Original article

Antibody responses to individual proteins of SARS coronavirus and their neutralization activities

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Abstract

A novel coronavirus, the severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV), was identified as the causative agent of SARS. The profile of specific antibodies to individual proteins of the virus is critical to the development of vaccine and diagnostic tools. In this study, 13 recombinant proteins associated with four structural proteins (S, E, M and N) and five putative uncharacterized proteins (3a, 3b, 6, 7a and 9b) of the SARS-CoV were prepared and used for screening and monitoring their specific IgG antibodies in SARS patient sera by protein microarray. Antibodies to proteins S, 3a, N and 9b were detected in the sera from convalescent-phase SARS patients, whereas those to proteins E, M, 3b, 6 and 7a were undetected. In the detectable specific antibodies, anti-S and anti-N were dominant and could persist in the sera of SARS patients until week 30. Among the rabbit antisera to recombinant proteins S3, N, 3a and 9b, only anti-S3 serum showed significant neutralizing activity to the SARS-CoV infection in Vero E6 cells. The results suggest (1) that anti-S and anti-N antibodies are diagnostic markers and in particular that S3 is immunogenic and therefore is a good candidate as a subunit vaccine antigen; and (2) that, from a virus structure viewpoint, the presence in some human sera of antibodies reacting with two recombinant polypeptides, 3a and 9b, supports the hypothesis that they are synthesized during the virus cycle.
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Keywords: SARS coronavirus; Recombinant protein; Protein microarray; Antibodies; Neutralizing antibodies

1. Introduction

An outbreak of atypical pneumonia, first emerged in Guangdong province of China and termed severe acute res-

piratory syndrome (SARS) by the World Health Organization, rapidly spread to many regions and countries from late 2002 to early 2003. Thousands of people got infected and hundreds of them were claimed. A novel coronavirus, the SARS coronavirus (SARS-CoV), was identified as the causative agent of SARS [1–3]. Since this virus is a novel pathogen for humans and its many aspects remain unknown, accurate diagnostic tools and protective vaccine will play an indispensable role in its control. The genome of the SARS-CoV was predicted to encode replicase 1a and 1b,4 structural proteins, including spike glycoprotein (S protein), envelope glycoprotein (E protein), membrane protein (M protein) and

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nucleocapsid protein (N protein), and some putative uncharacterized proteins [1,4,5]. Although these putative uncharacterized proteins have not been found in other known coronaviruses, it is possible for them to be present and play certain function in the SARS-CoV. For example, a unique novel protein 3a of the SARS-CoV was identified and characterized recently [6,7].

Humoral immunity is one of the immune responses for defense against viral infection. Hence, investigation of human immune responses to the pathogens will provide us important information about the mechanism of how the pathogens interact with their hosts. To understand the humoral immunity to the SARS-CoV, Li et al. [8] studied the profile of specific IgG and IgM antibodies in SARS patient sera by indirect ELISA. They found that specific IgG antibodies were present in all tested convalescent patient sera and persisted for a long time (until week 12), but specific IgM antibodies remained measurable for a much shorter period, suggesting that IgG antibodies to the SARS-CoV may represent the primary humoral immune response protecting patients against SARS. Although several articles have been published on the antibody response against the structural proteins of SARS-CoV and their contribution to protective immunity [9–13], more extensive study on the profile of specific antibodies to individual proteins of the SARS-CoV remains to be uncovered, and it could help us not only to understand the humoral immunity but also to develop specific diagnostic tools and vaccine.

Protein microarrays with the ability to detect antibodies in parallel have a wide range of potential applications in epidemiological research, vaccine development, diagnosis of allergies, autoimmune and infectious diseases [14–16]. Mezzasoma et al. [17] generated protein microarrays by printing five microbial antigens to simultaneously determine the specific antibodies in human sera. Human IgG and IgM bound to the printed antigens were detected by confocal scanning microscopy and quantified with internal calibration curves. The determination results were well concordant with those by ELISA. This suggested the feasibility of utilizing antigen microarrays to determine specific antibodies in human sera. In our previous study [18], to investigate the antigenicity of N protein of the SARS-CoV by protein microarray containing different recombinant truncated N proteins, human IgG at serial concentrations were printed onto slides to act as internal calibration standards. The fluorescence intensity (FI) values gave good linear correlation with human IgG concentrations, indicating that FI values could be used to assess the relative levels of specific antibodies captured by respective antigens on a microarray.

In the present study, to understand the profile of antibodies to individual proteins of the SARS-CoV, 13 recombinant proteins associated with four structural proteins (S, E, M and N) and five putative uncharacterized proteins (3a, 3b, 6, 7a and 9b) of this virus were prepared and used to screen and monitor their specific IgG antibodies in SARS patient sera using protein microarray, and the rabbit antisera to recombi-

nant proteins S3 (aa 241–591), N (full length), 3a (aa 125–274) and 9b (full length) were prepared and used to investigate their neutralizing activity to the SARS-CoV infection in Vero E6 cells.

2. Materials and methods

2.1. Virus strain

The SARS-CoV strain used for this study was isolated by another group in our institute (isolate BJ01; GenBank accession number AY278488) [5], and it was passaged in Vero E6 cells to generate a virus stock with a titer of 10^7 50% tissue culture infective doses ($TCID_{50}$) per ml. All work with infectious virus was performed inside a biosafety cabinet, in a Biosafety Level 3 (BSL3) laboratory.

2.2. Human sera

A total of 58 sera were collected from SARS patients at convalescent-phase in Beijing in 2003, and they were confirmed for the presence of anti-SARS-CoV IgG antibodies by an ELISA kit with whole virus lysate as coating antigen (Beijing GBI Biotechnology). A group of serial sera were also collected from 13 recovered patients from weeks 2 to 30 after their onset of SARS in Guangdong province. The normal human sera were collected from healthy people more than 1 year before 2003 SARS outbreak. All human sera were inactivated at 58 °C for 30 min.

2.3. Molecular cloning

The SARS-CoV was cultured in Vero E6 cells at 37 °C with 5% CO_2 for 48 h. The supernatant medium of the infected cells was collected as crude SARS-CoV virus, and the viral RNA was extracted and purified by QIAamp viral RNA mini kit (QIAGEN). cDNA synthesis was performed with SuperScript II system (Invitrogen) by using gene specific primer. Amplification of the full length or truncated fragments of the four structural proteins and five putative uncharacterized proteins (Table 1) was performed by PCR. The sequences of primers were referred to by their positions within the corresponding genes, with the note that 5′ ends of the forward and reverse primers contained *BamH* I, *EcoR* I or *Nco* I sites and *Sal* I, *Xho* I or *BamH* I sites, respectively, to facilitate cloning.

The amplified products were purified with Montage PCR kit (Millipore), digested with specific restriction enzymes (Promega), ligated into the plasmid pET32a vector (Novagen) using T4 DNA ligase (Promega) and finally transformed into *Escherichia coli* (*E. coli*) strain BL21 (DE3) (Novagen). The positive recombinant clones were first identified by PCR and further confirmed by enzymatic cutting and sequencing.

2.4. Protein expression and purification

The recombinant bacteria were grown at 37 °C to an optical density of 0.5–0.6 at 600 nm in Luria–Bertani medium

Table 1
The expressed proteins used for this study

Protein [a]	Genome location Start-End (nt) [a]	Number o of amino acids	Expressed protein Start-End (aa)	Qin et al. ORF [b]	Marra et al. ORF [c]
S	21,492–25,259	1255	S1: 1–668; S2: 669–1255; S3: 241–591; S4: 419–591; S5: 647–935	S	S
3a	25,268–26,092	274	125–274	PUP1	ORF3
3b	25,689–26,153	154	67–154	PUP2	ORF4
E	26,117–26,347	76	37–76	E	E
M	26,398–27,063	221	99–221	M	M
6	27,074–27,265	63	24–63	PUP3	ORF7
7a	27,273–27,641	122	Full length	PUP4	ORF8
N	28,120–29,388	422	Full length	N	N
9b	28,130–28,426	98	Full length	PUP5	ORF13

[a] Based on the sequence of the SARS-CoV Tor2 strain (GenBank accession number NC_004718).

[b] In Qin et al. [5], the ORFs encoding the putative uncharacterized proteins (PUPs) are designated as PUP1 to PUP5 and are numbered sequentially beginning at the 5′ terminus of the genome.

[c] In Marra et al. [4], all of the ORFs, including those encoding the predicted replicase protein and structural proteins, are numbered sequentially from the 5′ terminus of the genome. This table shows only ORFs we expressed and used for this study.

with ampicillin (final concentration, 100 µg/ml) and were induced with 1 mmol/l isopropyl-β-D-thiogalactoside (IPTG) for 6 h at 30 °C. The expressed recombinant fusion proteins were purified using Ni-NTA agarose (Qiagen) following the manufacturer's instructions. The purified proteins were dialyzed against PBS (pH 7.5) at 4 °C overnight, and then quantified by spectrophotometer (Beckman DU640). However, the insoluble proteins, which were dissolved in 8 mol/l of urea during purification, were renatured in a refolding solution (50 mmol/l Tris Cl, pH 8.0, 200 mmol/l L-arginine, 1 mmol/l EDTA, 100 mmol/l urea, 0.25 mmol/l glutathione oxidized, 1 mmol/l glutathione reduced) at 4 °C for at least 24 h before dialysis and quantification.

2.5. Protein microarray analysis

The purified fusion proteins were dissolved in 40% glycerol-PBS solution at a concentration of 200 µg/ml, and then printed approximately 40 pl in triplicate onto aldehyded glass slides (CEL Associates) using GSI Flexys arrayer (Genomic Solutions Inc.). Human IgG and the lysate of *E. coli* BL21 (DE3) were printed onto the slides as positive and negative controls, respectively. Printed slides were stored at 4 °C overnight and used within 2 weeks after being printed.

The serum samples were absorbed with lysate of *E. coli* BL21 (DE3) carrying pET32a to eliminate the corresponding antibodies. For serum reaction with the proteins on the slide, the slides were first blocked with 3% nonfat milk in PBS for 1 h and then 40 µl of 1:10 diluted absorbed sera were added onto the slides and covered with coverslips. Following a 1 h incubation at room temperature, the slides were rinsed three times for 3 min each with PBST (0.1% Tween-20 in PBS) and then PBS twice. To probe the captured human IgG, 40 µl of 1:250 diluted Cy5-labeled goat-anti-human IgG (goat-anti-human IgG was labeled with Cy5 dye using Cy5 antibody labeling kit, Amersham Biosciences) was applied to the slides, using the same incubation condition and rinsing protocol as described above. The slides were spun dry prior to

scanning at 635 nm using a Genepix Personal 4100A microarray scanner (Axon Instruments). The FI value of each spot was taken by subtracting the median intensity of the local background from that of the spot, utilizing the GenePix Pro 4.0 software provided by the same company. The triplicate spot signals for each protein were averaged and ready for further analysis [18,19]. Each sample was analyzed three times.

2.6. Preparation of rabbit antisera

One New Zealand White rabbit was immunized with each of the recombinant fusion proteins S3 (aa 241–591), N (full length), 3a (aa 125–274) and 9b (full length), respectively. 0.2–0.8 mg of these proteins in 1 ml of PBS were emulsified with an equal volume of Freund's complete adjuvant and 3-month-old rabbits were immunized by subcutaneous injection. After 3 weeks, boosters of each protein emulsified in Freund's incomplete adjuvant were given by subcutaneous injection; this was followed by another intravenous injection of protein alone in another 3 weeks. Antisera were collected 12 days after the last boost and the immunoreactivity titers were monitored by double agar diffusion precipitation performed in 0.8% agarose in PBS.

2.7. Neutralizing antibody assay

Twofold serial dilutions of heat-inactivated antiserum from each immunized rabbit were tested by a microneutralization assay for the presence of antibodies that neutralized the infectivity of 100 $TCID_{50}$ of the SARS-CoV in Vero E6 cell monolayers, with four wells per dilution on a 96-well plate. The presence of viral cytopathic effect was read on days 3 and 4. The dilution of serum that completely prevented cytopathic effect in 50% of the wells was calculated by the Reed–Muench formula [20,21].

3. Results

3.1. Cloning, expression and purification of proteins

Besides encoding replicase 1a and 1b, the genome of the SARS-CoV was predicted to encode four structural proteins and some putative uncharacterized proteins. The number of predicted open reading frames (ORFs) encoding putative uncharacterized proteins is not the same in different publications, depending on the analysis method used [1,4,5]. In this study, we cloned and expressed the five putative uncharacterized proteins larger than 50 amino acids (3a, 3b, 6, 7a and 9b) according to the prediction of Qin et al. [5] and all the four structural proteins (Table 1). All the positive recombinant clones were confirmed by DNA sequencing. Among the expressed proteins, S protein was expressed as five truncated fragments including S1 (aa 1–668), S2 (aa 669–1255), S3 (aa 241–591), S4 (aa 419–591) and S5 (aa 647–935); proteins 7a, N and 9b were expressed as full length; proteins 3a, 3b, E, M and 6 were expressed as truncated fragments because their full length could not be expressed with pET32a vector in *E. coli*, or the expression level was very low. All these recombinant proteins were expressed with Trx/6_His-tag at NH$_2$-terminus and their molecular weights were same as predicted (Fig. 1, S1 and S2 were not shown).

The 13 expressed proteins were in two forms, soluble (3a, 3b, E, M, 6, 7a, N and 9b) or insoluble (S1, S2, S3, S4 and S5). The soluble proteins were purified under native conditions using Ni-NTA agarose, while the insoluble ones were purified under denaturing conditions, and then renatured before dialysis and quantification.

3.2. Reactivity of IgG antibodies to individual proteins of the SARS-CoV in human sera

Thirteen recombinant proteins were printed in triplicate onto glass slide as protein microarray for screening their specific IgG antibodies in 58 sera from convalescent-phase SARS patients, with 23 sera from healthy people as control. The FI values for proteins S1, S2, S3, S4, S5, 3a, N and 9b to sera from SARS patients were statistically significantly more than those from healthy people ($P < 0.01$, using SPSS 10.0 software) (Fig. 2), but the FI values for proteins E, M, 3b, 6 and 7a to sera from SARS patients had no significant difference with those from healthy people ($P > 0.05$).

To establish the baseline for the assays, the cutoff value for each protein was calculated using the FI values for this protein to the normal human sera (cutoff = mean + 3S.D.). With these cutoff values, the specificities of the protein microarrays for antibody detections to proteins S1, S2, S3, S4, S5, 3a, N and 9b were all 100%, and the corresponding sensitivities were 44/58 (75.9%), 17/58 (29.3%), 57/58 (98.3%), 28/58 (48.3%), 37/58 (63.8%), 35/58 (60.3%), 58/58 (100%) and 24/58 (41.4%), respectively, with average FI values of 1733, 994, 3581, 1353, 924, 1122, 9848 and 721, respectively.

Five truncated recombinant S proteins showed different immunoreactivities to the patient sera, among them S3 could react with almost all the samples, indicating that it is the most sensitive antigen for detecting anti-S antibodies. In the one sample (No. 496) missing anti-S3 antibody, both anti-S2 and anti-S5 were detected (Fig. 3). Combining these results, anti-S antibodies were present in all the patient sera.
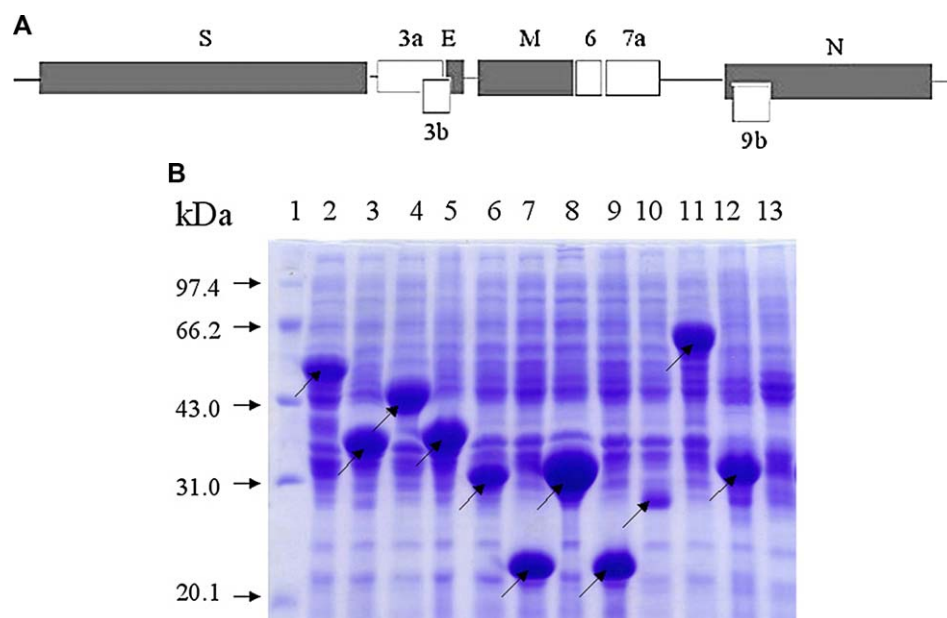

Fig. 1. Structural organization and expression of the ORFs of the SARS-CoV used for this study.
(A) ORFs corresponding to four structural proteins (S, E, M and N) and five putative uncharacterized proteins (3a, 3b, 6, 7a and 9b). (B) SDS-PAGE analysis of expressed fusion proteins with Trx/6_His-tag at NH$_2$-terminus from recombinant *E. coli* BL21 (DE3). Recombinant protein S3 (lane 2, 56.8 kDa), S4 (lane 3, 37.2 kDa), S5 (lane 4, 49.9 kDa), 3a (lane 5, 34.2 kDa), 3b (lane 6, 28.1 kDa), E (lane 7, 22.6 kDa), M (lane 8, 31.2 kDa), 6 (lane 9, 22.6 kDa), 7a (lane 10, 31.1 kDa), N (lane 11, 64.6 kDa), 9b (lane 12, 28.3 kDa) and plasmid pET32a vector control (lane 13) were induced by IPTG and analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250. Lane 1 shows the molecular weight markers, and arrows indicate the positions of target proteins. The polypeptides' molecular weights expressed in kDa were estimated by their relative mobility in SDS-PAGE.
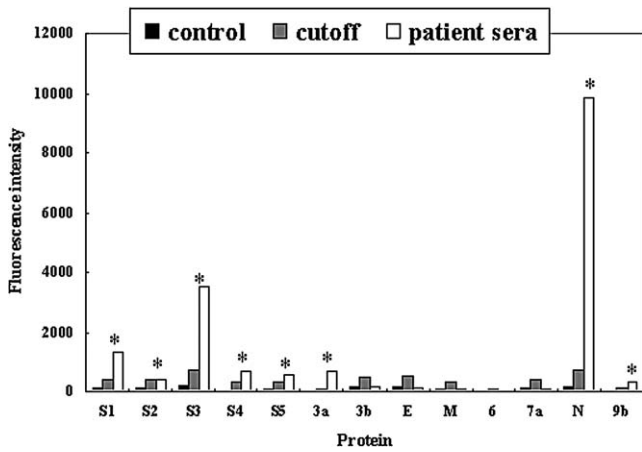
Fig. 2. The FI values for 13 recombinant proteins to human sera by protein microarray.
Thirteen recombinant proteins were applied to analyze human sera. The control and patient sera bands indicate the average FI values for each protein to the sera from 23 healthy people and 58 convalescent-phase SARS patients, respectively, and the cutoff values were calculated from the data from healthy people, cutoff = mean + 3S.D. * indicates statistically significant difference between patient sera and control.

3.3. Kinetics of the SARS-CoV antibodies in humans after onset of SARS

In order to investigate the kinetics of the IgG antibodies to recombinant proteins S3, 3a, N and 9b in humans after onset of SARS, a group of serial sera collected from 13 people from weeks 2 to 30 were analyzed. Both anti-N and anti-S3 antibodies were positive in all the serum samples, whereas anti-3a and anti-9b antibodies were only positive in part of these samples. As Fig. 4 shows, anti-N and anti-S3 antibodies persisted until week 30; their levels rapidly increased from weeks 2 to 4, and decreased from weeks 20 to 30. At week 30, the average FI values were about half of those at week 4. In addition, anti-3a and anti-9b antibodies remained at low levels all the while. Although it is regretable that we could not obtain the patient sera from weeks 5 to 19, the available data provided us valuable information, which is helpful in screening diagnostic markers and the development of vaccine and immunity protocol.
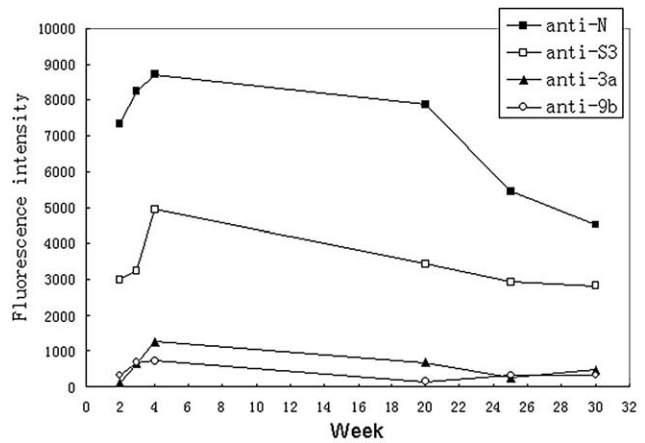

Fig. 4. Kinetics of the SARS-CoV IgG antibodies in human sera over time. The kinetics of average FI values for anti-N, anti-S3, anti-3a, anti-9b antibodies were monitored using a group of serial sera collected from people from weeks 2 to 30 after their onset of SARS. Anti-N and anti-S3 antibodies persisted until week 30, and the average FI values at week 30 were about half of those at week 4 but anti-3a and anti-9b antibodies remained at low levels all the while.

3.4. Neutralizing activity of rabbit antisera to four recombinant proteins of the SARS-CoV

The antisera were generated by immunizing New Zealand White rabbits with recombinant fusion proteins S3, N, 3a and 9b, respectively. Their immunoreactivity titers to respective recombinant proteins were not less than 16 using double agar diffusion precipitation (Table 2). These antisera were heat-inactivated and diluted serially twofold for testing neutralizing activity to the infectivity of 100 $TCID_{50}$ of the SARS-CoV in Vero E6 cells. Anti-S3 serum showed significant neutralizing activity to the SARS-CoV infection, with a neutralizing antibody titer of 708, whereas the other antisera and control serum from non-immunized rabbit lacked neutralizing antibodies (Table 2).

4. Discussion

Compared with ELISA-based assays, the protein microarrays have the advantage of parallelism, miniaturization and
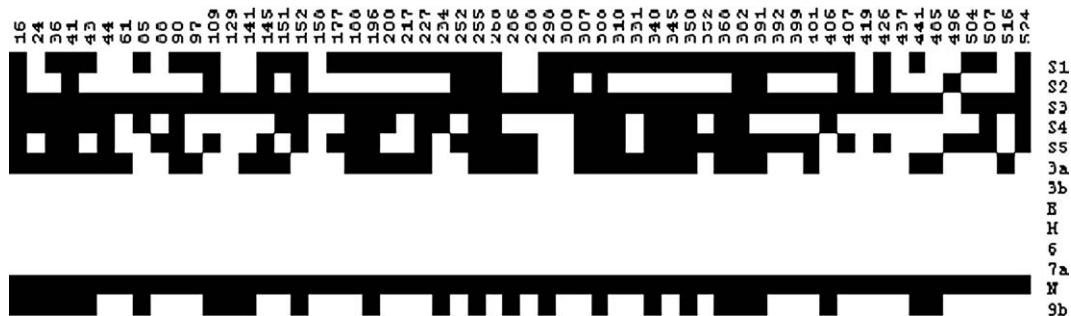

Fig. 3. Reactivity of specific IgG antibodies to individual proteins of the SARS-CoV in 58 sera from convalescent-phase SARS patients.
Thirteen recombinant proteins (vertical axis) were used to analyze 58 convalescent-phase SARS patient sera (horizontal axis). The black blocks indicate positive reactions and the white ones negative.

Table 2
The immunoreactivity and neutralizing activity of rabbit antisera to recombinant proteins of SARS-CoV

Recombinant protein	Immunoreactivity titer [a]	Neutralizing antibody titer [b]
S3	32	708
3a	32	<4
N	16	<4
9b	32	<4
Non-immunized control serum	<2	<4

[a] Titer of antibody that reacted with its respective recombinant protein, using double agar diffusion precipitation. A titre is the reciprocal value of the last dilution when the property was observed.

[b] Titer of antibody that neutralized infectivity of 100 $TCID_{50}$ of the SARS-CoV, using a microneutralization assay. A titre is the reciprocal value of the last dilution of serum that completely prevented cytopathic effect in 50% of the wells, calculated by the Reed–Muench formula [21].

high throughput to detect antibodies [19,22]. In this study, the recombinant proteins associated with four structural proteins (S, E, M and N) and five putative uncharacterized proteins (3a, 3b, 6, 7a and 9b) of the SARS-CoV were expressed in *E. coli* and were used to screen their specific IgG antibodies in 58 convalescent-phase SARS patient sera by protein microarray, and the antibodies to proteins S, N, 3a and 9b were detected with different positive rates. S and N are structural proteins present in the SARS-CoV and other known coronavirus [23], and a number of research groups have reported that the specific antibodies to S and N could be detected in SARS patient sera [13,23–28]. 3a and 9b are predicted proteins of the SARS-CoV that have not been found in other known coronaviruses, and our serological evidence implies that these two unique novel proteins may be actually present in the SARS-CoV. During completion of this manuscript, 3a protein has been identified and characterized by other laboratory and our group [6,7], respectively; and the antibody to 3a (also known as U274) has also been detected in SARS patient sera [13]. However, the possible presence of novel protein 9b, consisting of 98 amino acids residues, in the SARS-CoV needs to be further proved.

On the other hand, no significant antibodies against proteins E, M, 3b, 6 and 7a were detected in the tested sera. Proteins E and M are structural proteins of the SARS-CoV. Our results indicate that they may not induce antibody production in humans or the antibody concentrations may be too low to be detected. Another possible reasons that could not be ruled out are that the bacteria expressing proteins were not folded or post-translationally modified correctly to recognize the corresponding antibodies. Similar results for proteins E, M and 7a (also known as U122) could be seen in a newly published paper [13]. Tan et al. expressed full length proteins E, M and 7a in mammalian cells and the total protein lysates were analyzed by Western blotting with sera from three convalescent-phase SARS patients, but no antibodies to these proteins were detected.

So far, serological diagnosis for SARS has been the most reliable laboratory method. In contrast to the whole virus-based assays, recombinant protein-based assays have the

advantages of safety and economy. Several research groups have evaluated the possibility of using recombinant proteins N, S and/or 3a for serological diagnosis in the past year [13,18,24–28]. Anti-N has been proved to be the most frequent antibody in SARS patients and bacteria expressing N protein or even some truncated N proteins are highly immunogenic [13,18,24–27]. Although anti-S antibody was detected in 100% (74/74) of convalescent-phase sera by immunofluorescence with mammalian cells expressing S protein [13], it was only detected in 47.5% (19/40) of SARS patient sera by Western blotting with bacteria expressing S protein [24]. Anti-3a was detected in 73% (59/81) of convalescent-phase sera by Western blotting with bacteria expressing 3a protein (aa 134–274) [13]. In the present study, we used a protein microarray to qualitatively and quantitatively screen the serological diagnostic markers. Among the detectable antibodies to proteins S3, 3a, N and 9b, anti-N and anti-S3 were the dominant, with 100% and 98.3%, respectively, of positive rates and high-level of FI values (9848 and 3581, respectively) in the convalescent-phase SARS patient sera, and even in the sample missing anti-S3, both anti-S2 and anti-S5 were detected. This indicates that anti-N and anti-S antibodies are sensitive diagnostic markers for the SARS-CoV infection, and bacterial expressed recombinant proteins N and S3 (aa 241–591) could be used for antibody detection. However, anti-3a and anti-9b were only detected in 60.3% and 41.4%, respectively, of the sera, with low-level of FI values (1122 and 721, respectively), suggesting that there is limitation of using these two antibodies as diagnostic markers for the SARS-CoV infection.

Immunization with one or more SARS-CoV subunit antigens, either administered as purified protein or expressed from viral or DNA vaccine vectors, is one approach to designing a vaccine against SARS. This approach would be facilitated by knowledge of the relative importance of the various viral structural proteins in inducing protective immunity. In the present study, the rabbit antisera to recombinant fusion proteins S3 (aa 241–591), N (full length), 3a (aa 125–274) and 9b (full length) were tested for their neutralizing activity to the infectivity of 100 $TCID_{50}$ of the SARS-CoV in Vero E6 cells, but only anti-S3 serum showed significant neutralizing activity to the SARS-CoV infection, with a neutralizing antibody titer of 708. This suggests that S protein is a neutralization antigen for the SARS-CoV, and recombinant protein S3 (aa 241–591) may be used as a candidate subunit vaccine. Although anti-N antibody is also present in SARS patient sera at a high-level and persisted for a long time (until week 30), recombinant N protein could not induce a detectable neutralizing antibody in rabbit to the SARS-CoV infection. In other papers about the SARS-CoV vaccine studies, only S among the structural proteins could induce protective immunity in experimental animals [9,10]. For example, Buchholz et al. [9] investigated the contributions of the structural proteins (S, E, M and N) of the SARS-CoV to protective immunity by expressing them individually and in combinations from a recombinant parainfluenza virus (PIV) type 3 vector called BHPIV3,

then evaluating BHPIV3/SARS recombinants for immunogenicity and protective efficacy in hamsters. The results identified protein S among the structural proteins as the only significant neutralization antigen and showed that a single mucosal immunization is highly protective in an experimental animal that supported efficient replication of the SARS-CoV.

S protein is very promising in the development of a diagnostic antigen and vaccine. The antigenicity of five truncated recombinant fusion S proteins was preliminarily analyzed in this study, and anti-S antibodies were detected in all the convalescent-phase SARS patient sera using S3 and either S2 or S5. The sequence of individually expressed S1 (aa 1–668) and S2 (aa 669–1255) formed the whole sequence of S protein, but these two recombinant proteins only reacted with 75.9% and 29.3% of the patient sera, respectively. Although the sequence of S1 covered that of S3 (aa 241–591), S3 was much more sensitive to detect the patient sera, suggesting that some important epitopes appeared in the conformation of S3 but not in that of S1. In addition, S2 (aa 669–1255) and S5 (aa 647–935) could react with the sample missing anti-S3 antibody, suggesting one or more important epitopes may be present in the overlapping sequence of S2 and S5, aa 669–935. Further work should be done for full antigenicity analysis of protein S to elucidate its epitopes for diagnostic antigen and vaccine development.

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