

Serology of Severe Acute Respiratory Syndrome: Implications for Surveillance and Outcome

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Background. Severe acute respiratory syndrome (SARS) is a novel infectious disease. No information is currently available on host-specific immunity against the SARS coronavirus (CoV), and detailed characteristics of the epidemiology of SARS CoV infection have not been identified.

Methods. ELISA was used to detect antibody to SARS CoV. Reverse-transcriptase polymerase chain reaction was used to detect SARS CoV RNA. T cells in peripheral blood of patients were quantified by flow cytometry.

Results. Of 36 patients with probable SARS CoV infection, 30 (83.3%) were positive for IgG antibody to SARS CoV; in contrast, only 3 of 48 patients with suspected SARS CoV infection, 0 of 112 patients with fever but without SARS, and 0 of 96 healthy control individuals were positive for it. IgG antibody to SARS CoV was first detected between day 5 and day 47 after onset of illness (mean \pm SD, 18.7 \pm 10.4).

Conclusion. Detection of antibody to SARS CoV is useful in the diagnosis of SARS; however, at the incubation and initial phases of the illness, serological assay is of little value, because of late seroconversion in most patients.

Severe acute respiratory syndrome (SARS) is a novel infectious disease with global impact. Since November 2002, an outbreak of SARS has affected 33 countries on 5 continents, with 8435 reported cases and 789 deaths at the time when a World Health Organization (WHO) report was published in 2003 [1]. A virus from the family *Coronaviridae*, termed "SARS coronavirus" (SARS CoV), has been identified as the cause [2–7], and criteria for laboratory confirmation of SARS CoV infection have been provided by WHO, on the basis of the following methods: (1) detection of SARS CoV RNA by reverse-transcription polymerase chain reaction (RT-PCR); (2) serological detection of SARS CoV-related antibody; and (3) isolation of SARS CoV by cell culture [4].

Thus, an investigation of the profile and implications of the presence of specific anti-SARS CoV antibody would be likely to provide information that would be beneficial in diagnostics (confirmation and exclusion of SARS cases) and that could function as a valuable

indicator to be used in the analysis of host-specific immunity against SARS CoV and of the character of SARS CoV infection. The immunology and characteristics of SARS CoV infection have not yet been fully understood, because there has been such a short span of time since the outbreak of SARS and because of the unavailability of such tools as a detection reagent. Using an indirect immunofluorescence assay and parallel acute and convalescent serum samples obtained from patients with SARS, tested for IgG antibody to SARS CoV, Peiris et al. recently documented seroconversion of IgG antibody in 93% of patients, at a mean of 20 days [5]. The results of Peiris et al.'s study prompted us to study the serology and humoral immunity of SARS CoV infection. To gain a comprehensive understanding of antibody to SARS CoV, additional clarification, such as that which would be gained by more-detailed profiles of IgG and IgM antibodies to SARS CoV (by such convenient assays as ELISA), was required. We also sought to determine the implications of these antibody profiles. Toward this aim, we performed a study of patients with SARS who had been admitted to Shenzhen Municipal Hospital of Infectious Disease (Guangdong, People's Republic of China) from 9 February 2003 to 20 May 2003, where we examined their humoral and cellular immune responses to SARS CoV infection. The results are reported here.

Received 26 June 2003; accepted 26 September 2003; electronically published 16 March 2004.

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The Journal of Infectious Diseases 2004;189:1158–63

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0022-1899/2004/18907-0004\$15.00

PATIENTS, MATERIALS, AND METHODS

Patients and samples. Included in the present study were 36 patients with probable SARS CoV infection and 48 patients with suspected SARS CoV infection. SARS was diagnosed on the basis of the case definition provided by WHO [5]. Also included in the study were 112 patients with fever but without SARS who were admitted to our hospital during the study's time frame; in addition, 96 healthy individuals, all health-care workers, were included as controls. Of the 112 patients with fever but without SARS, 35 had an upper-respiratory-tract infection, 46 had pneumonia, 22 had influenza A, and 9 had pulmonary tuberculosis. The 96 individuals in the control group consisted of 36 physicians and 60 nurses, all of whom came into close contact with patients with SARS and routinely underwent isolation procedures prior to contact. All of the patients and control individuals who participated in the present study were negative for HIV. The characteristics of the study participants are shown in table 1. All patients and control individuals were informed of the purposes, procedures, and content of our clinical study, and all clinical samples were obtained from them after they had given written informed consent.

We obtained clinical specimens of serum, nasopharyngeal aspirate, feces, and whole blood from all patients with probable SARS CoV infection and all of the patients with suspected SARS CoV. Serum samples were obtained every 3–4 days during the

first month after the patients' admission to the hospital and, thereafter, every week until 60 days after the onset of fever. Samples of nasopharyngeal aspirate and of feces were obtained on day 0 (i.e., the day of admission) and on day 7 after the patients' admission. Whole blood for the measurement of CD4⁺ and CD8⁺ T cells was obtained on day 0.

For patients with fever but without SARS, serum samples were obtained on days 0 and 21, samples of nasopharyngeal aspirate and of feces on days 0 and 7, and samples of whole blood on day 0. For the control group, serum samples were obtained on days 7, 21, and 90 after their first contact with patients with SARS; and samples from nasal swabs and samples of feces were obtained on days 7 and 90.

Detection of IgG and IgM antibodies to SARS CoV in serum by ELISA. Complete SARS CoV particles purified from the supernatant of SARS CoV-infected Vero E6 cell cultures were used as antigen in the detection of IgG and IgM antibodies to SARS CoV, by use of an ELISA kit (Jibiai Biotech). All serum samples were stored at –30°C, and the ELISAs for all samples were performed in parallel. The procedures and the interpretation of the results strictly followed the ELISA supplier's instructions.

Detection of SARS CoV RNA by RT-PCR. Total RNA was extracted from the clinical samples, as described elsewhere [6], by use of a commercial RNA-extraction kit. In brief, a QIAamp Viral RNA Mini Kit (Qiagen) was used for samples from na-

Table 1. Characteristics of patients and of healthy control individuals (health-care workers).

Characteristic	Patients			Healthy control individuals (n = 96)	P
	With probable SARS CoV infection (n = 36)	With suspected SARS CoV infection (n = 48)	With fever but without SARS (n = 112)		
Age, mean ± SD, years	30.39 ± 12.15	25.30 ± 11.15	24.12 ± 13.52	32.35 ± 14.62	>.05
Sex					
Male	20 (55.6)	28 (58.4)	70 (62.5)	35 (36.5)	<.05
Female	16 (44.4)	20 (41.6)	42 (37.5)	61 (63.5)	<.05
Treatment					
Antibiotic	3 (100)	48 (100)	112 (100)
Ribavirin	36 (100)	48 (100)	57 (50.9)	96 (100) ^a	<.05
Steroids	24 (66.7)
Underlying condition(s)					
Diabetes	2 (5.6)	2 (4.2)	5 (4.5)	...	>.05
Hypertension and/or coronary heart disease	3 (8.3)	5 (10.4)	9 (8.1)	2 (2.1)	>.05
Asthma	3 (2.6)
Pregnancy	1 (2.7)	2 (4.2)	3 (2.6)	1 (1.0)	>.05
Emphysema	2 (5.6)	3 (6.25)	5 (4.5)	1 (1.0)	>.05
Pulmonary tuberculosis	...	3 (6.25)	9 (8.0)
Tetanus	1 (2.7)

NOTE. Data are no. (%) of patients or individuals, unless otherwise noted. SARS CoV, severe acute respiratory syndrome coronavirus.

^a For prophylaxis, all healthy control individuals (health-care workers) took 400 mg of ribavirin/day, whereas, for treatment of SARS, the dosage is 8 mg of ribavirin/kg of body weight every 8 h.

sal swabs and for samples of nasopharyngeal aspirate, and a QIAamp Stool Kit (Qiagen) was used for samples of feces. For nested RT-PCR, 5 μ L of total RNA obtained from each clinical sample was reverse transcribed by use of the oligonucleotide 5'-AATGTTTACGCAGGTAAGCG-3' (nt 15627–15608); then the cDNA was amplified by use of the outer primers 5'-CAGAGCCATGCCTAACATG-3' (nt 15239–15257) and 5'-AATGTTTACGCAGGTAAGCG-3' (nt 15608–15627) and, subsequently, by use of the inner primers 5'-TGTTAAACCAGGTGGAAC-3' (nt 15376–15393) and 5'-CCTGTGTTGTAGATTGCG-3' (nt 15515–15532) [7]. Real-time quantitative RT-PCR assays were performed as described elsewhere [6], by use of a commercial kit (RealArt HPA-Coronavirus LC RT-PCR Reagents; Roche Biomedical Laboratories). RT-PCR was performed on a LightCycler (Roche Biomedical Laboratories), in accordance with the manufacturer's instructions. All samples positive for SARS CoV RNA were confirmed when direct DNA sequencing, by use of a DNA sequence analyzer (ABI 3100; Applied Biosystems), indicated 99.4% nucleotide-sequence alignment with the SARS CoV sequence published by GenBank (accession numbers gi29826276, gi30027610, and gi30027610).

Flow-cytometry analysis of CD4⁺ and CD8⁺ T cells. In brief, samples of whole blood were collected in EDTA and were immunolabeled with monoclonal antibodies CD4-FITC, CD8-PE, and CD3-CY5 (Immunotech) at room temperature; mouse IgG1-FITC, mouse IgG1-PE, and mouse IgG1-CY5 (Immunotech) were incubated as isotype controls, to allow for subtraction of nonspecific staining. Red blood cells were lysed, for 10 min at room temperature, by use of 0.5 mL Optilyse C (Immunotech), and the cells were then washed 2 times with 0.5 mL of PBS and were resuspended for flow-cytometry analysis by use of a Coulter EPICS XL (Beckman-Coulter).

Statistical analysis. Continuous variables were compared by Student's *t* test, and correlation was assessed by Pearson correlation analysis, both by use of the software program SPSS version 10.0; *P* < .05 was considered to be statistically significant.

RESULTS

Appearance and persistence of IgG and IgM antibodies to SARS CoV. The production of IgG and IgM antibodies to SARS CoV were seen as early as day 3 and day 5, respectively, after the onset of fever. Of the 36 patients with probable SARS CoV infection, 9 (25.0%) had not produced anti-SARS CoV antibody by day 21, and 6 (16.7%) had not produced it by day 60 (figure 1). Of 48 patients with suspected SARS CoV infection, 3 (6.3%) were positive for IgG antibody to SARS CoV and 2 (4.2%) were positive for IgM antibody to SARS CoV. No anti-SARS CoV antibody was detected either in the patients with fever but without SARS or in the control individuals. The time of first appearance of IgM antibody to SARS CoV ranged

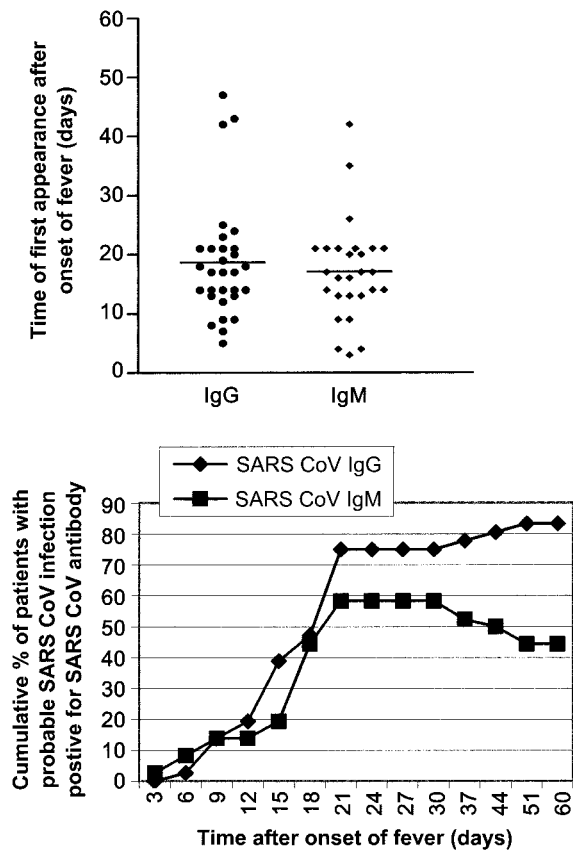


Figure 1. Time of first appearance and persistence of IgG and IgM antibodies to severe acute respiratory syndrome coronavirus (SARS CoV), determined by ELISA of serum samples obtained from patients with probable SARS CoV infection. *A*, Time of first appearance of IgG and IgM antibodies to SARS CoV; horizontal lines represent mean values. *B*, Cumulative percentage of patients positive for IgG and IgM antibodies during follow-up period.

from day 3 to day 42 (mean \pm SD, 17.1 \pm 8.5; figure 1A), and the time of first appearance of IgG antibody to SARS CoV ranged from day 5 to day 47 (mean \pm SD, 18.7 \pm 10.4; figure 1A); the difference was statistically significant (*P* < .05). At the end of our follow-up, IgG antibody to SARS CoV persisted in all patients; however, 12 patients initially positive for IgM antibody to SARS CoV became negative; in these patients, the mean \pm SD of the duration of persistence of IgM antibody to SARS CoV was 28.5 \pm 8.7 days (figure 1B).

Detection of SARS CoV RNA by RT-PCR. Among all the patients and control individuals, only 9 people were determined to be positive for SARS CoV RNA by RT-PCR, and all were in the group of 36 patients with probable SARS CoV infection. The RNA fragment detected by nested RT-PCR was 158 bp in length (figure 2) and is part of the RNA polymerase gene (nt 15377–15532). As mentioned above, all samples positive for SARS CoV RNA were confirmed when direct DNA sequencing indicated 99.4% nucleotide-sequence alignment with the

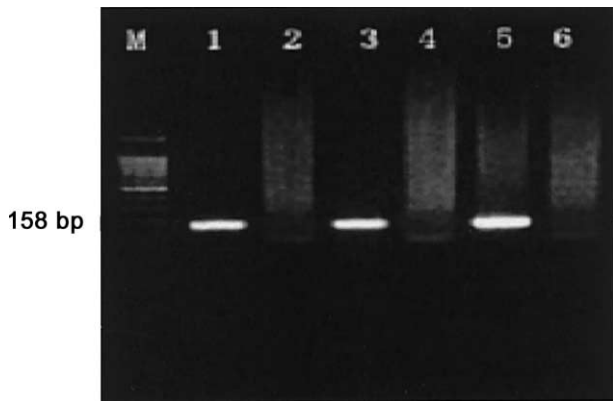


Figure 2. Electrophoresis of severe acute respiratory syndrome coronavirus (SARS CoV) RNA from clinical specimens positive (lanes 1, 3, and 5) and negative (lanes 2, 4, and 6) for SARS CoV RNA, amplified from samples of nasopharyngeal aspirate by nested reverse-transcription polymerase chain reaction. M, 100-bp DNA molecular standard.

SARS CoV sequence published by GenBank (accession numbers gi29826276, gi30027610, and gi30027610). Moreover, all samples positive for SARS CoV RNA were further confirmed when real-time PCR indicated a viral load of $>10^5$ copies/mL. No patients with suspected SARS CoV infection, no patients with fever but without SARS, and no control individuals were positive for SARS CoV RNA. In addition, there was no difference between the amount of anti-SARS CoV antibody produced by patients positive for SARS CoV RNA and that produced by patients negative for SARS CoV RNA.

The relationship between the production of IgG antibody to SARS CoV and T cell immunity. We quantified CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells in the peripheral blood of the patients with probable SARS CoV infection and of those with suspected SARS CoV infection. Our results indicated that all of these patients experienced a dramatic decrease in CD3⁺CD4⁺ T cell percentage (mean \pm SD, 19.72% \pm 7.78%) and CD3⁺CD8⁺ T cell percentage (mean \pm SD, 23.7% \pm 6.35%), compared with patients with fever but without SARS ($P < .05$), whose mean \pm SD CD3⁺CD4⁺ T cell percentage and CD3⁺CD8⁺ T cell percentage were 40.6% \pm 7.8% and 28.7% \pm 7.1%, respectively. In patients with probable SARS CoV infection, there was a significant difference between the CD3⁺CD4⁺ T cell percentage in those positive for IgG antibody to SARS CoV (mean \pm SD, 24.07% \pm 7.95%) and that in those negative for it (mean \pm SD, 13.60% \pm 10.19%) ($P < .05$); however, no significant difference was found between the CD3⁺CD8⁺ T cell percentages in these 2 groups (table 2). Also in patients with probable SARS CoV infection, there was no significant difference, in either the CD3⁺CD4⁺ or the CD3⁺CD8⁺ T cell percentage, between those positive for IgM antibody to SARS CoV and those negative for it ($P > .05$). A correlation was found between the day of IgG antibody seroconversion and the CD3⁺CD4⁺ T cell percentages

measured on the day of the patients' admission to our hospital ($r = -0.543$, $P < .05$), if we assume day 60 to be the day of seroconversion for those patients who had not actually seroconverted by then.

DISCUSSION

Much progress has been made in SARS research, including the identification of the etiologic agent [2–7], the complete sequencing of the SARS CoV genome [8], the establishment of such diagnostic laboratory methods as RT-PCR and indirect immunofluorescence assay [5, 6], and the establishment of measures for the prevention of SARS and for the management of probable SARS cases. However, it is still uncertain whether SARS is recurrent and what the impact of recurrence might be, because there is no information on the status of host-specific immunity against SARS CoV and because the detailed characteristics of the epidemiology of SARS CoV infection are not known [9, 10]. Given this uncertain background, we investigated, for its potentially significant clinical implications, the profile of anti-SARS CoV antibody in different populations, including patients with probable SARS CoV infection, patients with suspected SARS CoV infection, patients with fever but without SARS, and healthy control individuals (health-care workers) who came into close contact with patients with SARS.

The results of our investigations of the presence of anti-SARS CoV antibody in patients with either probable or suspected SARS CoV infection show that the use of ELISA to detect IgG antibody and/or IgM antibody to SARS CoV is a specific and useful method for the diagnosis of SARS, especially given that ELISA's sensitivity in the detection of anti-SARS CoV antibody (83.3%) is much better than RT-PCR's sensitivity in the detection of SARS CoV RNA (~25% in our study). Serological assay, however, is of little value during the incubation and initial phases of the illness, because of late seroconversion in most patients (figure 1A). In addition, our results indicated that 9 (25.0%) of 36 patients with probable SARS CoV infection had not produced detectable anti-SARS CoV antibody by day 21 after the onset of fever; this implies that 25.0% of patients with SARS might be misdiagnosed by the laboratory confirmation guidelines that WHO currently recommends [5]. However, this was not the case in our study, because (1) 3 of these 9 patients were later confirmed, by detection of seroconversion before day 47, to be infected by SARS CoV and (2) the 6 other patients, who had remained negative for anti-SARS CoV antibody until day 60, were later confirmed, by fulfillment of WHO criteria and by exclusion of other pathogenic infections, to be infected by SARS CoV. Specifically, all of the patients with probable SARS CoV infection came into contact with someone with SARS, had documented persistent fever ($>38^\circ\text{C}$), showed a consistent clinical course of the ill-

Table 2. Relationship between CD3⁺CD4⁺ and CD3⁺CD8⁺ T cell percentages and presence of IgG and IgM antibodies, in patients with probable severe acute respiratory syndrome coronavirus (SARS CoV) infection.

Parameter	Antibody to SARS CoV			
	IgG		IgM	
	Positive (n = 30)	Negative (n = 6)	Positive (n = 28)	Negative (n = 8)
CD3 ⁺ CD4 ⁺ , mean ± SD, %	24.07 ± 7.95 ^a	13.60 ± 10.19 ^a	23.43 ± 8.01	19.01 ± 13.04
CD3 ⁺ CD8 ⁺ , mean ± SD, %	26.12 ± 6.05	18.05 ± 12.34	25.34 ± 6.44	19.46 ± 12.24
CD3 ⁺ CD4 ⁺ percentage:CD3 ⁺ CD8 ⁺ percentage, mean ± SD, ratio	0.96 ± 0.32	0.88 ± 0.33	0.92 ± 0.33	0.99 ± 0.29

^a For the difference between the CD3⁺CD4⁺ T cell percentage in patients positive for IgG antibody to SARS CoV and that in patients negative for it, $P < .05$.

ness, and showed evidence of pneumonia, by plain radiography and/or computed tomography; in addition, there was no evidence of infection by other pathogens (including influenza viruses A and B, human parainfluenza viruses 1–3, respiratory syncytial virus, adenovirus, *Chlamdia pneumoniae*, *C. psittaci*, *Mycoplasma pneumoniae*, and *Mycobacterium tuberculosis*), and there was no conventional pathogenic bacterial infection and no response to antibiotic treatment (0.3 g of levofloxacin/day for 48–72 h). The discrepancy between our diagnosis of SARS in these patients and diagnosis on the basis of WHO guidelines likely reflects the evolution of the identification of definitive SARS cases, as more data on the disease accumulate; the current WHO guidelines are probably not 100% accurate. It is notable that, on 16 July 2003, the Centers for Disease Control and Prevention revised its laboratory criteria in its definition of SARS, to require that convalescent serum be collected >28 days, instead of >21 days, after the onset of symptoms [11]. The choice of day 28 presumably reflects data indicating that >95% of patients with SARS mount a detectable convalescent antibody response [11]. A more technical explanation for our identification of late-seroconverting patients may involve differences between the sensitivity of the ELISA used in the present study and the sensitivity of other serological assays, on which the WHO guidelines are based; this issue should be further clarified as more data are collected. In 6 of our patients with a late antibody response, there may have been other mitigating factors that could have led to a decrease in basic immunity. Of these 6 patients, 2 (who were >65 old) had emphysema, 1 had diabetes, 1 was hypertensive and had coronary artery disease, 1 had tetanus, and 1 was pregnant; these conditions may be associated with late seroconversion in these patients. It is notable that a few (2 of 36 [5.6%]) patients with probable SARS CoV infection were positive for IgG antibody to SARS CoV but, until day 60, were negative for IgM antibody to SARS CoV; the reason for this is uncertain, but one possible explanation is that, in these 2 cases, IgM antibody to SARS CoV persisted for a short time and had disappeared before the patients were admitted to the hospital.

We found production of IgG antibody to SARS CoV to be

associated with host T cell immunity, because patients who did not produce IgG antibody to SARS CoV had significantly lower CD3⁺CD4⁺ T cell percentages, compared with those in patients who seroconverted. In addition, the day of IgG antibody seroconversion correlated with CD3⁺CD4⁺ T cell percentages taken on the day of the patient's admission ($r = -0.543$; $P < .05$). These results suggest that the production of IgG antibody to SARS CoV is dependent on CD4⁺ T cells and that the appearance of IgG antibody to SARS CoV might be an indicator of the production of protective immunity against SARS CoV. Recently, we treated 1 pregnant patient with severe SARS CoV infection by using convalescent plasma in which the titer of IgG antibody to SARS CoV was >1:500; the IgG antibody could be detected until 60 days after the infusion, 2 days before her own IgG antibody to SARS CoV appeared. With combined treatment with the antiviral drug methylprednisolone and convalescent serum, she recovered fully. However, whether the IgG antibody to SARS CoV is itself a neutralizing antibody needs further study. Recently, Krokhn et al. reported that acute and early convalescent serum, obtained from several patients recovering from SARS, can react only with the 46-kDa nucleoprotein (which appears to be the major antigen) of SARS CoV [12]. Their results suggest that immune response to this nucleoprotein could serve as an early diagnostic indicator for infection; however, it is unlikely that immune response to this protein offers protection, because it is an internal protein and because neutralizing antibodies are more likely to target cell-surface proteins [13]. Nevertheless, it has been shown, for other CoVs, that some antigenic peptides of the nucleoprotein can be recognized on the surface of infected cells by host T cells [13]; thus, the appearance of IgG antibody in patients with SARS will more likely be an indicator of the production of protective immunity against SARS CoV.

Our results also indicate that all individuals positive for anti-SARS CoV antibody should present symptoms, whether severe or not. From 9 Feb 2003 to the time when this article was written, our hospital admitted 52 patients with probable SARS CoV infection and 48 patients with suspected SARS CoV infection; however, at our hospital there is not a single health-

care worker infected with SARS CoV—a situation that is in stark contrast to that at other hospitals in China, where nearly one-third of patients with SARS are health-care workers [14]. That our hospital has no health-care workers infected by SARS CoV was confirmed by both serological assay and lack of detection of SARS CoV RNA. The 96 health-care workers who participated in our study all came into close contact with patients with SARS, for a period of 3 months, but IgG and IgM antibodies to SARS CoV were not detected in the serum of the health-care workers. These results may be somewhat difficult to interpret—and it was certainly not the point of our study to investigate this matter—but their implications may be very important; they suggest that the possibility that people can be asymptotically infected by SARS CoV—and that such individuals (if they do exist) can transmit the virus—might be very small. The preventive measures taken at our hospital were almost exactly the same as those taken at other hospitals, with the only known difference being that, for prophylaxis, the health-care workers at our hospital took 400 mg of ribavirin/day. We must caution, however, that these are only anecdotal observations; further study is necessary to determine whether ribavirin has a prophylactic effect against SARS CoV infection.

Acknowledgments

We thank the staff of Shenzhen Municipal Hospital of Infectious Disease and the staff of Shenzhen Institute of Hepatology, for their technical assistance and management of patients; all of the patients and, especially, the health-care workers who participated in the present study, for their great cooperation; and Michael Graner (Dept. of Pediatrics, University of Arizona, Tuscon), for his helpful comments.

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