	**0.23**	**1.61**	0.06	**0.87**	0.17
No. 12	**2.96**	0.07	0.04	−0.01	0.03	0.11	**1.36**	**0.34**	**0.67**	0.09
No. 13	**1.68**	0.07	0.11	0.10	0.14	0.06	**1.41**	0.06	**0.85**	0.07
No. 14	**1.45**	0.08	**0.32**	0.04	0.03	0.01	**2.78**	0.07	**0.95**	0.07
No. 15	**1.42**	0.02	0.00	0.16	**0.57**	**0.34**	**2.48**	**0.52**	**0.60**	0.14
No. 16	**0.98**	0.08	0.03	0.08	0.06	0.05	**1.14**	−0.04	**0.78**	**0.45**
No. 17	**1.64**	**0.44**	**0.45**	**0.77**	**0.80**	**1.31**	**1.17**	**1.20**	**0.78**	**0.27**
No. 18	**1.48**	0.19	0.12	0.07	0.09	0.15	**2.54**	**0.50**	**0.59**	0.15
Control sera										
C-1	0.03	0.07	0.07	0.80	0.06	0.13	0.09	0.09	0.03	0.09
C-2	0.08	0.08	0.09	0.11	0.07	0.09	0.05	0.06	0.12	0.08

[a] Peptides were used at 10 μg/ml, and sera were tested at a 1:50 dilution. Positive values are in boldface.

SARS patients were collected to characterize the immunogenicity of SARS-CoV M protein. All samples were verified to be positive to SARS-CoV, as detected by ELISA with commercially available diagnostic kits with the mixture of proteins purified from viral lysates of SARS-CoV as the coating antigen (Fig. 2A). To map the immunodominant epitopes on the M protein, a set of 30 overlapping peptides that span the entire sequence of the M protein were used as coating antigens in ELISA, and serum samples were tested against each of the peptides. As shown in Fig. 2B, whereas most of the peptides did not significantly react with any of the serum samples from SARS patients, the peptide 7-23 (17-mer) from the N terminus and the peptide 140-157 (18-mer) from the C-terminal interior region reacted, respectively, with ca. 70% of serum samples. This result indicates that these two sites on the M protein contain the major immunodominant epitopes that induce antibody responses in humans. In addition, the peptide 164-180 was reactive with ca. 40% of serum samples, suggesting this site is a minor immunodominant epitope in the SARS patients.

Mapping of immunodominant epitopes on the M protein in immunized animals. Inactivated SARS-CoV, a major candidate SARS vaccine, can induce robust antibody responses in immunized animals. To map the antigenic sites of M protein, two mice and two rabbits were immunized with the inactivated SARS-CoV as immunogen, and their antisera were tested against each of the overlapping peptides derived from the entire M protein. Interestingly, both mouse and rabbit antisera significantly reacted with the N-terminal peptide 7-23 and C-terminal peptide 140-157 that were identified as immunodominant sites by human SARS sera (Fig. 3). This result suggests that these two sites also function as immunodominant epitopes in immunized mice and rabbits, highlighting the immunogenicity of M protein. In addition, peptide 97-111 reacted weakly with the mouse and rabbit antisera but not with sera of SARS patient, suggesting that this region may serve as a specific antigenic site in animals.

Identification of highly immunoreactive peptides for serologic diagnosis of SARS. Pepscan analyses against the convalescent-phase sera from SARS patients and the antisera from immunized animals revealed the N-terminal and C-terminal immunodominant epitopes on the M protein. To further characterize their epitopic structure, we designed a set of ten peptides that overlap with the N-terminal or C-terminal antigenic sequence as probes (Table 1). These peptides were used as coating antigens in ELISA to test their reactivity with 18 randomly selected serum samples from SARS patients. Strikingly, two longer peptides, M1-31 overlapping with the N-terminal epitope and M132-161 overlapping with the C-terminal epitope, were highly reactive with all of the SARS serum samples tested (Table 2). The peptides M1-23 and M4-26 had better reactivities with SARS sera than the peptide M7-23, which was used in Pepscan analyses, suggesting that the extended sequences at both N and C termini of the M7-23 (Table 1) are important to the constitution of the N-terminal immunodominant epitope; however, the reactivity of peptides was significantly improved by adding more residues at the C terminus of the peptide. These data suggest that both of the N-terminal and C-terminal immunodominant epitopes in the M protein may require longer sequences to maintain the integrity of their antigenic conformations.

The antigenicity of synthetic peptides was further evaluated by measuring their reactive titers to antiserum samples. As shown in Fig. 4, the peptides M1-31 and M132-161 reacted

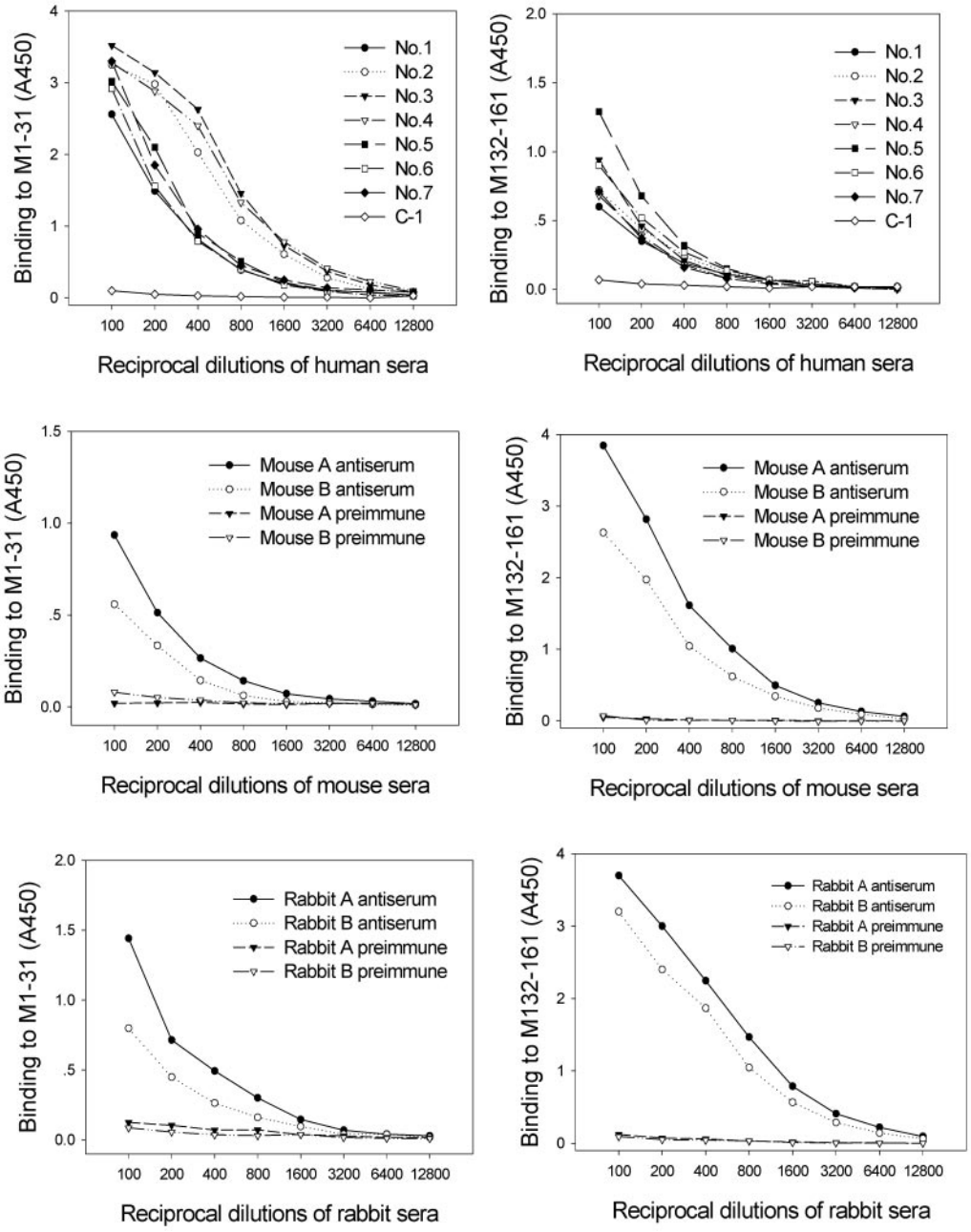

FIG. 4. Immunoreactivity of synthetic peptides (M1-31 and M132-161) derived from the N-terminal or C-terminal immunodominant epitopes on the M protein with the convalescent-phase sera from 40 SARS patients and antisera from immunized animals. Serum samples were tested as a series of twofold dilutions by ELISA.

with seven serum samples from SARS patients with mean endpoint titers of 1:3,628 and 1:1,020, respectively. The peptide M1-31 reacted with the mouse or rabbit antisera with mean titers at 1:1,460 or 1:1,788, respectively, whereas the peptide M132-161 reacted with the mouse or rabbit antisera with mean titers at 1:6,400 or 1:8,505, respectively. Obviously, peptide M1-31 had higher reactive titers with SARS patient sera but lower titers with mouse or rabbit antisera than M132-161 had. In contrast, the M132-161 strongly reacted with the animal antisera but not the patient sera. This result suggests that the N-terminal epitope is more immunogenic in infected patients but less immunogenic in immunized animals than the C-terminal epitope.

To develop immunoassays for SARS diagnosis, peptides M1-31 and M132-161 were used, respectively, as antigens for ELISA to detect site-specific antibodies in serum samples from SARS patients. As shown in Fig. 5, both peptides significantly reacted with each of the 40 patient sera, but none of the control sera from healthy blood donors. Notably, the peptide M1-31 had higher reactivity in ELISA than peptide M132-161.

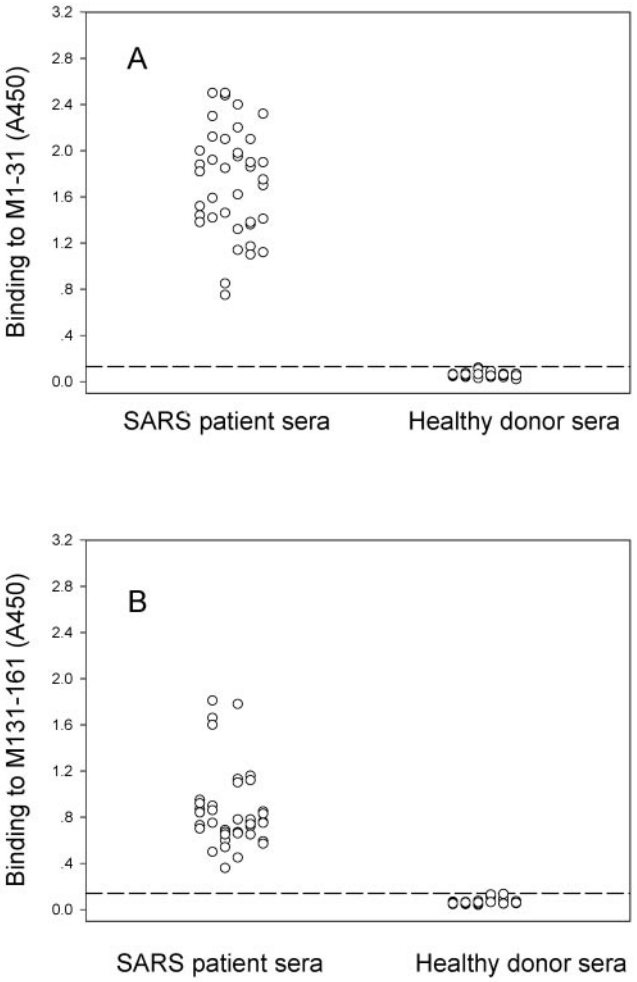

FIG. 5. Detection of M protein-specific antibodies in the convalescent-phase sera of SARS patients with synthetic peptides derived from the N-terminal epitope (A) and C-terminal epitope (B), respectively. Sera from 40 SARS patients and 30 healthy blood donors were tested at 1:50 dilutions. The dashed lines represent the cutoff values (mean A_{450} value of sera from health blood donors plus three standard deviations).

This result further indicates that these two peptides have potential to be developed as specific diagnostic antigens.

Synthetic peptides induced potent antibody responses in the rabbits. To evaluate the immunogenicity of synthetic peptides derived from the immunodominant epitopes of M protein, M1-31 and M132-161 were used as immunogens for the immunization of rabbits. Strikingly, the rabbits developed high titers of antibody responses after the first boosted immunization for both peptides and retained similar levels after the second and the third boosts. After the first boost, the endpoint titers of antisera from two rabbits immunized with M1-31 were 1:102,400 and 1:1,638,400, respectively (Fig. 6A and B), whereas those against M131-161 were 1: 26,214,400 and 1:6,553,600, respectively (Fig. 6C and D). This result indicated that both the N-terminal and C-terminal peptides were highly immunogenic in the immunized rabbits; however, the peptide M132-161 induced more potent antibody responses than the peptide M1-31, a finding consistent with the site-specific antibody responses in the rabbits immunized with the inactivated SARS-CoV.

DISCUSSION

Postgenomic characterization of SARS-CoV is important for exploring the mysteries of SARS. We sought to characterize the antigenicity and immunogenicity of SARS-CoV since this work is critical for developing effective SARS diagnostics and vaccines. Recently, we demonstrated that the S and N proteins, two major structural proteins of SARS-CoV, contain several immunodominant sites that induce appreciable antibody responses during viral infection and immunization (14, 15). We also found that the receptor-binding domain in S protein is a potent inducer of neutralizing antibodies (12, 13). In the present study, we focused on the M protein of SARS-CoV since little is known about its antigenic properties. Two immunodominant epitopes on the M protein were localized at the extreme N terminus (residues 1 to 31) and C-terminal region (residues 132 to 161), respectively, by Pepscan analysis against the convalescent-phase sera from SARS patients and the antisera from animals immunized with inactivated SARS-CoV. We showed that that the N-terminal epitope is more immunogenic in the infected patients but less immunogenic in the immunized animals than the epitope at the C-terminal region, highlighting the antigenic heterogenicity of SARS-CoV in infection and immunization. This finding is important for understanding the antigenicity and immunogenicity of SARS-CoV M protein.

The M protein of coronavirus is a peculiar glycoprotein, different from all other viral glycoproteins in its structural and biological features. Several lines of evidence indicated that the M protein, even largely buried within the viral membrane, was able to induce antibody responses during infection or immunization (8, 18, 26, 35, 39). Although the antigenic sites on the M protein of coronavirus have not been defined, it is presumed that its N-terminal hydrophilic ectodomain contains the major antigenic determinants that are responsible for immunological reactions (29). The exposed N-terminal region of transmissible gastroenteritis virus M protein has been shown to be an inducer of alpha interferon in lymphocytes, although the significance of alpha interferon action for immunity to viral infection has not been defined (4). Therefore, the discovery of immunodominant epitopes on the M protein of SARS-CoV will facilitate our understanding to the immunogenicity of M proteins from other coronaviruses.

Early recognition of SARS cases is critical in controlling future outbreaks. However, early diagnosis of SARS-CoV infection still mainly relies on a combination of clinical and epidemiologic features due to the lack of sensitive and specific laboratory tests. Since the emergency of the SARS outbreak in 2003, whole-virus or recombinant protein-based immunoassays have been developed for SARS diagnosis (14, 15, 20, 21, 38). However, there is serious concern over the sensitivity and specificity of these assays since SARS-CoV shares high sequence similarity with other coronaviruses. Recent studies have demonstrated that there exists antigenic cross-reactivity between SARS-CoV and other coronaviruses (17, 31, 40, 41). Woo et al. (41) reported that false-positive results were obtained from a recombinant SARS-CoV nucleocapsid protein-

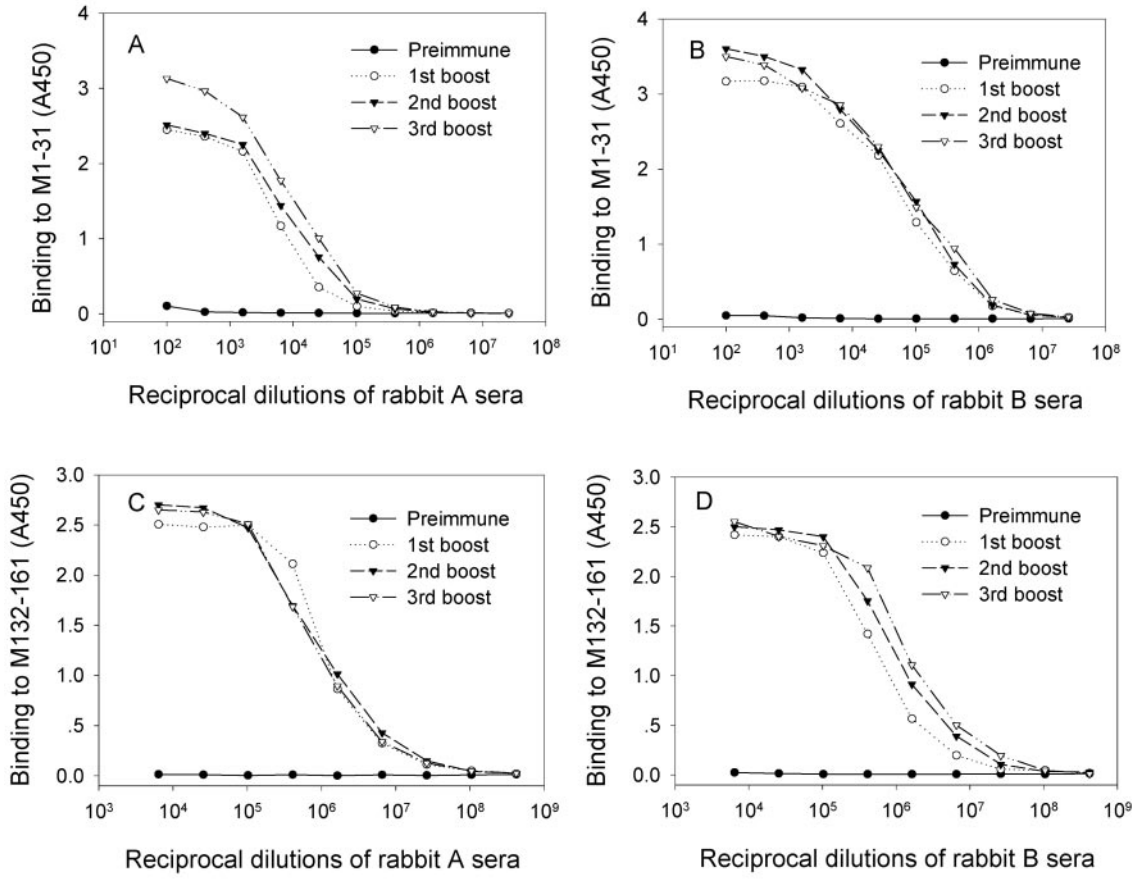

FIG. 6. Antibody responses in rabbits immunized with synthetic peptides derived from the N-terminal epitope (A and B) and C-terminal epitope (C and D), respectively. Rabbit sera were tested as a series of fourfold dilutions by ELISA.

based ELISA due to the cross-reactivity of SARS-CoV antigens with the antibodies against HCoV-OC43 and HCoV-229E. Prior experiences with the diagnosis of viral infection suggest that antigenic peptides may serve as ideal antigens for serologic detection of SARS-CoV infection. Wang et al. (36) tested several synthetic peptides derived from the M protein for their immunoreactivity with the serum samples from SARS patients and showed that a short peptide overlapping with the C-terminal epitope (residues 137 to 158) reacted weakly with most of the tested sera. In the present study, we found that synthetic peptides corresponding to the N-terminal or C-terminal epitopes of M protein (M1-31 and M132-161) were highly reactive with the convalescent-phase sera from SARS patients but did not react with the control sera from healthy blood donors, suggesting their potential application for serologic diagnosis of SARS. Furthermore, sequence alignment indicates that the full-length of SARS-CoV M protein has a sequence similarity of 41% with another human coronavirus (HCoV-OV43) (11), but the sequences of the N-terminal and the C-terminal epitopic sites are highly specific to SARS-CoV. This suggests that the synthetic peptides M1-31 and M132-161 can be used for specific detection of SARS-CoV infection.

Inactivated SARS-CoV is a major candidate for SARS vaccines and has been tested in clinical trials (13, 23, 32). However, its antigenic properties have not been clearly characterized. We previously showed that the inactivated SARS-CoV was able to induce high titers of antibody responses against the S and N proteins (13–15). Here, we further demonstrated that this vaccine also elicited high titers of antibodies against the M protein, especially against its N-terminal and C-terminal antigenic sites, in immunized mice and rabbits, suggesting that the M protein is a highly immunogenic component in an inactivated SARS vaccine preparation. It is important to study whether or not the anti-M antibodies can mediate neutralizing activity against SARS-CoV. The M protein of feline infectious peritonitis virus, a coronavirus, has been shown to be an inducer of protective immunity (35). Most recently, it was reported that a recombinant protein containing the interior domain (residues 106 to 221) of SARS-CoV M protein was able to induce protective humoral responses (24). We also showed that synthetic peptides (M1-31 and M132-161) derived from the immunodominant epitopes induced potent antibody responses in the immunized rabbits, highlighting the immunogenicity of M protein. Importantly, if these epitope-specific antibodies possess neutralizing activity to SARS-CoV, it will provide important information for designing a SARS subunit vaccine. The immunodominant sequences that elicit neutralizing antibodies should be included in a candidate subunit vaccine, whereas the immunodominant sequences that induce

non-neutralizing or enhancing antibodies should be eliminated from any potential vaccines.

REFERENCES

1. **Bisht, H., A. Roberts, L. Vogel, A. Bukreyev, P. L. Collins, B. R. Murphy, K. Subbarao, and B. Moss.** 2004. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. Proc. Natl. Acad. Sci. USA **101:**6641–6646.
2. **Buchholz, U. J., A. Bukreyev, L. Yang, E. W. Lamirande, B. R. Murphy, K. Subbarao, and P. L. Collins.** 2004. Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. Proc. Natl. Acad. Sci. USA **101:**9804–9809.
3. **Bukreyev, A., E. W. Lamirande, U. J. Buchholz, L. N. Vogel, W. R. Elkins, M. St. Claire, B. R. Murphy, K. Subbarao, and P. L. Collins.** 2004. Mucosal immunization of African green monkeys (*Cercopithecus aethiops*) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. Lancet **363:**2122–2127.
4. **Charley, B., and H. Laude.** 1988. Induction of alpha interferon by transmissible gastroenteritis coronavirus: role of transmembrane glycoprotein E1. J. Virol. **62:**8–11.
5. **Collins, A. R., R. L. Knobler, H. Powell, and M. J. Buchmeier.** 1982. Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology **119:**358–371.
6. **Drosten, C., S. Gunther, W. Preiser, W. S. van der, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kolesnikova, R. A. Fouchier, A. Berger, A. M. Burguiere, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Muller, V. Rickerts, M. Sturmer, S. Vieth, H. D. Klenk, A. D. Osterhaus, H. Schmitz, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
7. **Elia, G., G. Fiermonte, A. Pratelli, V. Martella, M. Camero, F. Cirone, and C. Buonavoglia.** 2003. Recombinant M protein-based ELISA test for detection of antibodies to canine coronavirus. J. Virol. Methods **109:**139–142.
8. **Fiscus, S. A., and Y. A. Teramoto.** 1987. Antigenic comparison of feline coronavirus isolates: evidence for markedly different peplomer glycoproteins. J. Virol. **61:**2607–2613.
9. **Fleck, F.** 2004. SARS virus returns to China as scientists race to find effective vaccine. Bull. W. H. O. **82:**152–153.
10. **Fleming, J. O., R. A. Shubin, M. A. Sussman, N. Casteel, and S. A. Stohlman.** 1989. Monoclonal antibodies to the matrix (E1) glycoprotein of mouse hepatitis virus protect mice from encephalitis. Virology **168:**162–167.
11. **Han, X., M. Bartlam, Y. H. Jin, X. Liu, X. He, X. Cai, Q. Xie, and Z. Rao.** 2004. The expression of SARS-CoV M gene in P. Pastoris and the diagnostic utility of the expression product. J. Virol. Methods **122:**105–111.
12. **He, Y., Y. Zhou, S. Liu, Z. Kou, W. Li, M. Farzan, and S. Jiang.** 2004. Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. Biochem. Biophys. Res. Commun. **324:**773–781.
13. **He, Y., Y. Zhou, P. Siddiqui, and S. Jiang.** 2004. Inactivated SARS-CoV vaccine elicits high titers of spike protein-specific antibodies that block receptor binding and virus entry. Biochem. Biophys. Res. Commun. **325:**445–452.
14. **He, Y., Y. Zhou, H. Wu, Z. Kou, S. Liu, and S. Jiang.** 2004. Mapping of antigenic sites on the nucleocapsid protein of the severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. **42:**5309–5314.
15. **He, Y., Y. Zhou, H. Wu, B. Luo, J. Chen, W. Li, and S. Jiang.** 2004. Identification of immunodominant sites on the spike protein of severe acute respiratory syndrome (SARS) coronavirus: implication for developing SARS diagnostics and vaccines. J. Immunol. **173:**4050–4057.
16. **Huang, L. R., C. M. Chiu, S. H. Yeh, W. H. Huang, P. R. Hsueh, W. Z. Yang, J. Y. Yang, I. J. Su, S. C. Chang, and P. J. Chen.** 2004. Evaluation of antibody responses against SARS coronaviral nucleocapsid or spike proteins by immunoblotting or ELISA. J. Med. Virol. **73:**338–346.
17. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
18. **Laude, H., J. M. Chapsal, J. Gelfi, S. Labiau, and J. Grosclaude.** 1986. Antigenic structure of transmissible gastroenteritis virus. I. Properties of monoclonal antibodies directed against virion proteins. J. Gen. Virol. **67**(Pt. 1)**:**119–130.
19. **Lee, N., D. Hui, A. Wu, P. Chan, P. Cameron, G. M. Joynt, A. Ahuja, M. Y. Yung, C. B. Leung, K. F. To, S. F. Lui, C. C. Szeto, S. Chung, and J. J. Sung.** 2003. A major outbreak of severe acute respiratory syndrome in Hong Kong. N. Engl. J. Med. **348:**1986–1994.
20. **Liu, X., Y. Shi, P. Li, L. Li, Y. Yi, Q. Ma, and C. Cao.** 2004. Profile of antibodies to the nucleocapsid protein of the severe acute respiratory syndrome (SARS)-associated coronavirus in probable SARS patients. Clin. Diagn. Lab. Immunol. **11:**227–228.
21. **Lu, L., I. Manopo, B. P. Leung, H. H. Chng, A. E. Ling, L. L. Chee, E. E. Ooi, S. W. Chan, and J. Kwang.** 2004. Immunological characterization of the spike protein of the severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. **42:**1570–1576.
22. **Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper.** 2003. The genome sequence of the SARS-associated coronavirus. Science **300:**1399–1404.
23. **Marshall, E., and M. Enserink.** 2004. Medicine: caution urged on SARS vaccines. Science **303:**944–946.
24. **Pang, H., Y. Liu, X. Han, Y. Xu, F. Jiang, D. Wu, X. Kong, M. Bartlam, and Z. Rao.** 2004. Protective humoral responses to severe acute respiratory syndrome-associated coronavirus: implications for the design of an effective protein-based vaccine. J. Gen. Virol. **85:**3109–3113.
25. **Poutanen, S. M., D. E. Low, B. Henry, S. Finkelstein, D. Rose, K. Green, R. Tellier, R. Draker, D. Adachi, M. Ayers, A. K. Chan, D. M. Skowronski, I. Salit, A. E. Simor, A. S. Slutsky, P. W. Doyle, M. Krajden, M. Petric, R. C. Brunham, and A. J. McGeer.** 2003. Identification of severe acute respiratory syndrome in Canada. N. Engl. J. Med. **348:**1995–2005.
26. **Pulford, D. J., and P. Britton.** 1991. Expression and cellular localization of porcine transmissible gastroenteritis virus N and M proteins by recombinant vaccinia viruses. Virus Res. **18:**203–217.
27. **Qin, E., Q. Zhu, M. Yu, B. Fan, G. Chang, B. Si, B. Yang, W. Peng, T. Jiang, B. Liu, Y. Deng, H. Liu, Y. Zhang, C. Wang, Y. Li, Y. Gan, X. Li, F. Lu, G. Tan, W. Cao, and R. Yang.** 2003. A complete sequence and comparative analysis of a SARS-associated virus (isolate BJ01). Chin. Sci. Bull. **48:**941–948.
28. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, S. Penaranda, B. Bankamp, K. Maher, M. H. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. Peret, C. Burns, T. G. Ksiazek, P. E. Rollin, A. Sanchez, S. Liffick, B. Holloway, J. Limor, K. McCaustland, M. Olsen-Rasmussen, R. Fouchier, S. Gunther, A. D. Osterhaus, C. Drosten, M. A. Pallansch, L. J. Anderson, and W. J. Bellini.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394–1399.
29. **Rottier, P.** 1995. The coronavirus membrane glycoprotein, p. 73–114. *In* S. G. Siddell (ed.), The *Coronaviridae*. Plenum Press, Inc., New York, N.Y.
30. **Shi, Y., Y. Yi, P. Li, T. Kuang, L. Li, M. Dong, Q. Ma, and C. Cao.** 2003. Diagnosis of severe acute respiratory syndrome (SARS) by detection of SARS coronavirus nucleocapsid antibodies in an antigen-capturing enzyme-linked immunosorbent assay. J. Clin. Microbiol. **41:**5781–5782.
31. **Sun, Z. F., and X. J. Meng.** 2004. Antigenic cross-reactivity between the nucleocapsid protein of severe acute respiratory syndrome (SARS) coronavirus and polyclonal antisera of antigenic group I animal coronaviruses: implication for SARS diagnosis. J. Clin. Microbiol. **42:**2351–2352.
32. **Takasuka, N., H. Fujii, Y. Takahashi, M. Kasai, S. Morikawa, S. Itamura, K. Ishii, M. Sakaguchi, K. Ohnishi, M. Ohshima, S. Hashimoto, T. Odagiri, M. Tashiro, H. Yoshikura, T. Takemori, and Y. Tsunetsugu-Yokota.** 2004. A subcutaneously injected UV-inactivated SARS coronavirus vaccine elicits systemic humoral immunity in mice. Int. Immunol. **16:**1423–1430.
33. **Tang, L., Q. Zhu, E. Qin, M. Yu, Z. Ding, H. Shi, X. Cheng, C. Wang, G. Chang, Q. Zhu, F. Fang, H. Chang, S. Li, X. Zhang, X. Chen, J. Yu, J. Wang, and Z. Chen.** 2004. Inactivated SARS-CoV vaccine prepared from whole virus induces a high level of neutralizing antibodies in BALB/c mice. DNA Cell Biol. **23:**391–394.
34. **Tsang, K. W., P. L. Ho, G. C. Ooi, W. K. Yee, T. Wang, M. Chan-Yeung, W. K. Lam, W. H. Seto, L. Y. Yam, T. M. Cheung, P. C. Wong, B. Lam, M. S. Ip, J. Chan, K. Y. Yuen, and K. N. Lai.** 2003. A cluster of cases of severe acute respiratory syndrome in Hong Kong. N. Engl. J. Med. **348:**1977–1985.
35. **Vennema, H., R. J. de Groot, D. A. Harbour, M. C. Horzinek, and W. J. Spaan.** 1991. Primary structure of the membrane and nucleocapsid protein genes of feline infectious peritonitis virus and immunogenicity of recombinant vaccinia viruses in kittens. Virology **181:**327–335.
36. **Wang, J., J. Wen, J. Li, J. Yin, Q. Zhu, H. Wang, Y. Yang, E. Qin, B. You, W. Li, X. Li, S. Huang, R. Yang, X. Zhang, L. Yang, T. Zhang, Y. Yin, X. Cui, X. Tang, L. Wang, B. He, L. Ma, T. Lei, C. Zeng, J. Fang, J. Yu, J. Wang, H. Yang, M. B. West, A. Bhatnagar, Y. Lu, N. Xu, and S. Liu.** 2003. Assessment

of immunoreactive synthetic peptides from the structural proteins of severe acute respiratory syndrome coronavirus. Clin. Chem. **49:**1989–1996.
37. **Wang, L. F., A. R. Gould, and P. W. Selleck.** 1997. Expression of equine morbillivirus (EMV) matrix and fusion proteins and their evaluation as diagnostic reagents. Arch. Virol. **142:**2269–2279.
38. **Wang, Y. D., Y. Li, G. B. Xu, X. Y. Dong, X. A. Yang, Z. R. Feng, C. Tian, and W. F. Chen.** 2004. Detection of antibodies against SARS-CoV in serum from SARS-infected donors with ELISA and Western blot. Clin. Immunol. **113:** 145–150.
39. **Wesseling, J. G., G. J. Godeke, V. E. Schijns, L. Prevec, F. L. Graham, M. C. Horzinek, and P. J. Rottier.** 1993. Mouse hepatitis virus spike and nucleocapsid proteins expressed by adenovirus vectors protect mice against a lethal infection. J. Gen. Virol. **74**(Pt. 10)**:**2061–2069.
40. **Woo, P. C., S. K. Lau, H. W. Tsoi, K. H. Chan, B. H. Wong, X. Y. Che, V. K. Tam, S. C. Tam, V. C. Cheng, I. F. Hung, S. S. Wong, B. J. Zheng, Y. Guan, and K. Y. Yuen.** 2004. Relative rates of non-pneumonic SARS coronavirus infection and SARS coronavirus pneumonia. Lancet **363:**841–845.
41. **Woo, P. C., S. K. Lau, B. H. Wong, K. H. Chan, W. T. Hui, G. S. Kwan, J. S. Peiris, R. B. Couch, and K. Y. Yuen.** 2004. False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OC43 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide. J. Clin. Microbiol. **42:**5885–5888.
42. **Yang, Z. Y., W. P. Kong, Y. Huang, A. Roberts, B. R. Murphy, K. Subbarao, and G. J. Nabel.** 2004. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature **428:**561–564.