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Profiles of IgG Antibodies to Nucleocapsid and Spike Proteins of the SARS-Associated Coronavirus in SARS Patients

YANBIN WANG,[1] ZHAORUI CHANG,[1] JING OUYANG,[1] HAIYAN WEI,[1,2] RENQUAN YANG,[1,3] YANGONG CHAO,[4] JIANGUO QU,[1] JIANWEI WANG,[1] and TAO HUNG[1]

ABSTRACT

To evaluate humoral immunity against the SARS-associated coronavirus (SARS-CoV), we studied the profiles of IgG antibodies to the nucleocapsid (N) and spike (S) proteins of SARS-CoV. Serum specimens from 10 SARS patients were analyzed by Western blotting and an enzyme-linked immunosorbent assay (ELISA) using purified recombinant N and truncated S (S1, S2, and S3) proteins as antigens. Western blotting results demonstrated that 100% of the SARS patients tested positive for N protein-specific antibodies, 50% for S1 protein-specific antibodies, 30% for S2 protein-specific antibodies, and 70% for S3 protein-specific antibodies. The ELISA results, which showed positive rates of IgG reactivity against recombinant proteins N, S1, S2, and S3, were, respectively, 28.57, 14.29, 14.29, and 14.29% at week 1, 77.78, 55.56, 44.44, and 66.67% at week 2, 100, 75, 75, and 87.5% at week 3, and 100, 77.78, 77.78, and 88.89% after 3 weeks. The average titers of IgG against recombinant proteins N, S1, S2, and S3 were, respectively, 691, 56, 38, and 84 after 3 weeks. These results suggest that the recombinant proteins N and S3 are potentially useful antigens for a serological diagnosis of SARS. In consideration of possible cross-reactivity among N proteins of SARS-CoV and other coronaviruses, immunoassays using recombinant N protein in combination with S3 as antigens might improve the specificity of SARS diagnoses.

INTRODUCTION

Coronaviruses are large, enveloped, RNA viruses that cause respiratory and enteric diseases in humans and animals. Based on amino acid sequence analysis, known coronaviruses have been divided into three groups: groups 1 and 2 infects various mammals and group 3 infects birds. A newly emerging coronavirus, severe acute respiratory syndrome-associated coronavirus (SARS-CoV) was identified as the causative agent of SARS in 2003 (Drosten *et al*., 2003; Peiris *et al*., 2003). SARS-CoV is not closely related to any of the previously characterized coronaviruses, and does not belong to any of the known groups of coronaviruses (Marra *et al*., 2003). The two previously identified human coronaviruses, HCoV-229E and HCoV-OC43, cause only mild upper respiratory infections (Lai and Holmes, 2001). However, SARS-CoV causes severe respiratory infection with a high mortality rate (10%). It is therefore imperative to understand the differences between SARS-CoV and other coronaviruses in terms of immune response to explore the pathogenesis of SARS-CoV as well as to develop effective diagnostic procedures.

The SARS-CoV genome is 29,727 nucleotides in length and encodes 23 putative proteins, including four major structural proteins: nucleocapsid (N), spike (S), membrane (M), and small envelope (E). S is a large glycoprotein of approximately 150 kDa containing 1255 amino acids (Marra *et al*., 2003; Rota *et al*., 2003). It may mediate membrane fusion and induce neutralizing antibody production (Gallagher and Buchmeier, 2001). Recent studies indicated that full-length S, expressed by an attenuated vaccinia virus vector, induces binding and neutralizing antibody production and protects immunized mice from subsequent infection with SARS-CoV (Bisht *et al*., 2004). N is a phosphoprotein containing 422 amino acids. The biological function of the coronavirus N protein is thought to be the bind-

[1]National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China.
[2]School of Medicine, Shandong University, Jinan, People's Republic of China.
[3]School of Animal Health Sciences, Nanjing Agricultural University, Nanjing, People's Republic of China.
[4]Beijing Jiuxianqiao Hospital, Tsinghua University, Beijing, People's Republic of China.

ing of the viral genome to form a helical nucleocapsid that participates in viral replication (Baric *et al*., 1988). It is a major viral antigen in several known coronaviruses, such as murine (Liu *et al*., 2001) and turkey (Wage *et al*., 1993). Previous studies with animal coronaviruses have shown that N is abundantly expressed during infection (Narayanan *et al*., 2003) and is the most abundant protein in purified SARS-CoV virions (Rota *et al*., 2003). These features imply that S and N proteins are probably useful in the early diagnosis of SARS.

Today, acute SARS outbreaks have, at least temporarily, ceased. However, with a wide range of animal reservoirs of the virus in nature, it is believed that SARS will likely resurface in the future (Guan *et al*., 2003; Martina *et al*., 2003). In the absence of antiviral drugs or vaccines available against SARS-CoV, early detection of virus-infected patients is critical for better control or prevention of future epidemics. In this paper, we studied the profiles of IgG antibodies against the S and N proteins by Western blotting and ELISA to evaluate the humoral immunity features of SARS-CoV and to select ideal antigens for early diagnosis and/or vaccine development.

MATERIALS AND METHODS

Serum specimens

A total of 50 specimens from 10 patients with SARS were collected during different stages of illness, including acute and convalescent sera. These patients fit the World Health Organization (WHO) definition for probable SARS infection (WHO, 2003)—fever of 38°C or higher, respiratory symptoms (e.g., cough, shortness of breath, and difficulty in breathing), hypoxia, and chest radiograph changes of pneumonia, and history of close contact with other SARS patients. Serum samples from healthy donors were collected as negative controls.

Cloning of cDNAs encoding N and truncated S proteins

Viral RNA was extracted with the TRIZOL® Reagent (Invitrogen, Carlsbad, CA) from SARS-CoV in a biosafety level 3 laboratory. The SARS-CoV genomic RNA was converted to cDNA by reverse transcription using random primers (Promega, Madison, WI). The coding regions for SARS-CoV N and truncated S proteins (S1, S2, and S3) were amplified by PCR using the resulting cDNA and *Pfu* polymerase (Stratagene, La Jolla, CA). The sequence-specific primers were designed according to the published cDNA sequences for SARS coronavirus strain BJ01 (GenBank accession No. AY278488). The oligonucleotide primers for nucleocapsid protein were NF: 5′-gaagatcttatgtctgataatggaccccaatc-3′ and NR: 5′-cggaattcttatgcctgagttgaatcag-3′. Primers for truncated S [S1 (amino acids 14–403), S2 (amino acids 370–770), S3 (amino acids 738–1196)] were S1F: 5′-ggggtaccgaccttgaccggtgcaccac-3′ and S1R: 5′-cggaattctcatcctggcgctatttgtc-3′; S2F: 5′-ggggtaccggcgtttctgccactaagttg-3′ and S2R: 5′-cggaattcctagacttgagcgaacacttc-3′; S3F: 5′-ggggtacccttctccaatatggtagcttttg-3′ and S3R: 5′-cggaattcctattgctcatattttccc-3′. The PCR products were then cloned into the pcDNAII vector (Invitrogen) and confirmed by DNA sequencing. For the expression of His-tagged proteins, the open reading frames of these positive clones were cloned into the pET-30a vector (Novagen) separately.

Protein expression and purification in E. coli

The pET-30a constructs were transformed into *E. coli* BL21 (DE3) cells (Novagen, Madison, WI), and the expression of recombinant proteins was induced by the addition of 0.1 mM isopropyl-l-thio-D-galactopyranoside (IPTG) at 37°C for 5 h. The cells were harvested by centrifugation and the pellet was resuspended in binding buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, 1 mM NaF, and 1 mM PMSF), sonicated, and centrifuged at 12,000 × *g* at 4°C for 30 min. The recombinant proteins were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and purified using Ni-nitrilotriacetic acid (Ni-NTA) columns according to the manufacturer's instructions (Qiagen, Hilgen, Germany). The purified protein concentration was determined using a BCA kit (Pierce, Rockford, IL), aliquoted, and stored at −80°C until use.

Western blotting

The recombinant SARS-CoV proteins S1, S2, S3, and N were separated by SDS–PAGE as described above and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked with a solution of 5% skim milk and 0.2% Tween

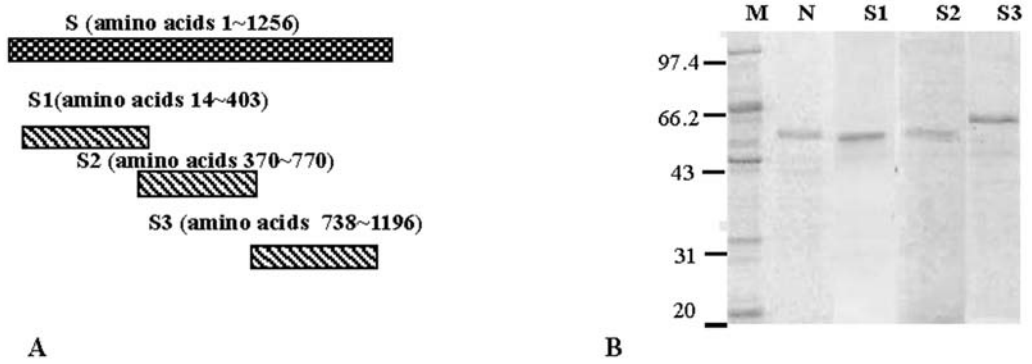

FIG. 1. Expression and purification of recombinant N, S1, S2, and S3 proteins in *E. coli*. (**A**) Schematic diagram of three overlapping fragments within the S protein. (**B**) SDS-PAGE of the expressed and purified recombinant proteins. Recombinant proteins were expressed in *E. coli* and purified using an Ni-NTA affinity column (Qiagen) and the purified recombinant proteins were separated by 15% SDS–PAGE, followed by Coomassie blue staining. Lane M denotes the protein markers.

20 in 50 mM Tris–HCl-buffered saline (TBS, pH 7.4) for 2 h at room temperature. Sera from convalescent patients with SARS (used at a dilution of 1:100) and anti-His-antibody (used at a dilution of 1:1000) (Sigma, St. Louis, MO) were allowed to react for 2 h at room temperature with the membranes, followed by incubation with an alkaline phosphatase-conjugated secondary antibody (Pierce) according to the manufacturer's instructions. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP; Pierce) was used as the substrate for membrane color development.

ELISA

Immulon-1 microtiter plates (Nunc, Naperville, IL) were coated overnight at 4°C with purified recombinant S1, S2, S3, or N protein (1 mg/ml) diluted in 0.05 M sodium carbonate buffer (pH 9.6; 100 ng protein/well), and blocked with 1% bovine serum albumin (BSA) for 2 h at 37°C . After the plates were washed with TBS containing 0.05% Tween-20 (TBST), the negative control sera and heat-inactivated sera from SARS patients, diluted in 0.1% BSA from 1:10 to 1:1280 (100 μl/well), were added to each well, and the plates were incubated for 60 min at 37°C. After three washes, 100 μl of horseradish peroxidase-conjugated goat antihuman IgG (Santa Cruz, Santa Cruz, CA), diluted 1:5000 in 0.1% BSA, was added to each well and the plates were incubated for 30 min at 37°C. The plates were rinsed six times with TBST and 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) solution was added to each well and the plates were incubated for 15 min at 37°C . The reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 to each well. Absorbance at 450 nm (A_{450}) was measured using a microplate reader (Model 550, Bio-Rad, Hercules, CA). The serum samples were examined in duplicate. The cutoff value was defined as the mean A_{450} of control samples plus two standard deviations (SD).

RESULTS

Expression and purification of recombinant SARS-CoV N, S1, S2, and S3 proteins in Escherichia coli

To acquire SARS-CoV recombinant proteins, the cDNAs encoding SARS-CoV structural proteins N and 3 truncated forms of the different domains of the S protein (S1, S2, and S3) were amplified by RT-PCR using total RNA from the lung tissue of a dead SARS patient. The open reading frames of these positive clones were inserted into the pET-30a vector and transformed into *E. coli* BL21 (DE3) cells for His-tagged recombinant protein expression. After purification using a Ni-NTA affinity column, as described in the Materials and Methods, the recombinant proteins were examined by 15% SDS–PAGE. As expected, the N, S1, S2, and S3 recombinant proteins were estimated to be approximately 53, 51, 51, and 57 kDa, respectively (Fig. 1). The purities of the four recombinant proteins were approximately 90%, and all reacted well with the anti-His antibody (data not shown). These results indicated that the N and truncated S proteins were expressed correctly and efficiently in *E. coli*. The recombinant proteins were used as antigens in subsequent studies, including Western blotting and ELISA.

Reactivity analysis of N and S proteins using sera from SARS patients

To characterize serological responses during SARS-CoV infection, a Western blotting assay was performed. We tested 50 serum specimens from 10 SARS patients using recombinant

TABLE 1. RESULTS FOR WESTERN BLOTTING FOR ANALYSIS OF IgG ANTIBODIES AGAINST SARS-CoV N AND S PROTEINS IN SERUM SPECIMENS FROM SARS PATIENTS

Patient no.	*Days after disease onset*	N	*S1*	*S2*	*S3*
1	7	+	−	−	−
	9	+	+	−	+
	12	+	+	−	+
	16	+	+	−	+
	18	+	+	−	+
	23	+	+	−	+
2	7	+	−	−	+
	12	+	−	−	+
	18	+	−	−	+
	25	+	−	−	+
3	9	+	−	−	−
	12	+	−	−	−
	15	+	−	−	−
	17	+	−	−	−
	22	+	−	−	−
4	9	+	+	−	+
	13	+	+	−	+
	15	+	+	−	+
	17	+	+	−	+
	22	+	+	−	+
5	9	−	−	−	+
	12	+	−	−	+
	16	+	−	−	+
	20	+	+	+	+
	24	+	+	+	+
6	7	−	−	−	+
	12	−	+	−	+
	14	−	+	+	+
	17	+	+	+	+
	24	+	+	+	+
7	12	−	−	+	−
	14	+	−	+	−
	17	+	+	+	+
	19	+	+	+	+
	25	+	+	+	+
8	6	−	−	−	−
	7	−	−	−	−
	9	−	−	−	−
	11	+	−	−	−
	14	+	−	−	−
9	7	−	−	−	−
	14	+	−	−	−
	18	+	−	−	−
	20	+	−	−	−
	22	+	−	−	−
10	7	−	−	−	−
	14	+	−	−	+
	17	+	−	−	+
	19	+	−	−	+
	22	+	−	−	+

proteins N, S1, S2, and S3 as antigens. After the purified proteins were transferred to nitrocellulose membranes, the immunoblot assay was performed as described above. At a 1:100 dilution, all 10 patients tested positive for the N-specific antibody, while five tested positive for S1, three for S2, and seven for S3 (Table 1). As shown in Figure 2, significant crossreactivities were observed between the sera and the recombinant antigens, although the patterns of reactivity were different among the three patients. Taken together, these results imply that: (1) the SARS-CoV N and S proteins are strong antigens and able to elicit humoral immunity during infection; and (2) the antigenicities of the recombinant proteins S1, S2, and S3 are different from one another. The antigenicity of the S3 protein was the highest, whereas that of S2 was the lowest among the three truncated S proteins. Tests for the presence of IgG antibody against S2 were negative in most cases, even in late clinical stages, but in patient 7 (P7), the S2 protein gave the strongest antigen signal among the 3 S proteins (Fig. 2). These results suggest an imbalanced reactivity or immunogenicity between the different domains of the S protein.

ELISA kinetics of IgG antibodies against SARS-CoV proteins N, S1, S2, and S3 in sera from SARS patients

To further characterize the immune response to SARS-CoV N and S proteins and to confirm the conclusions drawn by the results of the Western blotting assays, paired sera from the 10 SARS patients were also examined by ELISA. Using purified recombinant SARS-CoV N, S1, S2, and S3 proteins as coated antigens (1 mg/ml) separately, the 50 serum samples from the SARS patients and serum samples from healthy donors were serially diluted in 0.1% BSA from 1:10 to 1:1280 (100 μl/well) and were added to each well to measure the reactivity of the relevant IgG antibody. The positive rates of IgG reactivity against the four proteins at different time intervals after SARS infection were analyzed first. The proportion of patients with IgG reactivity against N, S1, S2, and S3 were, respectively, 28.57, 14.29, 14.29, and 14.29% at week 1, 77.78, 55.56, 44.44, and 66.67% at week 2, 100, 75, 75, and 87.5% at week 3, and 100, 77.78, 77.78, and 88.89% after 3 weeks (Fig. 3). These data demonstrated the stronger immunogenicity or antigenicity of the N and S3 proteins.

To evaluate the extent of immune responses to the proteins, the N, S1, S2, or S3 antigen-specific IgG titers from each patient's serum at different time points after the onset of illness were described as shown in Figure 3B–E. The data reveal that the titers increased with time. The average titers of IgG against recombinant proteins N, S1, S2, and S3 were, respectively, 3.50 ± 9.92, 1.69 ± 4.04, 1.69 ± 5.24, and 1.87 ± 5.24 at week 1, 95.92 ± 16.22, 18.82 ± 16.85, 10.56 ± 10.04, and 19.81 ± 14.33 at week 2, 697.92 ± 2.22, 66.44 ± 14.37, 36.23 ± 11.03, and 92.52 ± 8.39 at week 3, and 691.24 ± 2.24, 55.95 ± 11.25, 38.06 ± 10.04, and 84.29 ± 6.56 after 3 weeks (Fig. 4A and B). These data showed that the titer of antibody against antigen N is much higher than that against the S proteins, while the titer and positive rates for antibodies against the N and S3 proteins were higher than those against S1 and S2 during SARS-CoV infection. The results from the longitudinal dynamics analysis of the IgG antibodies to the recombinant N and S proteins match those of the Western blotting assay, implying that recombinant N and S3 might be ideal antigens for SARS-CoV antibody detection for diagnosis.

DISCUSSION

Viral infection elicits neutralizing antibodies in immunocompetent hosts (Holmes and Enjuanes, 2003). The detection of antibodies against SARS-CoV has been used to confirm the diagnosis of SARS. Presently, the most widely used methods for serodiagnosis of SARS-CoV infection are SARS-CoV-based ELISA and immunofluorescence assay (IFA), which require the preparation of viruses as a source of antigen. This requirement may reduce their accessibility for most clinical laboratories without a biosafety level 3 laboratory facilities (Ksiazek *et al*., 2003). The recombinant protein-based ELISA can be performed in general laboratories without containment facilities and allows the analysis of the immunogenic viral pro-

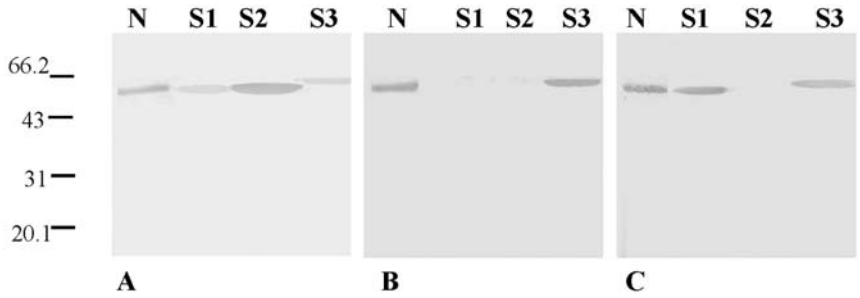

FIG. 2. Antigenic analysis of recombinant N, S1, S2, and S3 proteins by Western blotting. Partial typical reaction patterns between the recombinant antigens and the sera from different SARS convalescent patients are shown. The recombinant proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Each membrane was incubated with serum from a convalescent patient with SARS (used at a dilution of 1:100) followed by incubation with an alkaline phosphatase-conjugated secondary antihuman IgG (1:3,000 dilution) antibody. BCIP/NBT was used as the substrate for membrane color development. (**A**) Serum collected from patient 7 (P7) at day 25 after the onset of illness. (**B**) Serum collected from P10 at day 22. (**C**) Serum collected from P4 at day 22.

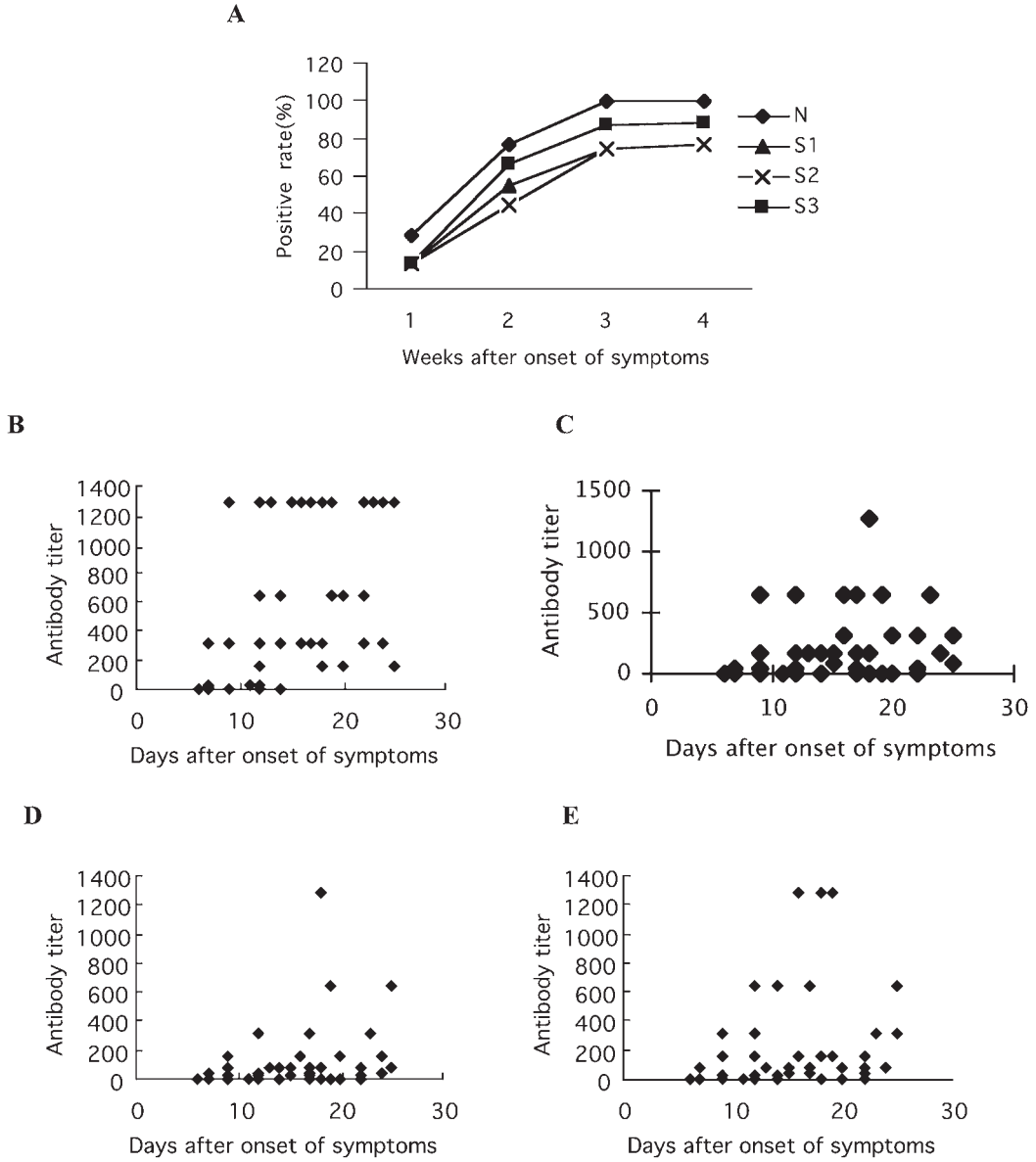

FIG. 3. Variability of positive rates and titers of IgG antibodies to recombinant N, S1, S2, and S3 proteins in sera from SARS patients. Using purified recombinant SARS-CoV N, S1, S2, and S3 proteins as coated antigens (1 mg/ml), 50 serum samples from 10 SARS patients at different time points after the onset of illness, and serum samples from healthy people, were diluted in 0.1% BSA from 1:10 to 1:1280 (100 μl/well) and added to each well to assay antibody reactivity against recombinant SARS-CoV proteins by ELISA. (**A**) Positive reactivity rates of IgG antibodies to recombinant N, S1, S2, and S3 proteins at different time points after SARS infection. (**B–E**) Kinetic of titers of IgG antibodies to recombinant N (**B**), S1 (**C**), S2 (**D**), and S3 (**E**) proteins in sera from SARS patients.

teins of SARS-CoV instead of using the highly infectious SARS-CoV itself.

In the present work, we expressed N and S proteins in an *E. coli* system. Due to the difficulty of expressing full-length S in *E. coli*, three overlapping fragments of S protein were expressed. We then assayed the antibodies against N and the 3 truncated fragments of the S protein in 50 serum samples from 10 SARS-infected patients by Western blotting and ELISA. Our data suggest that all of the SARS patients were positive for N protein-specific IgG. The IgG was detectable from day 7 after the onset of symptoms and was 100% at day 14 by Western blotting (Table 1). Most of the serum specimens showed increasing antibody titers to the N protein, reaching 1:697.92 $\pm$ 2.22 by week 3 (Fig. 4A). The positive rates and average titers of IgG antibodies against the recombinant truncated S proteins were lower than that of the N protein (Fig. 4A and B). Interestingly, in three SARS patients (P3, P8, P9), IgG antibodies against the S protein could not be detected, although they each

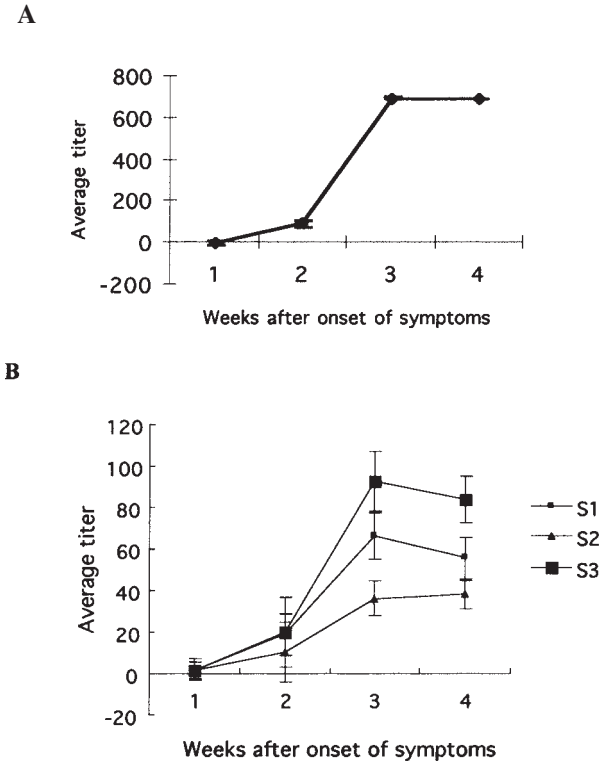

FIG. 4. Longitudinal profiles of IgG antibodies to recombinant N, S1, S2, and S3 proteins in SARS patients. (**A**) Titer of IgG antibodies to recombinant N; data are shown as the mean ± standard deviation. (**B**) Titers of IgG antibodies to recombinant S1, S2, and S3 proteins; data are shown as mean value ± standard deviation.

had anti-N protein IgG antibodies present (Table 1). One explanation for this may be that the current serological tests were unable to detect low antibody titers against the S protein and/or truncated S proteins expressed in an *E. coli* system lacking the correct posttranslational modifications, including complex folding, glycosylation, and oligomerization (Zeng *et al*., 2004). To our knowledge, this is the first report on the variability of IgG titers against the SARS-CoV S protein in SARS patients.

The antigenicity of the S1, S2, and S3 recombinant proteins were notably different from one another: the antigenicity of the S3 protein was higher than that of S1 or S2 in Western blotting assays, and the positive rate and average titer of anti-S3 IgG were also higher than the others. However, in patient 7, the S2 protein was the strongest antigen of the three truncated S proteins. Five serum IgG samples at different time frames after the onset of illness all recognized the S2 protein (Table 1) with a stronger reactivity than against the other S proteins (Fig. 2). These phenomena may be due to the different immune reactivities of SARS patients to S and/or the occurrence of nonconserved amino acid variations that may help the virus evade pressures from host immune responses. Further studies are needed to understand whether the differences between the immune responses against the three truncated S proteins are associated with the pathogenesis of SARS-CoV (Ruan *et al*., 2003).

Due to the strong antigenicity of the SARS-CoV N protein, it has been utilized in the serodiagnosis of SARS and studies on the seroprevalence of nonpneumonic SARS-CoV infections (Woo *et al*., 2004a, 2004b). However, very recent studies showed that the N protein IgG can be detected in some healthy people (Liu *et al*., 2004) and antigenic crossreactivities between the N protein of SARS-CoV and the polyclonal antisera of infectious peritonitis virus (FIPV), porcine transmissible gastroenteritis virus (TGEV), and canine coronavirus (CCoV) have been observed (Sun and Meng, 2004). The antibody against the SARS-CoV N protein also could be detected in sera from convalescent persons infected by HCoV-OC43 or HCoV-229E (Woo *et al*., 2004c). It has been reported that antibodies against human 229E- or OC43-like coronaviruses are widespread in the human population (McIntosh *et al*., 1970; Bradburne and Somerset, 1972). Therefore, it is possible that false positive cases could be detected using SARS-CoV N protein, or whole virus, as an antigen for SARS diagnosis and for identification of the SASR-CoV animal reservoirs.

The similarity between the amino acid sequences of the SARS-CoV S protein and other coronaviruses is quite low (20–27%) (Rota *et al*., 2003), and SARS-CoV S protein has specific T cell epitopes and a characteristic antigenicity compared with other coronaviruses (Liu *et al*., 2003). Thus, the possibility is slight that SARS-CoV S protein is antigenically crossreactive to other known coronaviruses. Although the titer of IgG antibodies against the S3 protein was much lower than against the N protein, the positive rate of antibodies against the S3 protein was similar to that of N, and in most cases, the antibody against S3 was detected earlier than (P5 and P6) or at least as early as (P2, P4, and P10) that of N (Table 1). Given such features, the S3 protein may be an ideal antigen for diagnostics, and in addition to the N protein, could eliminate the possible crossreactivity between SARS-CoV and other coronaviruses.

In conclusion, our studies suggest that using the recombinant protein N in combination with recombinant protein S3 as antigens may improve the specificity of SARS diagnosis and may serve as an effective, safe method for the serological diagnosis of SARS.

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Address reprint requests to:
Jianwei Wang, M.D., Ph.D.
National Institute for Viral Disease Control and Prevention
Chinese Center for Disease Control and Prevention
100 Ying Xin Jie, Xuan Wu Qu
Beijing 100052, People's Republic of China

E-mail: wangjw28@vip.sina.com

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