CLINICAL AND VACCINE IMMUNOLOGY, Feb. 2007, p. 146–149 Vol. 14, No. 2
1556-6811/07/$08.00+0 doi:10.1128/CVI.00360-06

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]

Received 2 October 2006/Returned for modification 15 November 2006/Accepted 20 December 2006

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.

▽ Published ahead of print on 3 January 2007.

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

This study was supported in part by a grant for research on emerging and reemerging infectious diseases from the Ministry of Health, Welfare, and Labor of Japan and the 21st Century Centers of Excellence program on Global Strategies for Control of Tropical and Emerging Infectious Diseases of Nagasaki University.

REFERENCES

1. **Chang, W. T., C. L. Kao, M. Y. Chung, S. C. Chen, S. J. Lin, W. C. Chiang, S. Y. Chen, C. P. Su, P. R. Hsueh, W. J. Chen, P. J. Chen, and P. C. Yang.** 2004. SARS exposure and emergency department workers. Emerg. Infect. Dis. **10:**1117–1119.
2. **He, Q., Q. Du, S. Lau, I. Manopo, L. Lu, S. W. Chan, B. J. Fenner, and J. Kwang.** 2005. Characterization of monoclonal antibody against SARS coronavirus nucleocapsid antigen and development of an antigen capture ELISA. J. Virol. Methods **127:**46–53.
3. **Hsueh, P. R., L. M. Huang, P. J. Chen, C. L. Kao, and P. C. Yang.** 2004. Chronological evolution of IgM, IgA, IgG and neutralisation antibodies after infection with SARS-associated coronavirus. Clin. Microbiol. Infect. **10:**1062–1066.
4. **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.
5. **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, L. J. Anderson, and the SARS Working Group.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
6. **Kuby, J.** 1997. Immunology, 3rd ed., p. 399. W. H. Freeman and Co., New York, NY.
7. **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. 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.
8. **Martin, D. A., D. A. Muth, T. Brown, A. J. Johnson, N. Karabatsos, and J. T. Roehrig.** 2000. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. J. Clin. Microbiol. **38:**1823–1826.
9. **Monath, T. P., R. R. Nystrom, R. E. Bailey, C. H. Calisher, and D. J. Muth.** 1984. Immunoglobulin M antibody capture enzyme-linked immunosorbent assay for diagnosis of St. Louis encephalitis. J. Clin. Microbiol. **20:**784–790.
10. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P.**

Icenogle, S. Peñaranda, B. Bankamp, K. Maher, M. Chen, S. Tong, A. Tamin, L. Lowe, M. Frace, J. L. DeRisi, Q. Chen, D. Wang, D. D. Erdman, T. C. T. 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. Günther, A. D. M. E. 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.

11. **Vazquez, S., G. Lemos, M. Pupo, O. Ganzon, D. Palenzuela, A. Indart, and M. G. Guzman.** 2003. Diagnosis of dengue virus infection by the visual and simple AuBioDOT immunoglobulin M capture system. Clin. Diagn. Lab. Immunol. **10:**1074–1077.
12. **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.
13. **Woo, P. C. Y., S. K. P. Lau, B. H. L. Wong, K. H. Chan, C. M. Chu, H. W. Tsoi, Y. Huang, J. S. M. Peiris, and K. Y. Yuen.** 2004. Longitudinal profile of immunoglobulin G (IgG), IgM, and IgA antibodies against the severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein in patients with pneumonia due to the SARS coronavirus. Clin. Diagn. Lab. Immunol. **11:**665–668.
14. **Woo, P. C. Y., S. K. P. Lau, B. H. L. Wong, H. W. Tsoi, A. M. Y. Fung, K. H. Chan, V. K. P. Tam, J. S. M. Peiris, and K. Y. Yuen.** 2004. Detection of specific antibodies to SARS coronavirus nucleocapsid protein for serodiagnosis of SARS coronavirus pneumonia. J. Clin. Microbiol. **42:**2306–2309.
15. **World Health Organization.** 2003. Severe acute respiratory syndrome (SARS). Wkly. Epidemiol. Rec. **78:**86–87.
16. **Yu, F., M. Q. Le, S. Inoue, H. T. Thai, F. Hasebe, M. D. C. Parquet, and K. Morita.** 2005. Evaluation of inapparent nosocomial severe acute respiratory syndrome coronavirus infection in Vietnam by use of highly specific recombinant truncated nucleocapsid protein-based enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **12:**848–854.