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A Serological Survey on Neutralizing Antibody Titer of SARS Convalescent Sera

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A seroepidemiologic study was conducted in North China in 2003 to determine the neutralizing antibody titer of severe acute respiratory syndrome (SARS) convalescent sera. A total of 99 SARS convalescent serum samples were collected from patients from the Inner Mongolia Autonomous Region, Hebei Province, and Beijing 35–180 days after the onset of symptoms. The anti-SARS antibodies were detected by enzyme-linked immunosorbent assay (ELISA), neutralization assay, and Western blot. Eighty-seven serum samples were confirmed to be positive for SARS antibodies. The neutralizing antibody titer of the 87 positive sera was analyzed quantitatively by neutralization assay. The geometric mean titer (GMT) of the 87 convalescent sera was 1:61. The Kolmogorov–Smirnov test showed that the neutralizing antibody titers conform to normal distribution, which suggests that the average anti-SARS antibody level in this study was representative of the convalescent antibody level of the SARS population. This result could be useful for the development and quality control of SARS vaccines. ***J. Med. Virol. 77: 147–150, 2005.***

KEY WORDS: geometric mean titer; immunity; neutralization assay; SARS; SARS-CoV

INTRODUCTION

Severe acute respiratory syndrome (SARS), the newly emerging severe acute respiratory syndrome caused by SARS coronavirus (SARS-CoV), is of importance to public health [Drosten et al., 2003; Kuiken et al., 2003]. As of July 2003, 8,096 cases of SARS had been reported and 774 of the infected patients died. From April to May 2004, China reported nine new laboratory-acquired SARS cases. To prevent future recurrence of SARS, more than a dozen SARS candidate vaccines are under development [Gao et al., 2003; Bisht et al., 2004; Marshall and Enserink, 2004; Yang et al., 2004]. There is an urgent need for an accurate measurement of SARS convalescent serum antibody levels as a reference to evaluate the efficacy of vaccine. Many efforts have been devoted to quantifying SARS-CoV specific antibody titer using enzyme-linked immunosorbent assay (ELISA) [Li et al., 2003; Shi et al., 2003; Huang et al., 2004], but the results are not comparable among different studies. In one report, the IgG antibody titer of convalescent serum measured by indirect ELISA was around 1:640 [Li et al., 2003]. In another report, the antibody measured was specific to recombinant SARS nucleocapsid (N) protein and the titer was expressed by the logarithm [Liu et al., 2004]. Since standardized reagents for SARS antibody detection have not become available, the data on SARS antibody titer reported from various researchers are not applicable for SARS vaccine evaluation. Neutralization assay measures the ability of sera to neutralize the infectivity of SARS-CoV in cell culture. It is a stable method and does not involve external reagents. To gain a comprehensive understanding of the antibody to SARS-CoV, we report the anti-SARS antibody titer of 87 SARS convalescent sera determined by neutralization assay. It provides important information for the development of SARS vaccine.

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MATERIALS AND METHODS

Serum Samples

Ninety-nine serum samples from convalescent SARS patients were collected 35–180 days after the onset of symptoms from May to September 2003. Seventy-five were collected from Inner Mongolia Autonomous Region, 8 from Hebei Province, and 16 from Beijing. All SARS patients were confirmed to be SARS cases according to the diagnosis criteria promulgated by the Ministry of Health of P.R. China [2003]. They all had a medical history of close contact with SARS patients; symptoms of fever higher than 38°C, coughing, difficulty in breathing; reticular change, flaky or striped infiltrative shadows on chest X-ray examination; no white blood cell (WBC) count rise; and no effect after treatment with antibiotics. A panel of serum samples were obtained from 10 healthy adults as negative controls. All serum samples were sterilized by a 0.22 μm filter and were heat-inactivated at 56°C for 30 min.

SARS-CoV and Cells

SARS-CoV Sino 1 strain was isolated from pharyngeal swabs of clinically confirmed SARS patients at Peking Union Hospital. The Sino 1 strain was well-characterized biologically and was confirmed by electron microscope observation and complete genome sequencing (GenBank accession No. AY485277). Vero cells, a line of African green monkey kidney cells, were obtained from ATCC (American Type Culture Collection). Vero cells were grown at 37°C in Minimum Essential Medium (MEM), containing 10% (w/v) new-born calf serum and 1% (w/v) glutamine. SARS-CoV was propagated in Vero cells in culture flasks at 37°C until 75% of the cells showed a cytopathic effect (CPE). After three freeze-thaw cycles, virus was harvested and characterized for the neutralization assay. A batch of SARS-CoV was inactivated by β-propiolactone in situ in flasks and then purified by centrifugation, ultrafiltration, and gel filtration. The purified SARS-CoV was used as antigen for Western Blot assay.

Titration of Infective Virus

Vero cells were cultivated in 96-well plates at 37°C to form a confluent monolayer. Cells were inoculated with 200 μl per well of viral suspension that was diluted serially in tenfold steps in MEM containing 2% new-born calf serum and 50 μg/ml penicillin, pH 7.2), with each dilution filling 8 wells. The 96-well plates were incubated at 37°C in a 5% CO_2 incubator. Virus titer was expressed as cell culture infectious dose (CCID), which is the dilution of virus causing half of the cultured cells to produce CPE. The virus batch had a titer of 6.7 Lg $CCID_{50}$/ml and was stored at −80°C for the neutralization assay.

Western Blot

Purified SARS-CoV Sino 1 strain proteins were separated by 12% SDS–PAGE and were transferred onto nitrocellulose membranes. The blocked membrane was incubated with the tested serum sample (diluted 1:200) as primary antibodies. The secondary antibody was goat anti-human IgG conjugated with HRP and the color developing substrate was DAB. A recombinant N protein was included in each test as positive antigen. When the N protein and a brand of purified SARS-CoV showing the molecular weight of about 46 kDa were detected, the serum used was considered SARS antibody positive.

ELISA

ELISA was performed using a commercial SARS Coronavirus IgG Antibody Diagnostic Kit (BGI-GBI Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. The tested sera were diluted ten times and added to the ELISA strip wells coated with SARS-CoV lysate. After the processes of washing, adding HRP-labeled anti-human IgG, color developing, and stopping reaction, the absorbance of the optical density at 450 nm (OD_{450}) was measured.

Neutralization Assay

The neutralization assay was conducted according to conventional procedures. Briefly, 100 μl diluted serum (twofold serial dilution from 1:2 to 1:2,048) was added to equal volume of virus (diluted to 100 $CCID_{50}$/0.1 ml) and incubated for 1 hr at 37°C. Then the mixture was added to confluent monolayer of Vero cells in 96-well plates and incubated at 37°C in a 5% CO_2 incubator for 4 days. On day 4, the presence of viral CPE was checked. The dilution of serum that completely inhibited CPE in 50% of the cells was calculated using the Reed-Muench formula [Reed and Muench, 1938]. Both positive and negative controls were included in each test. Meanwhile, in each assay, the SARS-CoV was re-titrated for its infectivity ($CCID_{50}$/0.1 ml) in parallel.

The neutralization assay experiment system was validated first. A well-titrated SARS in-house reference antiserum with the geometric mean titer (GMT) of 1:52.7 was used as a positive control [Zhang et al., 2005]. The test results showed that the neutralization antibody titer for 10 negative control sera were all below 1:8, therefore neutralization antibody titer equal to or higher than 1:8 was defined as positive. The neutralization assay was taken as valid only when the titer of the positive control was in the range of 1:34 to 1:68, and the titer of negative serum was below 1:8. The above experiment was repeated three times by three persons simultaneously.

Ninety-nine serum samples (in dilution 1:8) were firstly qualitatively tested by neutralization assay. Those positive sera were then serially diluted to be quantitatively tested for the neutralizing antibody titer.

Statistical Analysis

The normal distribution analysis was performed by one sample Kolmogorov–Smirnov test using SPSS software, with the level of significance set at $P < 0.05$.

TABLE I. Number of Positive SARS Antibody Detected by Different Methods

Results obtained by	No. of positive samples	No. of samples tested by Western blot	No. of positive confirmed by Western blot
ELISA	90	71	68
Neutralization	87[a]	68	68
ELISA and neutralization assay	86	67	67

SARS, severe acute respiratory syndrome; ELISA, enzyme-linked immunosorbent assay.
[a]Of these 87 sera, 86 were double positive for ELISA and neutralization assay, the remaining 1 serum was double positive for neutralization assay and Western blot.

RESULTS

Detection of Anti-SARS Antibodies

A total of 99 SARS convalescent sera were included in this research. All samples were tested qualitatively for antibody response to SARS-CoV by ELISA and neutralization assay in parallel, but only 80 samples were tested by Western blot. Of 99 samples, ELISA detected 90 positive sera and neutralization assay identified 87 positive sera. Eighty-six sera were positive both for ELISA and neutralization assay (Table I). Neutralization assay detected one more positive serum than the result obtained by ELISA and neutralization assay. This serum sample was confirmed further to be positive by the evidence of Western blot, though it was negative for ELISA. Of 87 neutralization positive samples, 68 were tested by Western blot and they were all positive for the anti-SARS antibody (Table I). These 87 serum samples were confirmed to be positive for anti-SARS antibodies with the combination of ELISA, neutralization, and Western blot, so they were pooled to form a convalescent sera database for the further analysis of neutralizing antibody titer. The remaining 12 serum samples were ruled out, as they were negative by the evidence of both neutralization assay and Western blot or ELISA.

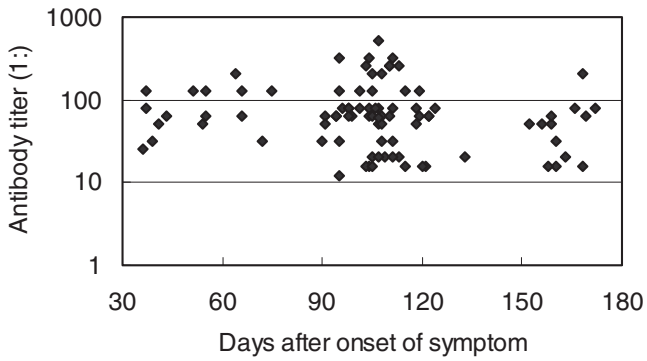

Fig. 1. Distribution of anti-SARS neutralizing antibody titer of 87 convalescent sera. The anti-SARS neutralizing antibody titer was measured by neutralization assay. Twofold serial dilutions of sera were tested for the presence of antibodies that neutralized the infectivity of 100 $CCID_{50}$ of SARS-CoV in a monolayer of Vero cells in 96-well plates. The dilution of serum that completely prevented CPE in 50% of the wells was calculated by the Reed–Muench formula.

Average Level of SARS Neutralizing Antibody

The anti-SARS neutralizing antibody titer of 87 positive convalescent sera was analyzed quantitatively by the neutralization assay. Titers collected from 35 to 180 days after the onset of symptoms were tested by the neutralization assay, and the results are shown in Figure 1. The lowest titer detected by the neutralization assay was 1:12 and the highest titer was 1:512. The GMT of these convalescent sera was 1:61. Test results showed that SARS specific neutralizing antibody level was relatively stable and persisted as long as 180 days with a slight decline from 1:67 to 1:40 after 121 days after the onset of the symptom (Table II).

A normal distribution test of anti-SARS titer was performed by one sample Kolmogorov–Smirnov test using SPSS software after the log transformation of titers. The test result showed that the P-value was 0.109 ($P \geq 0.05$), which revealed that the anti-SARS neutralizing antibody titer was in normal distribution. A conclusion can be drawn that the average anti-SARS neutralizing antibody level of convalescent sera obtained in our study is a representative of the convalescent neutralizing antibody level of the SARS population.

DISCUSSION

During the outbreak of SARS in 2003, ELISA, immunofluorescent assay, Western blot, and neutralization assay were used for serological diagnosis of SARS cases. ELISA, the most widely used assay, possesses a sensitivity and specificity of over 98% in some reports [Wu et al., 2004]. The objective of this investigation was to provide a profile of anti-SARS neutralizing antibody

TABLE II. Neutralizing Antibody Titer of SARS Convalescent Sera at Different Time Intervals

Days after onset	No. of patients	Geometric mean titer (1:)
31–60	10	62
61–90	6	69
91–120	53	67
121–150	5	40
151–180	13	43
Total	87	61

titer from convalescent patients, rather than establishing neutralization assay as a diagnostic method for SARS. In our laboratory, a combination of ELISA, neutralization assay, and Western blot were performed on 99 SARS convalescent sera. Using these methods, 87 sera were confirmed to be positive for anti-SARS antibody. The results from the neutralization assay accord with those from ELISA and Western blot. A high level of consistency was demonstrated for ELISA and the neutralization assay (86/87 [98.9%]) for these 87 positive samples, illustrating the high sensitivity and specificity of the neutralization assay. The GMT of anti-SARS neutralizing antibody was quantitated to be 1:61, and the normal distribution of the neutralizing antibody titer was shown by statistical analysis. The neutralization assay could, therefore, become a useful tool for the surveillance of SARS serological epidemiology.

It has been demonstrated in a mouse model that the neutralizing antibody elicited by primary infection of SARS-CoV can protect animals from re-infection. In addition, passive transfer of this immune serum to naïve mice can prevent SARS-CoV replication in the respiratory tract [Subbarao et al., 2004]. Yang et al. showed that a DNA vaccine encoding the spike (S) glycoprotein of the SARS-CoV induced mice to generate T-cell and neutralizing antibody responses, as well as a protective immune response. They demonstrated that the protection was mediated by a humoral but not a T-cell-dependent immune mechanism by T-cell depletion and passive transfer of purified IgG. Although more investigations on humoral and cellular immunity are needed in humans, it is reasonable to conclude that human neutralizing antibody is protective against SAR-CoV.

The development of SARS vaccine entails methods that best reflect the immune response in humans to evaluate the vaccine efficacy. The neutralization assay satisfies this requirement. It is specific and correlates with the humoral immune response in SARS convalescent patients. In addition, unlike some serological tests based on subunits (such as N or S protein), the neutralization assay detects all effective neutralizing antibodies having specificities for both characterized and uncharacterized epitopes.

Although the neutralization assay is a gold standard, several disadvantages limit its wide use. It is cumbersome, expensive, and in need of biosafety containment, because of the risk of infection. The manipulation of SARS-CoV might bring risk not only to the researchers but also to the public, as SARS laboratorial infection cases have been reported in Taiwan, Singapore, and the Chinese mainland [Watts, 2004]. The average neutralizing antibody titer against SARS-CoV in convalescent serum is undoubtedly one of the most important indexes as a reference for SARS candidate vaccine assessment.

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