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

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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
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 a 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 antigenic 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 (NucleiSens 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 (urban 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 Souav 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 in 5 ml of 20 mM Tris/HCl buffer (pH 7.4) containing 200 mM NaCl, 100 mM PMSF, and lysed by sonication with an ultrasonic processor (Misonix, 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 guanidine 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 Ni²⁺-NTA resin equilibrated with 5 volumes of 20 mM Tris/HCl buffer (pH 8.0) containing 300 mM NaCl, 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 buffer (pH 7.4) containing 100 mM imidazole, 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.

(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 pET21b opened with the same restriction 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 His₆ tag proteins to facilitate their purification by using Ni²⁺-nitrilotriacetic acid (NTA) agarose resin (QIAGEN S.A., Courtabeuf, 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 Souav 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 in 5 ml of 20 mM Tris/HCl buffer (pH 7.4) containing 200 mM NaCl, 100 mM PMSF, and lysed by sonication with an ultrasonic processor (Misonix, 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 (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 buffer (pH 7.4) containing 100 mM imidazole, 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 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 then probed with 1:1,000 dilution of anti-SARS-CoV 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

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

| Gene | Residues | Primers (5'-3')<sup>a</sup> | Cloning sites |
|------|----------|----------------------|--------------|
| S1   | 14–760   | TCTCTCTCTAGAATTGGACCTTGACCGGACACACCCTATTAGGTGATGTTGAGAGAACCCCTCATGTGTGTTGGAGATCTGTTACACATACC | XbaI BamHI |
| S2   | 761–1190 | TCTCTCTCTAGAATTGGATAGTGTTGATGTTGAGAGAACCCCTCATGTGTGTTGGAGATCTGTTACACATACC | XbaI BamHI |
| N    | 1–1305   | TCTCTCTCTAGAATTGGACCTTGACCGGACACACCCTATTAGGTGATGTTGAGAGAACCCCTCATGTGTGTTGGAGATCTGTTACACATACC | XbaI BamHI |

* Boldface type indicates restriction enzyme sites.
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<sub>6</sub>-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 and incubated separately with each of 78 serum samples (lanes A1, A2, A3) and cell lysate supernatants (lanes B1, B2, B3) of the S1, S2, and N proteins, respectively, are shown.

**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<sup>2+</sup>-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
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               | +++                              | +++                              | +++    |

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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).

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 higher 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
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 serodiagnostics, 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 of 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.

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