Two-Year Prospective Study of the Humoral Immune Response of Patients with Severe Acute Respiratory Syndrome

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In a cohort study of 56 convalescent patients with severe acute respiratory syndrome (SARS), titers of immunoglobulin G (IgG) antibodies and neutralizing antibodies (NAbs) against SARS-associated coronavirus were assessed at regular intervals (at 1, 4, 7, 10, 16, and 24 months after the onset of disease) by use of enzyme-linked immunosorbent assay and neutralization assay. IgG antibody and NAb titers were highly correlated, peaking at month 4 after the onset of disease and decreasing thereafter. IgG antibodies remained detectable in all patients until month 16, and they became undetectable in 11.8% of patients at month 24. The finding that NAbs remained detectable throughout follow-up is reassuring in terms of protection provided against reinfection; however, NAb titers decreased markedly after month 16.

Severe acute respiratory syndrome (SARS) is an emerging infectious disease caused by a newly identified coronavirus (CoV) known as "SARS-associated CoV" (SARS-CoV) [1]. Although the first SARS epidemic (2002–2003) was brought under control, reemergence of the disease from infected animals or from

laboratories handling SARS-CoV has already been documented on several occasions [2, 3]. Although many studies have addressed the short-term humoral response to SARS-CoV [4, 5], no studies have provided detailed information beyond 1 year of follow-up. However, on the basis of findings for other human coronavirus (OC43 and 229E) infections, it is known that immune protection is not long-lasting [6–8]. Therefore, it is important to study the long-term immune response of patients with SARS, to determine whether protection would potentially last for several years. This would have important practical implications for (1) hospital staff who might have had SARS in the past and who would again be exposed in the event of reemergence of the disease, and (2) optimization of the vaccines under development to provide long-term immunity. The current report presents the results from a 2-year cohort study of the humoral immune response of patients with previous cases of SARS.

Study participants. This prospective study was conducted at the Beijing Hospital of Armed Forces Police, which was one of the major hospitals in Beijing, People's Republic of China, that was designated to treat cases of SARS during the SARS epidemic of 2003 [9]. Patients with SARS had probable cases of the disease, according to a modified definition of SARS provided by the World Health Organization [10]; the patients had high fever, respiratory symptoms, and chest radiographic findings of pneumonia, and they had either SARS-CoV RNA detected by reverse-transcription polymerase chain reaction or a positive serum antibody level detected by ELISA and IFA. Of the 86 patients who had SARS diagnosed during the outbreak, 6 patients died. Of the 80 remaining patients with SARS, 63 were health care workers at the hospital and were invited to participate in the study.

Collection of specimens and information. After informed consent was obtained from each patient, epidemiological data and past clinical information were retrieved from medical records and through patient interviews. Serum samples were collected from each patient at regular intervals (at 1, 4, 7, 10, 16, and 24 months after the onset of disease) and were frozen at $-20°C$ until analysis could be performed. The study protocol was approved by the Chinese National Center for AIDS Prevention and Control institutional review board.

ELISA. Serum titers of IgG antibodies to SARS-CoV were measured using a commercially available ELISA kit (product no. S20030003; BJI-GBI Biotechnology). This whole-virus lysate-based kit was the first kit approved by the China Food and Drug Administration for the detection of SARS-CoV an-

tibody in China, and it has been widely used in previous studies [5, 11]. In brief, 100 μL of serially diluted serum (2-fold dilutions, beginning with an initial dilution of 1:10) was added to wells coated with SARS-CoV antigens. The plate was incubated at 37°C for 30 min and then was washed 5 times with washing buffer. A total of 100 μL of diluted enzyme-labeled anti–human IgG was added to each well, and the plate was then incubated for an additional 30 min, followed by washing performed as described above. Next, 100 μL of tetramethylbenzidine substrate was added, and the plate was incubated at 37°C for 20 min. The reaction was terminated by adding hydrochloric acid, and absorbance at 450 nm was measured. A cutoff OD value of 0.18 was calculated as the mean +2 SDs of the OD values for control serum samples obtained from 1000 donors. The maximum dilution of a serum sample for which the OD was >0.18 was considered to be the antibody titer. For the whole process, internal positive and negative controls were included on each plate. All serum samples obtained from individual patients were analyzed on the same ELISA plate.

Neutralization assay. The serum titers of neutralizing antibodies (NAbs) were measured by neutralization assay, according to the procedures described by Guan et al. [12], with modifications. In brief, serum samples obtained from convalescent patients with SARS were incubated at 56°C for 30 min and then were diluted serially in cell-culture medium. Aliquots of diluted serum samples were mixed, on a 96-well microtiter plate, with 100-fold the $TCID_{50}$ of the SARS-CoV (BJ01 strain), and they then were incubated at 37°C for 1 h in 5% CO_2. Finally, the mixture was inoculated onto 96-well plates with Vero E6 cells and was incubated at 37°C in 5% CO_2. Daily microscopic examination was performed for the assessment of cytopathic effects. On day 5 after the onset of disease, the highest dilution of serum that completely inhibited 100-fold $TCID_{50}$ of SARS-CoV was recorded as the NAb titer. Neutralization assays were performed in triplicate with negative control serum samples obtained from healthy people.

Statistical methods. All data were processed using SPSS software (version 10 for Windows; SPSS) and Microsoft Excel (version 7.0; Microsoft). Samples were grouped by the month that the samples were obtained. The antibody reciprocal titers were not normally distributed, so they were log-transformed to allow for comparison of geometric mean reciprocal titers (GMRTs) across groups by use of the t test. The GMRTs of IgG antibodies and NAbs were calculated and plotted against the months after the onset of disease. Random-effects models were used to study the predictive value of the characteristics of the study participants (i.e., age, sex, severity of disease, presence of comorbid conditions, and steroid use) for the GMRTs for both IgG antibodies and NAbs. Correlations between IgG antibody and NAb reciprocal titers were analyzed using Spearman's cor-

relation coefficients. $P < .05$ was considered to be statistically significant throughout the study.

Results. A total of 63 patients with SARS who met the selection criteria were invited to participate in the study. Seven of the patients failed to provide a sufficient number of blood samples, so only 56 participants who contributed at least 3 blood specimens during the follow-up were included in the analysis. The mean patient age was 29 years (range, 18–59 years), and 27 patients were men. Nine patients had underlying disease, including tuberculosis ($n = 3$), heart disease ($n = 2$), *Pseudomonas aeruginosa* infection ($n = 2$; 1 patient had coinfection with tuberculosis), high blood pressure ($n = 1$), diabetes ($n = 1$), and septicemia ($n = 1$). Seven patients had a severe clinical condition, such as pulmonary aggravation that required oxygen ventilation and transfer of the patient to an intensive care unit. No patients died. Fifty patients received steroids during hospitalization, with cumulative doses of prednisolone ranging from 2600 to 30,000 mg.

A total of 216 serum samples were tested by ELISA for the presence of specific IgG antibody. The number of study participants tested at each follow-up visit varied from 32 to 41 (figure 1). IgG serological findings remained positive throughout follow-up for all patients, except at the last visit (at month 24), when findings for 4 (11.8%) of 34 serum samples changed from positive to negative findings. Figure 1 shows IgG antibody GMRTs by month after the onset of disease, with a peak GMRT occurring at month 4, before a significant decrease occurred over time until month 24.

A total of 136 serum samples were analyzed for the presence of NAbs (25 of 36 samples available at month 1 after the onset of disease were analyzed, and all samples available at months
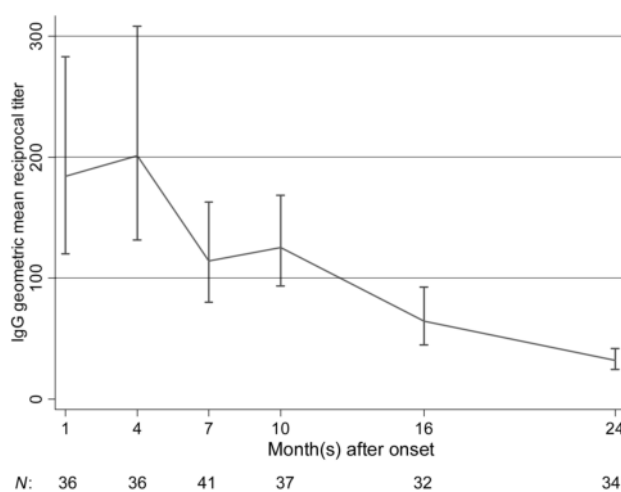

Figure 1. Kinetics of IgG antibody titers, by month after the onset of disease symptoms, for 56 patients with severe acute respiratory syndrome in Beijing, People's Republic of China. *N,* no. of study participants tested at each visit.

4, 7, and 24 were analyzed). All samples tested positive for NAbs at all visits. Figure 2 shows the GMRTs of NAbs by month after the onset of disease. NAb GMRTs peaked at month 4, decreased at month 7, and decreased again at month 24. NAb and IgG antibody titers were strongly correlated (Spearman's correlation coefficient, 0.58; $P < .001$).

By use of random-effects models, no difference was found in IgG antibody or NAb GMRTs, on the basis of age, severity of symptoms, presence of comorbid conditions, and steroid use (with both the maximal and cumulative dose considered). The only difference that was noted was a more rapid decrease in NAb GMRTs in men, compared with those in women ($P < .001$, for the interaction between time and sex in random-effects models), with a more pronounced decrease in GMRTs noted for men, compared with women, at month 24 (32.8 vs. 222.2 for men and women, respectively; $P < .001$). This difference was not associated with other patient characteristics (e.g., age, severity of symptoms, presence of comorbid conditions, and steroid use), because it remained unchanged in multivariate analysis after controlling for other factors.

Discussion. The present study reports the kinetics of specific IgG antibodies and NAbs in a cohort of 56 convalescent patients with SARS who were monitored for 2 years after the onset of illness. Although some previous studies reported longitudinal profiles of IgG antibodies or NAbs in patients with SARS, the duration of follow-up in these studies was limited to 1 year [5, 13]. To our knowledge, this is the first published prospective study involving a series of serum samples obtained from participants for a 2-year follow-up. Samples could not be obtained from all patients at all visits, for reasons of convenience, but all patients provided at least 3 samples, and 32–41 samples were available from each visit.

A major concern regarding the reemergence of SARS is the duration and quality of natural immunity after infection, which may influence both the risk to hospital staff on reexposure to the virus and the strategies for vaccine development. Primary experiments involving mice found that NAb titers in serum samples were sufficient to protect against challenge with live SARS-CoV [14], and a recent study indicated that IgG antibodies in the serum samples of patients who had recovered from SARS played a major role in neutralizing SARS-CoV in vitro [5]. These findings suggest that both specific IgG antibodies and NAbs are important to protect hosts from reinfection, often in combination with cellular immunity [15]. The present study adds to this existing knowledge by focusing on the dynamic changes in the serum levels of IgG antibodies and NAbs in convalescent patients with SARS for up to 2 years after infection. Not all samples could be analyzed for NAbs, because of both the time-consuming nature of the experiment and the need to work in a biosafety level 3 laboratory because of safety concerns. However, by systematic analysis of all samples avail-
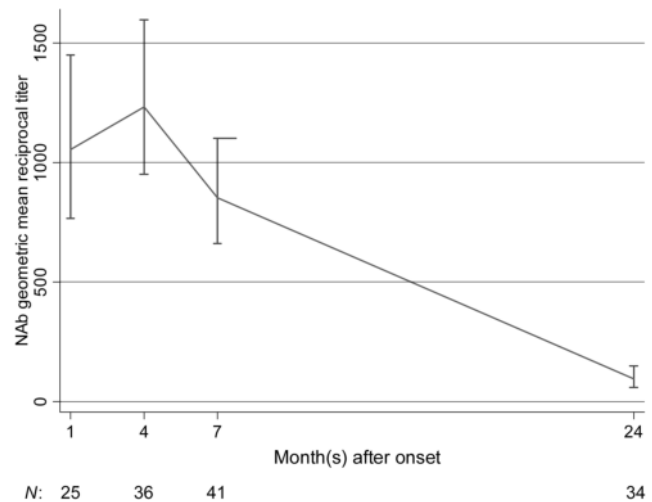

Figure 2. Kinetics of neutralizing antibody (NAb) titers, by month after the onset of disease symptoms, for 56 patients with severe acute respiratory syndrome in Beijing, People's Republic of China. *N*, no. of participants tested at each visit.

able at months 4, 7, and 24 after the onset of disease, it was possible to show that the dynamics of IgG antibodies and NAbs were similar, with peak titers measured at month 4 and a dramatic decrease in titers noted at month 24. All samples tested were positive for NAbs throughout follow-up, including at month 24; this finding is reassuring in terms of protection against reinfection. Of the serum samples that originally had positive results of ELISA detection for IgG antibodies, few samples (11.8%) were negative for IgG antibodies at month 24, indicating that population-based surveys of past exposure to the virus may fail to detect antibodies among some subjects who were infected more than 2 years ago.

Factors that may influence the dynamics of antibody responses were also investigated. The only factor statistically associated with the decrease in NAb titers was sex. The antibody levels of men decreased significantly faster than did those of women. Although this could be a chance finding, because 5 different factors were tested in the model, the level of significance of the association between NAb titers and sex at month 24 was $P < .001$, giving some credibility to the finding. Other factors known for their immunomodulatory role, such as steroids, did not influence the level of the humoral immune response.

In conclusion, the present study shows a marked decrease in IgG antibody and NAb titers 2 years after the onset of SARS. Still, all study participants had detectable NAbs after 2 years; this finding is comforting, considering that low levels of NAbs are compatible with protection on reexposure to infection. Prolonged follow-up of NAb titers in the same cohort for >2 years is required to assess whether humoral immunity will eventually vanish.

Acknowledgments

We thank all of the patients and health care workers who participated in the present study.

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