Comparison of Immunoglobulin G Responses to the Spike and Nucleocapsid Proteins of Severe Acute Respiratory Syndrome (SARS) Coronavirus in Patients with SARS[▽]

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Both the nucleocapsid (N) and the spike (S) proteins of severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) are able to induce strong humoral responses in humans following an infection. To compare the immunoglobulin G (IgG) responses to the S and N proteins of SARS-CoV in SARS patients during the manifestation/convalescent period with those during the postinfection period, serum samples were collected from hospitalized SARS patients within 6 weeks after the onset of illness (set 1; 57 sequential samples from 19 patients) or 2 to 3 months after their recovery (set 2; 33 postinfection samples from 33 subjects). Serum samples from 100 healthy blood donors (set 3), collected in 2002, were also included. The specific IgG response to whole virus, the fragment from positions 450 to 650 of the S protein (S450-650), and the full-length N protein of SARS-CoV were measured by enzyme-linked immunosorbent assays (ELISAs). Western blot assays were carried out to confirm the ELISA results. Fifty-one of the serum samples in set 1 (89%) bound to the N protein, a proportion similar to that which recognized whole virus (79%) and the S-protein fragment (77%). All 33 serum samples from set 2 were strongly positive for N-protein-specific IgG, while 27 (82%) were positive for anti-S450-650 IgG. Two of the serum samples from set 3 were strongly positive for anti-N-protein IgG but not anti-S450-650 IgG. Similar levels of IgG responses to the S and N proteins were observed in SARS patients during the manifestation and convalescent stages. In the postinfection period, however, a number of patients had much lower serum IgG levels against S450-650 than against the N protein.

Severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV), a positive-stranded RNA virus of the family *Coronaviridae*, is the causative agent of SARS (8, 9). Its genome encodes several structural and nonstructural proteins, including the spike (S) glycoprotein, the nucleocapsid (N) protein, the membrane protein, RNA polymerase, and the main protease 3C-like (3CL) protein (2, 7, 9). The 49-kDa, 422-amino-acid-residue N protein of SARS-CoV shares 32% homology to the N proteins of the other human CoV strains (7, 9). It is abundantly expressed in cells infected by SARS-CoV and induces strong antibody responses in patients (2, 5, 10). The S glycoprotein, which mediates membrane fusion between the virus and the host cell (4, 13, 14, 16), consists of 1,267 amino acid residues and has approximately 25% homology to the S glycoproteins of the other human CoVs (7, 9). We have previously documented that a 31-kDa fragment comprising amino acid residues 450 to 650 (S450-650) of the S protein of SARS-CoV contains immunodominant epitopes and can be used with high specificity and sensitivity as the coating antigen in enzyme-linked immunosorbent assays (ELISAs) for the detection of anti-S-protein immunoglobulin G (IgG) in patient sera (18, 19).

However, the levels of IgG antibodies against the S and N proteins in serum in the postinfection period in SARS patients have not been fully characterized. In this study, we used recombinant protein antigens in ELISA systems to compare the serum IgG responses to the S and N proteins of SARS-CoV in the manifestation/convalescent period with those in the postinfection period of SARS patients.

MATERIALS AND METHODS

Reagents. High-fidelity *Taq* DNA polymerase was purchased from TaKaRa Biotech Co. Ltd (Shiga, Japan), restriction enzymes and T4 ligase were from Invitrogen (Carlsbad, CA), and a kit for DNA extraction and purification was from QIAGEN (Hilden, Germany). *Escherichia coli* BL21(DE3) was obtained from Stratagene (La Jolla, CA.). Nickel-nitrilotriacetic acid agarose was from Novagen (Darmstadt, Germany). Horseradish peroxidase (HRP)-labeled goat anti-human IgG was obtained from Zhongshan Biotech Co. (Beijing, China), and complementary DNAs encoding the full lengths of the S and N proteins of SARS-CoV were from the China CDC. Purified recombinant 3CL protein of SARS-CoV (17) was kindly provided by Zihe Rao, Tsinghua University, Beijing, China.

Subjects and blood samples. Table 1 summarizes the three sets of serum samples used in this study. A major outbreak of SARS took place in Beijing, China, beginning on 24 March 2003. We collected sequential venous blood samples (set 1; 57 samples in total) from 19 patients (both sexes; age range, 18 to 51 years; average age, 35.5 years) who fulfilled the WHO definition of SARS (a temperature of 38°C or higher, cough, new pulmonary infiltrates on chest radiography in the absence of an alternative diagnosis to explain the clinical presentation). All blood samples were collected within 6 weeks after the onset of illness. Thirteen of the patients in set 1 became infected during the major outbreak of SARS in 2003 and were admitted to the First Affiliated Hospital of Peking University, Beijing, China. Blood samples from these patients were collected between 15 April and 5 June 2003. A smaller outbreak of SARS took place in April 2004 and involved nine patients in Anhui and Beijing, China. Sequential

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TABLE 1. Summary of serum samples used in this study

Serum sample group	Donors	Serum samples	Collection dates
Set 1	19 hospitalized patients with SARS confirmed by the presence of anti-SARS-CoV IgG antibodies	57 sequential serum samples from 19 patients	April to June 2003 (13 patients); April to June 2004 (6 patients); all samples were collected within 6 wk of the onset of illness
Set 2	33 subjects, 2–3 mo after their recovery from SARS	33 samples from 33 subjects	July and August 2003
Set 3	100 healthy blood donors	100 samples from 100 donors	May and July 2002
Control pooled HDS	10 randomly selected healthy individuals from set 3	A mixture of sera from 10 healthy blood donors	May and July 2002

serum samples from six patients who were confirmed to have SARS (second- or third-generation cases) and who were admitted to Ditan Hospital between 15 April and 10 June 2004 were therefore also included in set 1. All infections included in this study were confirmed by the presence of IgG antibodies against SARS-CoV by using the Huada ELISA kit (see below). Informed consent was obtained from the patients before blood collection.

Sera for set 2 were from 33 patients who had recovered from SARS and were collected between July and August 2003 (2 to 3 months after their recovery and subsequent discharge from hospital) by the Beijing Red Cross Blood Center. The blood samples were processed within 18 h of collection, and the sera were stored at −80°C. Set 3 comprised serum samples from 100 healthy blood donors (both sexes; age range, 22 to 45 years) that were collected between May and July 2002 and that were also provided by Beijing Red Cross Blood Center. In addition, a control serum sample (pooled healthy donor sera [HDS]) was prepared by mixing sera from 10 randomly selected healthy individuals and was used throughout the study.

Expression and purification of recombinant proteins. Construction of expression plasmids for the S-protein fragment (S450-650) and the method used for its subsequent expression have been described previously (20). DNA encoding the full-length N protein was cloned into the pET28a vector (Novagen). The recombinant His-tagged fusion protein was expressed in *E. coli* BL21(DE3). Briefly, bacterial colonies harboring the plasmid were cultured to the appropriate density in 2× yeast extract-tryptone medium containing kanamycin (25 μg/ml) with continuous shaking at 37°C. Isopropyl-β-D-thiogalactopyranoside was then added to induce the expression of fusion proteins. After a further 3 h of incubation at 22°C, the bacterial cell suspension was centrifuged at 5,000 × g for 15 min. The cell pellets were resuspended and subjected to sonication in an ice bath for 8 min. The lysed cells were then centrifuged at 12,000 × g for 30 min at 4°C, and the supernatants were subsequently applied to an Ni column. The column-bound proteins were eluted with elution buffer containing 500 mM imidazole and were then examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue or transferred to nitrocellulose membrane for Western blotting.

Western blot assays. The nitrocellulose membranes (Pierce, Rockford, IL) to which the recombinant proteins were transferred were blocked at room temperature for 2 h with 5% nonfat dried milk in Tris-buffered saline (TBS; pH 7.5) and were then incubated with the serum samples for 2 h at room temperature. After the membranes were washed in TBS containing 0.05% Tween 20, they were incubated with HRP-labeled goat anti-human IgG. The reaction was visualized by using the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO).

ELISAs. ELISA plates were coated at 4°C overnight with recombinant proteins (2.5 pmol/well) in carbonate buffer (pH 9.6). Each well of the plates was then incubated with blocking solution (2% bovine serum albumin in phosphate-buffered saline [PBS]) for 2 h at 37°C. The wells were washed five times with PBS containing 0.05% Tween 20 (PBS-T). One hundred microliters of serially diluted sera or human IgG was added in triplicate, followed by further incubation for 90 min at 37°C. After five washes with PBS-T, the plates were incubated with HRP-labeled goat anti-human IgG antibody for 1 h at 37°C. *ortho*-Phenylenediamine (100 μl/well; Sigma) was added after five washes with PBS-T, and the wells were incubated for 2 min at room temperature. Fifty microliters of 2 M H_2SO_4 was added to each well to terminate the reaction, and the optical density (OD) was immediately read at 492 nm.

A SARS-CoV-specific ELISA kit, developed by the Huada Institute, China, has been widely used in China for SARS-CoV-specific antibody testing, with reliable results. For the ELISAs with the kit produced by the Huada Institute, the manufacturer's instructions were followed. Briefly, dilution buffer (100 μl/well) was added to the precoated wells, followed by the addition of 10 μl serum and incubation for 30 min at 37°C. After the washes, HRP-labeled detection antibody (1/2,000 dilution) was added (100 μl/well) and the plates were incubated for 20 min at 37°C before further washes. Substrate buffer containing 2,2′-azino-di-(3-ethylbenzothiazoline sulfonate) was then added, and the reactions were allowed to develop for 10 min before stop buffer was added. The plates were read at 450 nm.

Preparation of pseudovirus. Pseudovirus expressing the SARS-CoV S protein was prepared as described previously (19). Briefly, 5 × 10⁶ 293T cells maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum were seeded in 10-cm-diameter tissue culture dishes. One day later the cells were cotransfected with defective human immunodeficiency virus type 1 (HIV-1) genome pNL4.3-Luc-R⁻E⁻ (20 μg) and either plasmid pVSV-G (10 μg), which encodes the vesicular stomatitis virus (VSV) G protein, or plasmid pCMV-S, which encodes the S protein of SARS-CoV, by using a transfection reagent (Vigorous Biotech Co., Beijing, China). The defective HIV-1 genome contains the gene for luciferase, which can be used as a reporter of successful infection by the pseudovirus. The culture medium was replaced with fresh medium at 24 h posttransfection, and the cells were cultured for an additional 24 h. The culture supernatant, which contained VSV G-protein-expressing (VSV-G) pseudovirus or SARS-CoV S-protein-expressing (SARS-CoV-S) pseudovirus, was then harvested and filtered through a 0.45-μm-pore-size filter, followed by centrifugation at 35,000 rpm for 3 h at 4°C with an XL-90 ultracentrifuge (Beckman Coulter, Fullerton, CA). The pseudoviral pellets were resuspended in PBS, titrated, aliquoted, and stored at −80°C until use.

Neutralization assays with the SARS-CoV pseudovirus system. SARS-CoV-S pseudovirus and VSV-G pseudovirus infection of cells and neutralization of the infection were described previously (19). Briefly, Vero E6 cells maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum were seeded in 96-well plates at a density of 8 × 10³ cells/well and cultured overnight. Serially diluted serum samples were mixed with the pseudovirus preparations for 30 min at 37°C, and then the mixtures were added to the wells containing monolayers of Vero E6 cells. After incubation at 37°C for 1 h, the mixture in the wells was replaced with fresh medium and the cells were cultured for an additional 48 h. After washes, the cells were lysed with the luciferase assay reagent (Promega, Madison, WI), and the luciferase activity in the cell lysate was determined with a Veritas microplate luminometer (Turner Biosystems). The luciferase activity of the reference group (Vero E6 cells treated with pseudovirus alone) was taken as 100% infection. Cells not treated with pseudovirus were included as a specificity control, and their luciferase readings were at least 3 log units lower than that for the reference group in all experiments. The luciferase activities of the experimental groups (Vero E6 cells treated with pseudovirus preparations in the presence of serum antibodies) were compared with that of the reference group; and the results, expressed as percent infection, were calculated as follows: 100 × (luciferase activity of the experimental groups/luciferase activity of the reference group).

Statistical analysis. All experiments described here were carried out at least three times. The results obtained by the N- and S-based ELISAs and with the Huada ELISA kit were compared by using the CORREL module of Microsoft Excel software. The Cohen kappa test was performed to analyze the agreement between the results obtained with the ELISA kits. Comparison of the data was performed by the Student t test. Significance was defined as a P value <0.05.
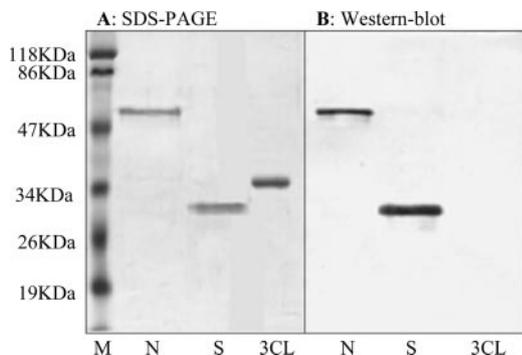
FIG. 1. SDS-PAGE and Western blot analysis of recombinant proteins. Affinity-purified recombinant N protein (lanes N), S450-650 (lanes S), and 3CL protein (lanes 3CL) were run in two identical SDS–12% polyacrylamide gels. One of the gels was stained with Coomassie blue (A). The protein bands in the unstained gel were transferred onto a nitrocellulose membrane for Western blotting with convalescent-phase serum sample PT18 as the first antibody (B). The detecting antibody was HRP-labeled goat-anti-human IgG. Protein molecular weight markers (lane M) were run in the left-hand lane.

RESULTS

Recombinant protein-based ELISAs. The recombinant S-protein fragment S450-650 (31 kDa) and the full-length N protein (49 kDa) were expressed in *E. coli* and purified to more than 90% purity, as determined by SDS-PAGE analysis (Fig. 1A); and their antigenicities were confirmed by Western blot assays (Fig. 1B). The recombinant proteins were then used as coating antigens to develop ELISA systems for the detection of S-specific and N-specific antibodies. The results presented in Fig. 2 demonstrate that these ELISA systems are able to readily detect specific IgG antibodies in patient sera. To confirm the assay specificity, 100 individual serum samples from healthy blood donors (set 3) were screened at a dilution of 1 in 100 by the S450-650-based and N-protein-based ELISAs. None were positive by the S450-650-based ELISA (Fig. 3B). Interestingly, serum samples N20 and N49 were positive by the N-protein-based ELISA (Fig. 3A), which was subsequently confirmed by the results of the Western blot assays (see below). Serum samples N20 and N49 remained positive in the N-protein-based and also the Huada ELISAs even after dilution 400-fold (data not shown).

FIG. 3. Screening of serum samples from healthy donors. Serum samples from 100 healthy blood donors (set 3) were diluted 1:100 and tested by using the N-protein-based (A) and the S450-650-based (B) ELISAs. The results are expressed as the absorbance readings at 492 nm. The cutoff values were calculated as the mean absorbance readings of the serum samples from all the 100 blood donors plus 3 standard deviations.

N-protein-specific IgG antibodies in patient sera. The N protein-based ELISA exhibited better sensitivity than the virus-based kit in analyzing the sera from set 1. As illustrated in Fig. 4A and Table 2, 51 of the 57 (89%) serum samples in set 1 were positive for anti-N-protein IgG antibodies, while 45 (79%) serum samples were positive by the Huada test (Fig. 4B, Table 2). All serum samples in set 2 were strongly positive for anti-N-protein IgG antibodies, which is consistent with the results of the Huada ELISA (Fig. 4A and B; Table 2). When the N-protein-based and virus-based ELISA data were plotted against each other, a close linear correlation (first-degree regression, $r = 0.768$) was observed (Fig. 5A). The Cohen kappa test also confirmed a strong agreement between the results obtained with these two ELISA systems ($\kappa = 0.8$).

Anti-S-protein IgG antibodies in patient sera. Of the 57 serum samples in set 1, 44 (77%) were positive by the S450-650-based ELISA, which is slightly less than the proportions positive by the virus-based (79%) and N-protein-based (89%)

FIG. 2. Sensitivities of the ELISA systems based on N protein and S450-650. ELISA plates were coated with recombinant N protein (A) or S450-650 (B). Convalescent-phase sera from three SARS patients and the pooled HDS control were serially diluted and dispensed, in triplicate, into the wells. HRP-labeled goat anti-human IgG was used as the second antibody, with *ortho*-phenylenediamine used as the substrate. The results are expressed as the absorbance readings at 492 nm.

FIG. 4. Antibodies against the N protein and S450-650 in patient sera. The N-protein-based (A), S450-650-based (B), and whole-virus-based (C) ELISAs were carried out to analyze the IgG antibodies in serum samples from sets 1 and 2. The results are expressed as the absorbance readings at 492 nm. The same sets of sera were also analyzed by using the Huada ELISA kit, and the results are expressed as the absorbance readings at 450 nm (C). Each bar represents a single serum sample; samples collected at different time points from each individual subject are grouped together. A negative control serum sample (a mixture of serum samples from healthy subjects) was included in each plate for calculation of cutoff values (mean + 3 standard deviations). The cutoff values were 0.30, 0.46, and 0.13 for panels A, B, and C, respectively.

TABLE 2. IgG antibodies against SARS-CoV in patients and healthy controls

Specificity	No. (%) of serum samples in:					
	Set 1 (n = 57)		Set 2 (n = 33)		Set 3 (n = 100)	
	Positive	Negative	Positive	Negative	Positive	Negative
Whole virus[a]	45 (79)	12 (21)	33 (100)	0 (0)	2 (2)	98 (98)
N protein[b]	51 (89)	6 (11)	33 (100)	0 (0)	2 (2)	98 (98)
S protein[c]	44 (77)	13 (23)	27 (82)	6 (18)[d]	0 (0)	100 (100)

[a] Based on the Huada ELISA results.
[b] N-protein-based ELISA results were confirmed by Western blotting assays.
[c] Fragment S450-650 ELISA.
[d] Including five samples (samples PT35, PT40, PT51, PT52, and PT60) that were weakly positive by ELISA but negative by Western blotting assays.

ELISAs (Fig. 4C, Table 2). Different patterns of anti-S-protein and anti-N-protein IgG profiles were observed in SARS patients. For example, patient PT20 was a strong responder to the S protein and a relatively weak responder to the N protein. In contrast, patient PT19 was a strong responder to the N protein but a nonresponder to the S-protein fragment (Fig. 4A and C).

Of the 33 serum samples in set 2, 27 (82%) were positive by the S450-650-based ELISA (Fig. 4C; Table 2). The Cohen kappa test confirmed the correlation between S-protein-based and N-protein-based ELISAs (first-degree regression, $r = 0.493$) or the S-protein-based and the whole-virus-based ELISAs (first-degree regression, $r = 0.558$) (Fig. 5B and C).

Representative serum samples, including serum samples
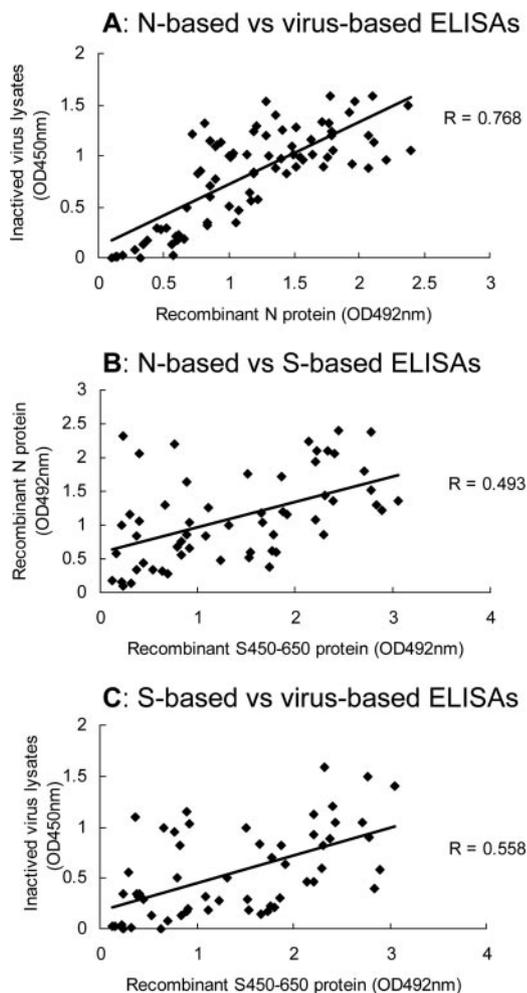
FIG. 5. Correlation among the virus-based, N-protein-based, and S450-650-based ELISA results. The absorbance readings of the virus-based ELISA (OD at 450 nm; serum dilution, 1/11), the N-protein-specific ELISA (OD at 492 nm; serum dilution, 1/100), or the S450-650-specific ELISA (OD at 492 nm; serum dilution, 1/100) were plotted against each other. First-degree regression (r) shows a linear correlation between the results of each two ELISAs. (A) Correlation between virus-based and N-protein-based ELISAs; (B) correlation between virus-based and S450-650-based ELISAs; (C) correlation between N-protein-based and S450-650-based ELISAs.

from strong (serum samples PT31 and PT53), medium (serum samples PT45 and PT50), and weak or negative (serum samples PT52 and PT55) responders, as determined by the S450-650-based ELISA (Fig. 4), were further analyzed for their ability to recognize the recombinant N and S450-650 proteins by Western blot assays (Fig. 6). The results were consistent with those obtained by the N-protein-based and the S450-650-based ELISAs, although Western blotting appeared to be less sensitive for the detection of S-protein-specific antibodies. For instance, PT52, a sample weakly positive by the S450-650-specific ELISA, was unable to recognize S450-650 by Western blotting (Fig. 6C).

Serum samples N20 and N49 (from healthy donors) were also assayed for their abilities to recognize the recombinant N and S450-650 proteins by Western blotting. They clearly re-

acted with the N protein but not the S450-650 fragment (Fig. 6D), consistent with the data shown in Fig. 3.

Some of the serum samples in set 1 were collected at the early phase of the infection, which allowed us to assess the kinetics of the antiviral IgG response in vivo. As illustrated in Fig. 7, specific IgG antibodies were detected by the N-based (Fig. 7A) and S450-650-based (Fig. 7B) ELISAs as early as 8 days after the onset of illness, 3 to 4 days earlier than the time to detection by the whole virus-based kit (Fig. 7C).

Correlation between the anti-S450-650 antibody titer and the neutralizing abilities of serum samples. By cotransfecting 293T cells with a SARS-CoV S-protein-expressing vector and a defective HIV-1 genome expressing luciferase, we prepared S protein expressing pseudovirus. The pseudovirus thus obtained was able to infect angiotensin-converting enzyme 2 (ACE2)-positive Vero E6 cells in vitro (19); ACE2 is a known receptor for SARS-CoV. The system was used to test the neutralization capacities of the serum samples. As shown in Fig. 8A, the serum samples that responded to the S450-650 fragment (serum samples PT31, PT53, PT45, and PT50) could dramatically block SARS-CoV-S pseudovirus infection of Vero E6 cells, while the weak responder serum sample (sample PT52) was much less efficient at doing so. The serum samples from the two healthy blood donors (serum samples N20 and N49) which did not react with the S450-650 fragment showed no neutralizing ability (Fig. 8B).

DISCUSSION

Whole virus-based ELISAs have the advantage of being able to detect antibodies specific for all structural components of the virus. However, protein-based assays permit a more detailed analysis of the antiviral humoral responses. Our N-protein-based and S-protein-based ELISA systems appeared to be more sensitive than the virus-based Huada ELISA kit, since sera diluted 100-fold (instead of 11-fold) were used in the protein-based assays. Several groups have reported that the N protein is an immunodominant antigen of SARS-CoV and is capable of inducing strong antibody responses in humans (5, 10). In this study, a close correlation between the N-protein-based and the virus-based ELISA results was clearly established (Fig. 5).

Since the S450-650 polypeptide covers less than one-fifth of the S-protein sequence, a negative result by our S450-650-based assay does not necessarily rule out the possible presence of anti-S-protein antibodies in the samples under investigation. However, ample evidence suggests that S450-650 contains a dominant epitope(s) for anti-S-protein antibodies in sera from most patients confirmed to have SARS. For instance, we have previously shown that the S450-650-based ELISA could detect IgG antibodies specific for two immunodominant B-cell epitopes, S511-545 and S596-600, in convalescent-phase sera from most SARS patients in a sensitive and specific fashion (18, 19). Lu and coworkers showed that patient sera mainly recognized epitopes contained within residues 441 to 700 of the S protein of SARS-CoV, as determined in Western blot assays (6). In addition, Zhou and colleagues documented in 2004 that residues 485 to 625 of the S protein of SARS-CoV elicited neutralizing antibodies against the virus (21).

Comparison of the anti-N-protein and the anti-S-protein
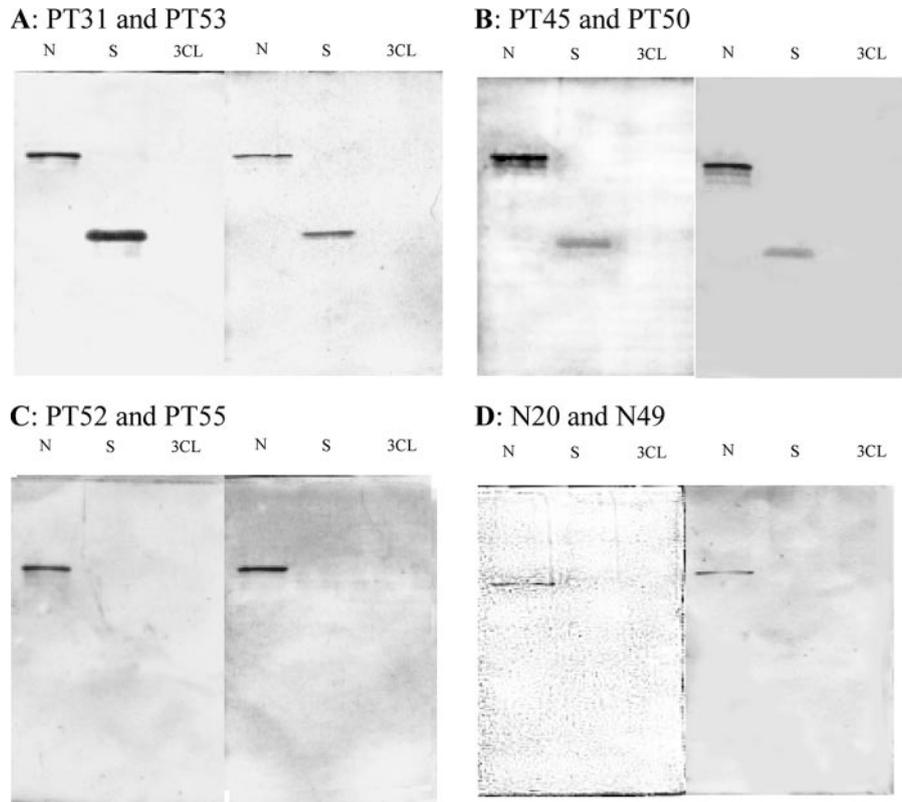
FIG. 6. Western blot assays of sera from patients and healthy subjects. Recombinant N protein (lanes N), S450-650 (lanes S), and 3CL protein (lanes 3CL) were run in SDS–12% polyacrylamide gels. After electrophoresis, the protein bands were transferred onto nitrocellulose membranes for probing with sera from patients in the convalescent phase (set 2) (A to C) or serum samples N20 and N49 from healthy blood donors (set 3) (D). Bound antibodies were then detected by using HRP-labeled goat anti-human IgG.

antibody profiles in the manifestation and convalescent periods with those in the postinfection period could provide valuable insights into the humoral responses following SARS-CoV infection in humans. In the serum samples from set 1, anti-S-protein and anti-N-protein IgG levels were similar (Fig. 4; Table 2), suggesting that antibodies against both structural proteins were successfully induced soon after the infection. However, clear segregation between the anti-N-protein and anti-S-protein IgG levels was observed in the postinfection sera, with the rate of positivity for anti-S-protein antibody lower than that for anti-N-protein antibody (100% versus 82%

positive for anti-N-protein and anti-S-protein IgG, respectively). This observation may have important implications for our understanding of the humoral immunity against SARS-CoV and also future SARS vaccination programs. For example, a combination of priming with an inactivated virus vaccine and boosting with recombinant S protein may be necessary to maintain high-titer S-protein-specific neutralizing antibodies in recipients.

SARS-CoV binds to the ACE2 receptor on the cell surface by using the S protein; thus, S-protein-specific rather than N-protein-specific antibodies are able to neutralize the infec-
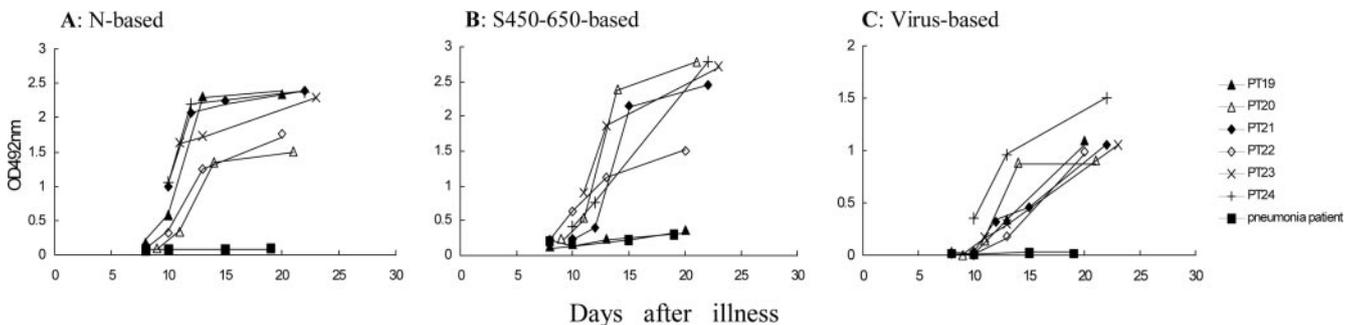
FIG. 7. Kinetics of IgG responses in SARS patients. Sequential serum samples from six SARS patients were tested by using the N-protein-based (A), S450-650-based (B), or virus-based (C) ELISA. Sera were diluted 100-fold (A and B) or 11-fold (C), and the results are expressed as the absorbance readings at 492 nm and 450 nm, respectively.
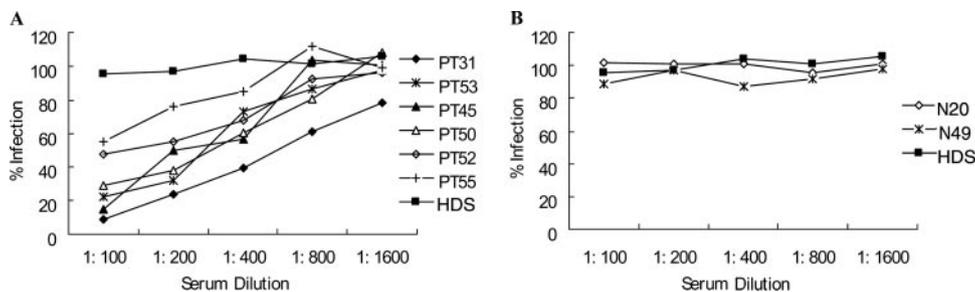
FIG. 8. Neutralization assays. Serially diluted serum samples from patients in the convalescent phase (A) or from healthy blood donors (B) were mixed with medium containing SARS-CoV-S pseudovirus and were incubated for 30 min at 37°C. The mixtures were then distributed into triplicate wells in 96-well plates containing a monolayer of Vero E6 cells seeded at 8×10^3/well the day before. The luciferase activities of the infected cells were determined 48 h later, and the results are expressed as percent infection compared with that for the control group (Vero E6 cells treated only with pseudovirus preparations).

tivity of the virus. However, it should be emphasized that anti-S-protein antibodies detected by S-protein-based ELISAs or Western blot assays are not necessarily all neutralizing antibodies. For instance, our previous studies demonstrated that murine antibodies against the S450-510 fragment were unable to block a S-protein-expressing pseudovirus infection in vitro (19). The data in Fig. 8 indicate a positive correlation between the titers of S450-650-specific IgG antibodies and their neutralization ability. Even though the number of serum samples ($n = 6$) included here was too small for a statistically meaningful analysis of any correlation between S450-650-specific antibody titers and their neutralization activity, the results were nonetheless supportive of such a notion. Presumably, all individuals make neutralizing antibodies following SARS-CoV infection; however, the S-protein-specific neutralizing antibodies seem to last in the host for a relatively shorter period of time than the N-protein-specific antibodies, as evidenced by the clear segregation between the anti-N-protein and anti-S-protein IgG antibodies in the serum samples from set 2.

Two of the 100 serum samples from set 3 were strongly positive by the N-protein-based ELISA and Western blot assays (Fig. 3 and 6D) and also by the Huada ELISA (Table 2). This is epidemiologically intriguing, as these serum samples were collected more than 1 year before the first reported outbreak of SARS in humans. These two individuals did not recall having a "mild" infection with symptoms similar to those associated with SARS prior to blood sample collection. This study also illustrated that these N-protein-specific sera did not have neutralizing abilities in our pseudovirus neutralization assays (Fig. 8B). It is possible that antibodies were elicited in the sera of these individuals by an infection(s) with human CoV strains such as 229E, OC43, NL63 (12), and HKU1 (15). It has been documented that human CoV strains 229E and OC43 are responsible for ~30% of all common colds (11), and the recently identified strains NL63 and HKU1 also contribute significantly to the overall spectrum of CoV infection (1). The N proteins of known CoVs are relatively more conservative than other structural proteins (3). The N protein of human CoV strain NL63 shared the highest amino acid sequence identity with that of human CoV strain 229E (12), while the N protein of human CoV strain HKU1 had less than 40% homology with those of other non-group 2 CoVs (15). Furthermore, Sun and Meng showed that polyclonal antisera against

known animal CoVs cross-reacts with the N protein of SARS-CoV (11). Since serology remains the "gold standard" for the diagnosis of SARS, it is important to explore serological cross-reactions between SARS-CoV and other CoVs.

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