CLINICAL AND VACCINE IMMUNOLOGY, Mar. 2006, p. 409–414 Vol. 13, No. 3
1556-6811/06/$08.00+0 doi:10.1128/CVI.13.3.409–414.2006

False-Positive Results in a Recombinant Severe Acute Respiratory Syndrome-Associated Coronavirus (SARS-CoV) Nucleocapsid-Based Western Blot Assay Were Rectified by the Use of Two Subunits (S1 and S2) of Spike for Detection of Antibody to SARS-CoV

Mimoun Maache,[1]* Florence Komurian-Pradel,[1] Alain Rajoharison,[1] Magali Perret,[1] Jean-Luc Berland,[1] Stéphane Pouzol,[1] Audrey Bagnaud,[1] Blandine Duverger,[1] Jianguo Xu,[2] Antonio Osuna,[3] and Glaucia Paranhos-Baccalà[1]

Emerging Pathogens Department of bioMérieux, IFR128 BioSciences Lyon Gerland, CERVI, 21, Avenue Tony Garnier, 69365 Lyon Cedex 07, France[1]; State Key Laboratory for Infectious Disease Prevention and Control (China CDC), National Institute for Communicable Disease Control and Prevention, Changping, Beijing 100026, China[2]; and Biotechnology Institute, University of Granada, Cp 18071, Granada, Spain[3]

Received 20 September 2005/Returned for modification 31 October 2005/Accepted 27 December 2005

To evaluate the reactivity of the recombinant proteins expressed in *Escherichia coli* strain BL21(DE3), a Western blot assay was performed by using a panel of 78 serum samples obtained, respectively, from convalescent-phase patients infected with severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (30 samples) and from healthy donors (48 samples). As antigen for detection of SARS-CoV, the nucleocapsid protein (N) showed high sensitivity and strong reactivity with all samples from SARS-CoV patients and cross-reacted with all serum samples from healthy subjects, with either those obtained from China (10 samples) or those obtained from France (38 serum samples), giving then a significant rate of false positives. Specifically, our data indicated that the two subunits, S1 (residues 14 to 760) and S2 (residues 761 to 1190), resulted from the divided spike reacted with all samples from SARS-CoV patients and without any cross-reactivity with any of the healthy serum samples. Consequently, these data revealed the nonspecific nature of N protein in serodiagnosis of SARS-CoV compared with the S1 and S2, where the specificity is of 100%. Moreover, the reported results indicated that the use of one single protein as a detection antigen of SARS-CoV infection may lead to false-positive diagnosis. These may be rectified by using more than one protein for the serodiagnosis of SARS-CoV.

The severe acute respiratory syndrome (SARS) is a viral infectious disease caused by the human SARS-associated coronavirus (SARS-CoV) (5, 17, 20).

The SARS-CoV is an enveloped positive-stranded RNA virus with a genome of about 29.740 kb in length (2, 9). Its genomic organization is typical of that of coronaviruses, but the phylogenetic analysis and sequence comparison show that SARS-CoV is not closely related to any of the previously characterized coronaviruses with only an approximate 25 to 30% identity (23). In addition to the nonstructural proteins, the SARS-CoV genome encodes four structural proteins: envelope, membrane glycoprotein, nucleocapsid (N), and spike (S) (19). Each of these proteins plays a key role in the virus infection cycle and pathogenicity, especially the two major structural proteins such as nucleocapsid and spike proteins (7, 13, 14, 15).

Spike, a major structural glycoprotein of coronaviruses, is cleaved for many of them into two noncovalently associated subunits: S1 and S2 (15). The distal subunit (S1) contains the receptor-binding domain, which interacts with a cellular receptor ACE2 (angiotensin I converting enzyme 2), and the membrane-anchored subunit S2 contains a putative internal fusion peptide inducing membrane fusion to allow viral entry into a susceptible target cell. However, this phenomenon of cleavage is not yet clear for the spike of SARS-CoV (10, 15). The S protein is a main surface antigen, a factor of virulence, and a major neutralizing antigen capable of inducing protective immunity and eliciting immune responses during viral infection (3, 9, 10, 12, 24, 33, 34). For the known coronaviruses, the spike protein is recognized by antibodies to SARS-CoV, and it is considered one of the candidate antigens for the detection of SARS-CoV, owing to its high antigenicity (11).

The nucleocapsid protein appears to be the more conserved antigen among other viral structural proteins (6, 36) and is involved in important functions, such as the formation of helical nucleocapsid during the viral life cycle, and it has also been reported to activate the AP1 (activator protein1) signal transduction pathway (26). In addition to its physiological and structural roles, the nucleocapsid protein appears to be the major immunogenic antigen. Nucleocapsid protein is abundantly expressed during viral infection and is readily recognized by acute-phase sera from SARS patients and by T cells on the infected cell surface (4, 21, 25, 37). In addition, the involvement of N protein in the generation of primary humoral immune response was suggested (1, 28).

Antigenicity studies in other coronaviruses indicated that the N protein is one of the immunodominant antigens that

* Corresponding author. Mailing address: Emerging Pathogens Department of bioMérieux, CERVI, 21 Avenue Tony Garnier, 69365 cedex 07, Lyon, France. Phone: 33 04 37 28 24 13. Fax: 33 04 37 28 24 11. E-mail: mmaache@ugr.es.

TABLE 1. Primers used for amplification of genes coding for S1, S2, and N recombinant proteins

Gene	Residues	Primers (5′–3′)[a]	Cloning sites
S1	14–760	TCTCTC**TCTAGA**ATGGACCTTGACCGGAGCACCAC	XbaI
		CTCTCT**GGATCC**TTAGTGGTGATGGTGATGGTGAGAACCCCTCATTGT GTTGCGATCCTGTTCAGCAATACC	BamHI
S2	761–1190	TCTCTC**TCTAGA**ATGCGTGAAGTGTTCGCTCAAGTC	XbaI
		TCTCTC**GGATCC**TTAGTGGTGATGGTGATGGTGAGAACCCCTCATTTG CTCATATTTTCCCAATTCTTG	BamHI
N	1–1305	TCTCTC**TCTAGA**ATGTCTGATAATGGACCCCAATCAAACCAACGTAGTGC	XbaI
		CTCTCT**GGATCC**TTAGTGGTGATGGTGATGGTGAGAACCCCTCATTGC CTGAGTTGAATCAGCAGAAGCTCCA	BamHI

[a] Boldface type indicates restriction enzyme sites.

induce cross-reactive antibodies in high titers, whereas the S glycoprotein induces the serotype-specific and cross-reactive antibodies (21, 25).

Early detection and identification of SARS-CoV-infected patients is absolutely critical to prevent another SARS-CoV outbreak and the spread of SARS. However, the choice of a suitable system for the epidemiological study may allow an effective survey and control of the already infected and convalescent-phase patients. In this study, and by using Western blot assays, our results revealed that the S1 and S2 subunits of spike protein reacted only with confirmed positive serum samples and without any cross-reactivity with any of the healthy donors, which indicated that the S1 and S2 proteins are specific antigens for the diagnosis of SARS-CoV. The nucleocapsid protein has been reported to be a sensitive marker for the serodiagnosis of SARS-CoV (8). However, our results, while confirming its high sensitivity, also showed the nonspecific nature of this protein and indicated that the N protein reacted strongly to all healthy serum samples, giving a significant rate of false positives. In addition, the use of one single antigen for the detection or diagnosis of SARS-CoV gives limited information and might lead to false-positive results. Therefore, this study provides very useful information for choosing a suitable antigen system for the serodiagnosis of SARS-CoV infection.

MATERIALS AND METHODS

RNA extraction. RNA extraction was performed in a biosafety level 3 laboratory. RNA was extracted directly from plasma samples according to the manufacturer's instructions by using the miniMAG viral RNA mini kit (NucliSens bioMérieux, Boxtel, The Netherlands).

Constructions of plasmids for expression of S1, S2, and N genes of SARS-CoV.
(i) Spike protein. After computer analysis to predict and delete hydrophobic regions (hydrophobic cluster analysis), the spike protein of SARS-CoV (urbani strain) was divided into the S1 (residues 14 to 760) and S2 (residues 761 to 1190) subunits because the complete protein could not be well expressed in *Escherichia coli* strain BL21(DE3) (Novagen, Merck Eurolab, Fontenay Sous Bois, France).

By using reverse transcription and PCR (Invitrogen, Cergy-Pontoise, France) with a specific pair of primers (Table 1), the genes encoding the S1 and S2 proteins were amplified. Both expected fragments coding for S1 and S2 were digested with XbaI/BamHI and separately cloned into the expression plasmid pET21b opened with the same restriction enzymes to generate the expression vectors pET21b-S1 and pET21b-S2.

(ii) Nucleocapsid protein. The complete gene coding for N protein was amplified by using reverse transcription-PCR (Invitrogen, Cergy-Pontoise, France) and specific primers (Table 1). After being digested with XbaI/BamHI, the PCR product coding for the N protein was inserted into the expression vector digested with the same enzymes to generate the expression vector pET21b-N.

The three inserts coding for the three proteins were sequenced to confirm the exactness of the S1, S2, and N protein sequences and proper in-frame ligation. In addition, all proteins were expressed as C-terminal His_6 tag proteins to facilitate their purification by using Ni^{2+}-nitrilotriacetic acid (NTA) agarose resin (QIAGEN S.A., Courtaboeuf, France).

Expression and purification of recombinant S1, S2, and N proteins. The three expression vectors pET21b-S1, pET21b-S2, and pET21b-N were separately transformed into *Escherichia coli* strain BL21(DE3) (Novagen, Merck Eurolab, Fontenay Sous Bois, France). The transformed BL21(DE3) host cells were incubated for 6 to 8 h at 37°C in 4 ml of Luria-Bertani (LB) medium broth (bioMérieux, Lyon, France) containing 100 μg/ml of ampicillin (Roche Diagnostics, Meylan, France) as an antibiotic. The cultures were then diluted 1:25 (vol/vol) in LB medium and incubated overnight at 37°C under agitation (225 rpm). After overnight growth, the cultures were diluted with LB medium 1:50 (vol/vol), and after being shaken, the cells grew to an optical density at 600 nm of 0.6 to 0.8. For expression, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM and then the bacteria were incubated at 37°C at 250 rpm for an additional 4 to 5 h, followed by centrifugation at 3,200 × *g* for 15 min to get the cell pellets. The pellets were suspended separately in 10 ml of 20 mM Tris/HCl buffer (pH 7.4) containing 200 mM NaCl, 100 mM PMSF (paramethyl sulfonyl fluoride), 1 mM Benzonase, and 5 mM β-mercaptoethanol, and lysed by sonication with an ultrasonic processor (Mi-sonix, Inc., Farmingdale, N.Y.). The resulting lysates were centrifuged at 10,400 × *g* for 13 min at 4°C. In addition, the pellets and the supernatants were analyzed by 12% SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). All three recombinant proteins expressed in *E. coli* BL21(DE3) mainly formed inclusion bodies and released limited soluble forms in the cytoplasm. Consequently, the recombinant proteins could not be purified directly from the soluble fractions. To obtain a high protein yield and to facilitate their purification, the bacterial pellets were treated separately with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 5 mM β-mercaptoethanol, and 8 M urea for S1 and N and 6 M guanidine for S2, followed by a strong probe sonication to completely dissolve the inclusion bodies. After centrifugation at 10,400 × *g* for 13 min at 4°C, the supernatants were applied separately to Ni^{2+}-NTA resin equilibrated with 5 volumes (5 ml/ml) of binding buffer (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 1 mM PMSF, 5 mM β-mercaptoethanol, and 8 M urea) (pH 8.0) at room temperature. For the three proteins, the columns were then washed successively with 10 volumes of binding buffer containing 20 mM imidazole. In the case of the polyhistidine-tagged S1 and S2 proteins, the elution was finally performed with 20 mM Tris-HCl (pH 7.4) containing 100 mM imidazol, 300 mM NaCl, and 1 mM PMSF. However, the tagged N protein was eluted with sodium phosphate buffer (pH 4.0) containing 8 M urea. To eliminate imidazole, the eluates were dialyzed overnight against 50 mM sodium phosphate buffer (pH 8.0). The high purity of purified proteins was analyzed by SDS-PAGE and confirmed by Western blot assay.

Verification of the expressed and purified S1, S2, and N proteins by using SDS-PAGE and Western blot analysis. The Western blot analysis was performed to verify the protein expressions and antigenicity and, principally, to test the sensitivity of the purified recombinant S1, S2, and N proteins to the human convalescent-phase serum samples with SARS-CoV. The recombinant proteins were separated by 12% SDS—PAGE, and the protein bands were then transferred electrophoretically to nitrocellulose membranes (Whatman, Gerbershausen, Germany). The membranes were subsequently blocked in blocking buffer (phosphate-buffered saline, pH 7.4, 0.1% Tween 20, and 5% skim milk) and probed at room temperature for 1 h with rabbit anti-SARS-CoV polyclonal antibody or with anti-His-tagged monoclonal antibody raised in mouse. After being rinsed for 20 min in phosphate-buffered saline containing 0.05% Tween 20, the bound antibodies were detected either with anti-rabbit or with anti-mouse immunoglobulin G (IgG) conjugated with alkaline phosphatase at a dilution of

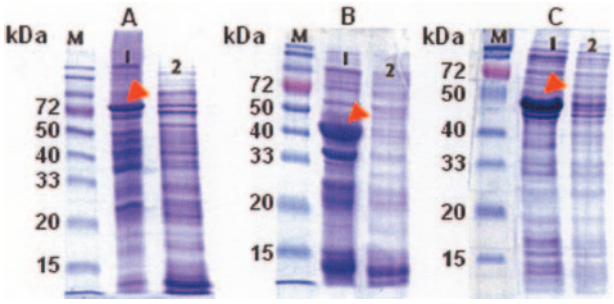

FIG. 1. Expression of the SARS-CoV S1, S2, and N recombinant proteins. SDS-PAGE and Coomassie blue staining of the expressed SARS-CoV S1, S2, and N recombinant proteins in *E. coli* BL21(DE3) after the IPTG addition are shown in panels A, B, and C, respectively. The arrows indicate the expressed recombinant S1 (74 kDa), S2 (47 kDa), and N (49 kDa) proteins. Protein markers (M), cell lysate pellets (lanes A1, B1, C1), and cell lysate supernatants (lanes A2, B2, C2) of the S1, S2, and N proteins, respectively, are shown.

1:10,000. The immunoprecipitated bands were developed by using a substrate mixture of *O*-dianisidine tetrazotized and beta-naphthyl acid phosphate (Sigma-Aldrich, Lyon, France) in borate buffer (pH 9.5) or with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, Lyon, France) followed by chemiluminescence reagents (Amersham Biosciences Europe GmbH, Orsay, France) and exposed to X-ray film for 1 to 3 min. To test the serum reactivities, the Western blot assay was performed in a biosafety level 3 laboratory by using the purified His_6-tagged recombinant S1, S2, and N proteins. After being loaded separately into each continuous well of 12% SDS–PAGE, the purified recombinant proteins were electroblotted onto a nitrocellulose membrane. The blot was cut into strips, and the strips were incubated separately with each of 78 serum samples diluted 1:1,500 for 5 h. The incubation with peroxidase-conjugated secondary anti-human antibody (1:10,000) and the strips revelation were performed by following the protocol detailed above.

Specimens and patients. A panel of 78 serum samples was used in a Western blot assay. This panel includes 30 convalescent-phase serum samples from Beijing and inner Mongolia (collected 20 to 25 days after disease onset) obtained from the Chinese Center of Disease Control and Prevention (Beijing, China) and confirmed for SARS-CoV infection clinically by the World Health Organization criteria (World Health Organization case definition of surveillance of severe acute respiratory syndrome [http://www.who.int/CSR/SARS/casedefinition/en/]) and 10 serum samples obtained from healthy Chinese donors provided by the same center. In addition, another 38 negative serum samples were purchased from EFS (Etablissement français du sang, Lyon, France), corresponding to healthy French donors and collected 2 years before the outbreak of SARS.

Rabbit anti-SARS-CoV polyclonal antibodies were prepared by immunizing rabbits with the SARS-CoV and were kindly provided by Sanofi-Pasteur (Marcy l'Etoile, Lyon, France). All serum samples were stored at −80°C until use.

RESULTS

Expression and purification of recombinant S1, S2, and N proteins. SDS-PAGE analysis of cell extracts from strains producing recombinant S1, S2, and N proteins revealed that the three proteins were successfully and abundantly expressed after IPTG addition. The size of each protein approximately corresponds to the predicted molecular mass, which were determined to be about 74 kDa, 47 kDa, and 49 kDa for the S1, S2, and N proteins, respectively (Fig. 1A, B, and C). The expression of recombinant S1, S2, and N proteins was confirmed by a Western blot analysis showing a positive reaction against monoclonal antihistidine antibody at the level of the expected molecular mass (Fig. 2A). By using Ni^{2+}-NTA resin, the recombinant polyhistidine-tagged proteins were successfully purified, and as expected, the SDS-PAGE analysis showed that each single pure band corresponded to the predicted size of the S1, S2, and N proteins (Fig. 2B). Antigenicity analyses of purified proteins were performed and confirmed by Western blot assay against SARS-CoV polyclonal antibodies raised in rabbits, as shown in (Fig. 2C) where the recombinant proteins reacted strongly. According to our finding, different recombinant SARS-CoV proteins may be used for the diagnostic test of SARS-CoV infection. However, the effectiveness of each protein depends on its specificity.

Western blot performance of recombinant purified S1, S2, and N proteins against SARS-CoV and healthy serum. The analysis of 78 serum samples by Western blot (Table 2) showed that almost all convalescent-phase specimens with SARS-CoV developed antibodies against the purified recombinant S1, S2, and N proteins. However, the degree of reactivity varied according to the antigen and the serum sample.

The results revealed that the S1 protein showed strong immunoreactivity (+++) with 21 of 30 serum samples, moderate immunoreactivity (++) with 4 of 30 samples, and weak immunoreactivity (+) with 5 of 30 samples. In addition, no signal (−) was observed with any negative serum samples from healthy donors, neither with those obtained from China (10 serum samples) nor with those obtained from France (38 serum samples) (specificity of 100%). The S2 protein showed strong reactivity (+++) with 16 of 30 samples, moderate reactivity (++) with 6 of 30 samples, weak reactivity (+) with 4 of 30 samples, no signal (−) with 4 of 30 samples (sensitivity of

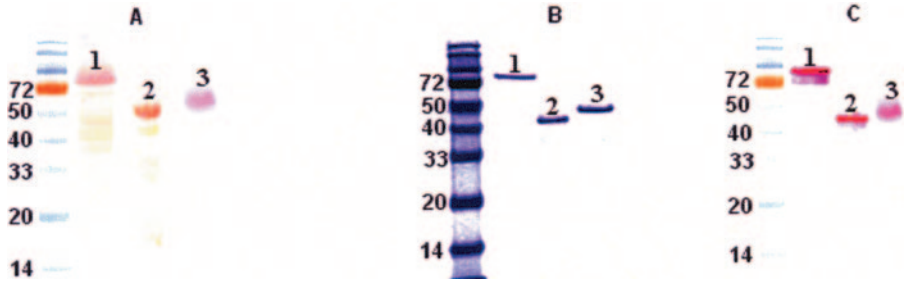

FIG. 2. Expression and purification of SARS-CoV S1, S2, and N recombinant proteins. (A) Western blot analysis of the expressed SARS-CoV S1, S2, and N recombinant proteins performed by using His_6-tagged monoclonal antibody. Protein markers (M) and cell lysate pellets of the expressed S1 (74 kDa), S2 (47 kDa), and N (49 kDa) proteins (lanes A1, A2, A3, respectively) are shown. (B) Purity of purified proteins analyzed by SDS-PAGE and Coomassie blue staining. Protein markers (M) and purified S1, S2, and N proteins (lanes B1, B2, B3, respectively) are shown. (C) Western blot analysis of purified recombinant proteins detected by polyclonal antibody to SARS-CoV raised in rabbits. Protein markers (M) and purified S1, S2, and N proteins (lanes C1, C2, and C3, respectively) are shown.

TABLE 2. Individual reactivity of serum samples obtained from convalescent-phase patients with SARS-CoV infection against S1, S2, and N recombinant proteins by Western blot assays[a]

Serum sample no.	Reactivity with recombinant protein		
	S1[b]	S2[b]	N[c]
1	+++	+++	+++
2	+++	+++	+++
3	+++	+++	+++
4	+++	+	+++
5	+++	++	+++
6	+++	+++	+++
7	+++	+++	+++
8	+++	+++	+++
9	+++	+++	+++
10	+++	+++	+++
11	+++	++	+++
12	+++	+++	+++
13	+++	+++	+++
14	+++	+++	+++
15	+++	++	+++
16	+++	+++	+++
17	+++	+	+++
18	+++	++	+++
19	+++	+++	+++
20	+++	++	+++
21	+++	++	+++
22	+	+++	++
23	+	+++	++
24	++	+++	+++
25	++	−	+++
26	++	−	+++
27	++	+	+++
28	+	+	+++
29	+	−	+++
30	+	−	+++

[a] Table represents only the immunoreactive patterns shown by the confirmed convalescent-phase patients with SARS-CoV (30 serum samples). +++, strong immunoreactivity; ++, moderate immunoreactivity; +, weak immunoreactivity; −, negative (no signal was observed).

[b] No cross-reactivity (−) was shown with any of the healthy serum samples in western blot assay based on recombinant S1 and S2 proteins.

[c] Western blot assay based on N recombinant protein showed a moderate reaction (++) with all serum samples from healthy donors from France (38 serum samples) and from China (10 serum samples).

86.6%), and no cross-reactivity (−) with any of healthy serum samples (specificity of 100%). For the nucleocapsid protein (N), the results demonstrated that this protein evoked a strong immunoreactivity (+++) with 28 of 30 serum samples and moderate immunoreactivity (++) with 2 of 30 samples. In addition, we have found that the recombinant N protein also reacted either with all healthy serum samples obtained from China (10 of 10 samples tested) or with all those obtained from healthy French donors (38 of 38), giving then false-positive results. Hence, reacting with all negative serum samples indicated the nonspecific nature of recombinant N protein, which is not the case when using both recombinant S1 and S2 proteins as diagnostic antigens.

The band intensities of three recombinant proteins against the same serum tested indicated that both S1 and N proteins generally showed an equivalent and a stronger reactivity (+++) than S2, except for positive serum samples (no. 22 and 23) where the intensity of the reaction against S2 protein is higher (+++) than that showed in the case of N (++) and S1 (++) proteins. For the other positive serum samples (no. 25, 26, 29, and 30), the reactivity is high (+++), moderate (++), and negative (−) against N, S1, and S2, respectively. These results indicate that the S1 (100% of sensitivity) and N (100% of sensitivity) proteins are more sensitive than the S2 subunit (86.6% of sensitivity). However, S1 and S2 recombinant proteins (100% of specificity) are much more specific than N recombinant protein (0% of specificity) with the tested sera.

DISCUSSION

To evaluate the specificity and sensitivity of each recombinant protein produced in *Escherichia coli* BL21(DE3) and the diagnostic efficacy of Western blot assay for SARS-CoV, we used a total of 78 serum samples, where 30 sera were obtained from convalescent-phase patients with SARS-CoV infection, 10 sera were collected from healthy Chinese donors, and other 38 healthy serum samples obtained from healthy French donors.

Because of the difficulties in expressing the full-length protein and according to computer analysis, the predicted hydrophobic cluster was deleted and the spike glycoprotein was divided in two portions called S1 and S2. Separately, the two portions of spike were expressed abundantly in *E. coli* strain BL21(DE3). By using Western blot assay, the expressed proteins showed high antigenicity and have been shown to be recognized by antibodies to SARS-CoV raised in rabbits and by convalescent-phase patient serum samples infected with SARS-CoV.

With regard to the nucleocapsid, the full-length protein was expressed in *E. coli* BL21(DE3). As with the spike protein, the nucleocapsid was recognized by antibodies raised against SARS-CoV and collected during the convalescent phase of infection from patients infected with SARS-CoV, indicating that the N protein is also antigenic.

The Western blot analysis revealed that almost all convalescent-phase patients had antibodies against S1 and S2. By comparing the reaction intensities of the two subunits of spike, we found that S1 showed a stronger immunoreactivity than S2. Therefore, some serum samples that reacted strongly to the S1 domain did not recognize or reacted weakly with the S2 domain. This may be due either to the antigenic dependence conformation of the S2 and S1 domains or to the high antigenicity of the S2 protein. These observations are consistent with previous findings that the full spike protein of SARS-CoV contains multiple linear immunodominant sites that are capable of inducing site-specific antibody responses during infection (10). In addition, the S1 protein appears with six antigenic sites, whereas only two antigenic sites are located in S2 (8, 35). Both S1 and S2 reacted with different convalescent-phase sera, and no reactivity was shown with any of healthy serum samples, which indicated that the S1 and S2 are specific antigens, in particular the S1 protein, owing to its reactivity to all positive sera (30 of 30 samples) and the existence of the major immunodominant epitope (residues 528 to 635 of S1), as proven by He and coworkers (10). The comparison study of the reactivity of serum samples obtained from convalescent-phase patients against S1 and S2 may provide useful information for the serodiagnosis of SARS-CoV and indicated that the S1 portion could be a primary and primordial target for the specific antibodies which could be induced abundantly and persist for a

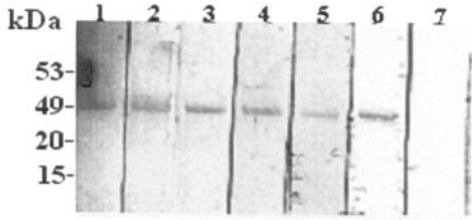

FIG. 3. Western blot analysis showing an example of cross-reactivity by serum samples from healthy donors to N protein. Lanes 1 to 6 show strips that were incubated with serum samples from healthy donors; lane 7 shows a strip incubated only with the peroxidase-conjugated anti-human IgG as a secondary control.

long time. The entire spike protein is a major requirement for the induction of protective immunity, and this property is probably associated with S1, as is the case for other coronavirus strains such as infectious bronchitis virus (15, 18, 35). These results strongly suggest that S1 is a specific antigen and could be the ideal marker for the diagnosis of SARS-CoV infection.

The high sensitivity of N protein in detecting antibodies to SARS-CoV may be due to the abundance of IgG anti-N which is the first and most abundantly released immunoglobulin during the acute and convalescence phases of disease (22, 25). In addition, the N protein is considered free of glycosylation; subsequently, its immunogenicity is not modified during expression in a prokaryotic system. On the other hand, spike is a major viral component protein with 24 glycosylation sites which may affect its immunological characteristics (31). The analysis of Western blot assay based on recombinant N protein revealed that all healthy serum samples cross-reacted with N protein. Thus, we suspected that the cross-reactivity may be due to various factors. Among them, we can first highlight the serum origin (China). We supposed that the healthy donors from China could be in contact with SARS-infected patients or with people with SARS contact history. However, the use of serum samples obtained from healthy French donors who had never been in a risk area and were collected 2 years before the outbreak of SARS did not affect the profile reactivity and indicated that the N protein reacted to all healthy serum samples with moderate protein band intensities, such as that observed for SARS-CoV-related serum samples (Fig. 3). Second, we suspected the high amount of antigen used to perform the Western blot analysis. However, when reducing the quantity of protein, we noted that the cross-reactivity with healthy serum samples still persists, independent of the quantity of protein used in the Western blot assay. Finally, we speculated about the integrity and the purity of the recombinant N protein. We supposed that the protein may be altered during the expression or purification process. However, mass spectrometry analysis (data not shown) indicated that the N protein was pure and corresponded to the mass of the nucleocapsid protein of SARS-CoV. The false-positive results with healthy donor serum samples may be explained as follows. First, there is serological cross-reactivity with other known coronaviruses such as human coronavirus OC43 (HCoV-OC43) and human coronavirus 229E (HCoV-229E). Indeed, the nucleocapsid is the most conserved antigen among other structural proteins of coronaviruses (9, 32). Previous reports expected that the antibodies against these human coronaviruses (HCoV-229E and HCoV-OC43) are widespread in the human population, which could produce a cross-reactivity in SARS diagnosis (16). Second, there is cross-reactivity with an eventual undetected pathogen or with human tissue antigens where N protein may share sequence homologies. Third, the positive reaction may be due not only to cross-reactivity with known coronaviruses or with other undetected pathogens but to the avidity of IgG to recombinant nonglycosylated protein, knowing well that the N protein is a nonglycosylated protein (29).

The false-positive results are not limited to the diagnosis of SARS-CoV strain but they have also been detected in the diagnosis of other coronavirus strains like HCoV-OC43 and HCoV-229E by using the nucleocapsid protein as the diagnostic antigen (30). In this way, our results are consistent with others in the literature that the use of N protein in the diagnosis of SARS-CoV could produce false-positive results. In addition, Guan and coworkers demonstrated that the N protein of coronaviruses reacted to 15 of 18 healthy serum samples used as negative controls (8).

The nucleocapsid was reported to be a sensitive marker for SARS-CoV detection (27). The data obtained in this study confirm its high sensitivity but also revealed the nonspecific nature of the N protein. Moreover, the false-positive rate of the antibodies to N protein showed by immunoblot assay was significantly high, which might pose problems for serodiagnosis, epidemiological survey, and control of SARS-CoV. Thus, we suppose that the use of one single antigen, such as N protein, remains insufficient and does not appear to provide good and trustworthy diagnostic information. We suggest that, if it is necessary to use N recombinant protein to diagnose SARS-CoV infection, owing to its high sensitivity, it is important to identify the main immunoreactive N epitope instead the complete N protein, to obtain a better specificity. In addition, it is preferable to improve by using other SARS-CoV antigens, such as S1 and S2, as a suitable system for specific and sensitive serodiagnosis and epidemiological study of SARS-CoV.

REFERENCES

1. **Ai, X. R., H. X. You, Y. Y. Kong, G. Z. Yang, Y. Z. Zhang, Y. Wang, and X. F. Wu.** 2004. Expression, purification and sublocalization of SARS-CoV nucleocapsid protein in insect cells. Acta Biochim. Biophys. Sin. **36:**754–758.
2. **Bi, S., E. Qin, Q. Zhu, M. Yu, B. Fan, G. Chang, B. Si, Z. Zhenpeng, Z. Haiqing, Z. Yilin, Z. Hui, Z. Jun, Z. Xiuqing, L. Bin, Z. Changqing, L. Songgang, T. Xuehai, L. Siqi, D. Wei, W. Jun, K. W. Gane, Y. Jun, W. Jian, Z. Qingyu, and H. Yang.** 2003. Complete genome sequences of the SARS-CoV: the BJ Group (Isolates BJ01-BJ04). Genomics Proteomics Bioinformatics **1:**180–192.
3. **Cavanagh, D.** 1995. The coronavirus surface glycoprotein. *In* S. G. Siddell, Jr. (ed.), The *Coronaviridae*. Plenum Press, New York, N.Y.
4. **Che, X. Y., W. Hao, L. W. Qiu, Y. X. Pan, Z. Y. Liao, H. Xu., J. J. Chen, J. L. Hou, P. C. Woo, and S. K. Lau.** 2003. Antibody response of patients with severe acute respiratory syndrome (SARS) to nucleocapsid antigen of SARS-associated coronavirus. Di Yi Jun Yi Da Xue Xue Bao **23:**640–642.
5. **Drosten, C., S. Gunther, W. Preiser, S. V. Werf, H. R. Brodt, S. Becker, H. Rabenau, M. Panning, L. Kesnikova, R. A. Fouchier, A. Berger, A. M. Burguière, J. Cinatl, M. Eickmann, N. Escriou, K. Grywna, S. Kramme, J. C. Manuguerra, S. Müller, V. Rickerts, M. Stürmer, S. Vieth, H. D. Klenk, and H. W. Doerr.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1967–1976.
6. **Fiscus, S. A., and Y. A. Teramoto.** 1987. Antigenic comparison of feline coronavirus isolates: evidence for markedly different peplomer glycoproteins J. Virol. **61:**2607–2613.
7. **Gallagher, T. M., and M. J. Buchmeier.** 2001. Coronavirus spike proteins in viral entry and pathogenesis. Virology **279:**371–374.
8. **Guan, M., H. Y. Chen, P. H Tan, S. Shen, P. Goh, Y. Tan, P. H. Pang, Y. Lu, P. Y. Fong, and D. Chin.** 2004. Use of viral lysate antigen combined with

recombinant protein in Western immunoblot assay as confirmatory test for serodiagnosis of severe acute respiratory syndrome. Clin. Diagn. Lab. Immunol. **11:**1148–1153.
9. **Han, D. P., H. G. Kim, Y. B. Kim, L. L. Poon, and M. W. Cho.** 2004. Development of a safe neutralization assay for SARS-CoV and characterization of S-glycoprotein. Virology **326:**140–149.
10. **He, Y., Y. Zhou, H. Wu, B. Luo, J. Chen, W. Li, and S. Jiang.** 2004. Identification of immunodominant sites on the spike protein of severe acute respiratory syndrome (SARS) coronavirus: implication for developing SARS diagnostics and vaccines. J. Immunol. **173:**4050–4057.
11. **Ho, T. Y., S. L. Wu, S. E. Cheng, Y. C. Wei, S. P. Huang, and C. Y. Hsiang.** 2004. Antigenicity and receptor-binding ability of recombinant SARS coronavirus spike protein. Biochem. Biophys. Res. Commun. **313:**938–947.
12. **Hofmann, H., K. Hattermann, A. Marzi, T. Gramberg, M. Geier, M. Krumbiegel, S. Kuate, K. Uberla, M. Niedrig, and S. Pohlmann.** 2004. S protein of severe acute respiratory syndrome-associated coronavirus mediates entry into hepatoma cell lines and is targeted by neutralizing antibodies in infected patients. J. Virol. **78:**6134–6142.
13. **Holmes, K. V.** 2003. SARS coronavirus: a new challenge for prevention and therapy. J. Clin. Investig. **111:**1605–1609.
14. **Holmes, K. V.** 2003. SARS-associated coronavirus. N. Engl. J. Med. **348:** 1948–1951.
15. **Holmes, K. V., and L. Enjuanes.** 2003. Virology: the SARS coronavirus: a postgenomic era. Science **300:**1377–1378.
16. **Huang, L. R., C. M. Chiu, S. H. Yeh, W. H. Huang, P. R. Hsueh, W. Z. Yang, J. Y. Yang, I. J. Su, S. C. Chang, and P. J. Chen.** 2004. Evaluation of antibody responses against SARS coronaviral nucleocapsid or spike proteins by immunoblotting or ELISA. **73:**338–346.
17. **Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson.** 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. **348:**1953–1966.
18. **Lu, L., I. Manopo, B. P. Leung, H. H. Chng, A. E. Ling, L. L. Chee, E. E. Ooi, S. W. Chan, and J. Kwang.** 2004. Immunological characterization of the spike protein of the severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. **42:**1570–1576.
19. **Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, W. A. Brooks, Y. S. N. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, and R. L. Roper.** 2003. The genome sequence of the SARS-associated coronavirus. Science **300:**1399–1404.
20. **Peiris, J. S., S. T. Lai, L. L. Poon, Y. Guan, L. Y. Yam, W. Lim, J. Nicholls, W. Yee, W. W. Yan, M. T. Cheung, V. C. Cheng, K. H. Chan, D. N. Tsang, R. W. Yung, K. Y. Yuen, and members of the SARS study group.** 2003. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet **361:**1319–1325.
21. **Poon, L. L., O. K. Wong, K. H. Chan, W. Luk, K. Y. Yuen, and J. S. Peiris.** 2003. Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS). Clin. Chem. **49:**953–955.
22. **Ren, Y., Z. Zhou, J. Liu, L. Lin, S. Li, H. Wang, J. Xia, Z. Zhao, J. Wen, C. Zhou, J. Wang, J. Yin, N. Xu, and S. Liu.** 2003. A strategy for searching antigenic regions in the SARS-CoV spike protein. Genomics Proteomics Bioinformatics **1:**207–215.
23. **Rota, P. A., M. S. Oberste, S. S. Monroe, W. A. Nix, R. Campagnoli, J. P. Icenogle, et al.** 2003. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science **300:**1394–1399.
24. **Saif, L. J.** 1993. Coronavirus immunogens. Vet. Microbiol. **37:**285–297.
25. **Shi, Y., Y. Yi, P. Li, T. Kuang, L. Li, M. Dong, M. Qingjun, and C. Cao.** 2003. Diagnosis of severe acute respiratory syndrome (SARS) by detection of SARS coronavirus nucleocapsid antibodies in an antigen-capturing enzyme-linked immunosorbent assay. J. Clin. Microbiol. **41:**5781–5782.
26. **Surjit, M., L. Boping, J. Shahid, T. K. Vincent, and S. K. Lal.** 2004. The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. Biochem. J. **383:**13–18.
27. **Tan, Y. J., P. Y. Goh, B. C. Fielding, S. Shen, C. F. Chou, J. L. Fu, H. N. Leong, Y. S. Leo, E. E. Ooi, A. E. Ling, S. G. Lim, and W. J. Hong.** 2004. Profile of antibody responses against SARS-coronavirus recombinant proteins and their potential use as diagnostic markers. Clin. Diagn. Lab. Immunol. **11:**362–371.
28. **Wege, H., A. Schliephake, H. Korner, E. Flory, and H. Wege.** 1993. An immunodominant CD4+ T cell site on the nucleocapsid protein of murine coronavirus contributes to protection against encephalomyelitis. J. Gen. Virol. **74**(Pt 7)**:**1287–1294.
29. **Wei, L., X. D. Wu, M. Shi, R. F. Yang, Y. Y. He, C. Bian, T. L. Shi, S. Yang, X. Zhu, W. Jiang, Y. X. Li, L. Yan, Y. Y. Ji, Y. Lin, G. Lin, L. Tian, J. Wang, H. X. Wang, Y. H. Xie, G. Pei, J. R. Wu, and B. Sun.** 2005. Synthetic peptides derived from SARS coronavirus S protein with diagnostic and therapeutic potential. FEBS Lett. **579:**2130–2136.
30. **Woo, P. C., S. K. Lau, B. H. Wong, H. Tsoi, A. M. Fung, K. H. Chan, W. T. Hui, G. S. Kwan, K. P. Victoria, J. S. Peiris, R. B. Couch, and K. Yuen.** 2004. False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OC43 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide. J. Clin. Microbiol. **42:**5885–5888.
31. **Woo, P. C., S. K. Lau, B. H. Wong, H. Tsoi, A. M. Fung, K. Chan, K. P. Victoria, J. S. Peiris, and K. Yuen.** 2004. Detection of specific antibodies to severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein for serodiagnosis of SARS coronavirus pneumonia. J. Clin. Microbiol. **42:** 2306–2309.
32. **Wu, X. D., B. Shang, R. F. Yang, H. Yu, Z. H. Ma, X. Shen, Y. Y. Ji, Y. Lin, Y. D. Wu, G. M. Lin, L. Tian, X. Q. Gan, S. Yang, W. H. Jiang, E. H. Dai, X. Y. Wang, H. L Jiang, Y. H. Xie, X. L. Zhu, G. Pei, L. Li, J. R. Wu, and B. Sun.** 2004. The spike protein of severe acute respiratory syndrome (SARS) is cleaved in virus infected Vero-E6 cells. Cell Res. **14:**400–406.
33. **Yu, H., Y. Yang, W. Zhang, Y. H. Xie, J. Qin, Y. Wang, H. B. Zheng, G. P. Zhao, S. Yang, and W. H. Jiang.** 2003. Expression and purification of recombinant SARS coronavirus spike protein. Acta Biochim. Biophys. Sin. **35:**774–778.
34. **Yuxian, H., H. Lu, S. Pamela, Z. Yusen, and J. Shibo.** 2005. Receptor-binding domain of severe acute respiratory syndrome coronavirus spike protein contains multiple conformation-dependent epitopes that induce highly potent neutralizing antibodies. J. Immunol. **174:**4908–4915.
35. **Zhang, H., G. Wang, J. Li, Y. Nie, X. Shi, G. Lian, W. Wang, X. Yin, Y. Zhao, X. Qu, M. Ding, and H. Deng.** 2004. Identification of an antigenic determinant on the S2 domain of the severe acute respiratory syndrome coronavirus spike protein neutralizing antibodies J. Virol. **78:**6938–6945.
36. **Zhao, P., J. Cao, L. J. Zhao, Z. L. Qin, J. S. Ke, W. Pan, H. Ren, J. G. Yu, and Z. T. Qi.** 2005. Immune responses against SARS-coronavirus nucleocapsid protein induced by DNA vaccine. Virology **331:**128–135.
37. **Zhu, M. S., Y. Pan, H. Q. Chen, Y. Shen, X. C. Wang, Y. J. Sun.** 2004. Induction of SARS-nucleoprotein-specific immune response by use of DNA vaccine. Immunol. Lett. **92:**237–243.