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B-Cell Responses in Patients Who Have Recovered from Severe Acute Respiratory Syndrome Target a Dominant Site in the S2 Domain of the Surface Spike Glycoprotein

Xiaofen Zhong,[1] Huanghao Yang,[1,2] Zu-Feng Guo,[1,2] Wan-Yee Fion Sin,[3] Wei Chen,[4] Junjie Xu,[4] Ling Fu,[4] Jie Wu,[1] Chun-Kit Gannon Mak,[1] Chak-Sum Samuel Cheng,[3] Yanzhen Yang,[1] Shuyong Cao,[1] Tin-Yau Wong,[5] Sik-To Lai,[5] Yong Xie,[3]* and Zhihong Guo[1,2]*

Department of Chemistry,[1] Biotechnology Research Institute,[2] and Department of Biology,[3] The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, and Princess Margaret Hospital,[5] Hong Kong SAR, and Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing,[4] China

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Severe acute respiratory syndrome (SARS) is a recently emerged infectious disease caused by a novel strain of coronavirus. Examination of the immune responses of patients who have recovered from SARS should provide important information for design of a safe and effective vaccine. We determined the continuous viral epitopes targeted by antibodies in plasma samples from convalescent SARS patients through biopanning with a vast M13 phage display dodecapeptide library. These epitopes converged to very short peptide fragments, one on each of the structural proteins spike and nucleocapsid and the nonstructural proteins 3a, 9b, and nsp 3. Immunoassays found that most of the patients who had recovered from SARS developed complementary antibodies to the epitope-rich region on the spike S2 protein, indicating that this is an immunodominant site on the viral envelope comprising the spike, matrix, and small envelope glycoproteins. These S2-targeting antibodies were shown to effectively neutralize the coronavirus, indicating that they provided protective immunity to help the patients recover from the viral infection. These results suggest that the SARS coronavirus might have an antigenic profile distinct from those of other human or animal coronaviruses. Due to the tested safety and protective effects of the convalescent-phase serological antibodies, identification of their complementary antigens may enable the design of an epitope-based vaccine to prevent potential antibody-mediated immunuopathology.

Severe acute respiratory syndrome (SARS) has emerged as a new infectious disease and claimed 8,098 victims, including 774 lives, in the last outbreak, which ended in July 2003 (40). A novel coronavirus (CoV) was identified as the etiological agent (9, 13, 23, 26). Unlike the known human HCoV-229E and OC43, which infect the upper respiratory tract and cause common colds (18), the new SARS CoV predominantly causes infection in the lower respiratory tract, causing lung lesions with high morbidity and mortality (14, 31). This new pathogen was first shown not to belong to any of the three serological groups of the coronavirus genus of the *Coronaviridae* family by phylogenetic analysis (16, 27), but later it was classified as an early split-off of group 2 (29), which includes HCV-OC43, mouse hepatitis virus, and bovine coronavirus; this was supported by the conserved cysteine distribution pattern of the major surface spike glycoprotein (S) (10).

Conventionally, the most effective prevention measure against a pathogen is vaccination. Candidate vaccines using various components of the SARS CoV have been developed to induce neutralizing humoral and cellular immunity in mouse and rhesus macaque models (1, 11, 42). These animal studies indicate that a protective vaccine against the life-threatening coronavirus is possible. However, caution in vaccine development is urged because of the immunopathology associated with immune responses to a number of animal coronaviruses (7, 17). Both humoral and T-cell-mediated responses to animal coronaviruses have been known to be capable of exacerbating the disease or causing new health problems. T-cell responses have been implicated in the demyelination of the brain and spinal cord following infection with neurotropic mouse hepatitis virus (2, 41), a group 2 coronavirus closely related to the SARS CoV. Adverse humoral responses to another group 2 coronavirus, bovine coronavirus, have also been linked to the development of "shipping fever" in cattle (19). Moreover, previous exposure to or active or passive immunization against the feline infectious peritonitis virus, a group 1 coronavirus, was found to cause the "early death syndrome" instead of providing immune protection (22, 33, 38). This disease exacerbation was due to the virus-specific antibodies that facilitated and enhanced uptake and spread of the virus, causing an antibody-dependent enhancement (ADE) of infectivity (25, 33, 37). Detailed analysis showed that antibodies directed against specific sites on the spike protein mediated the ADE (5, 6, 20, 21). Thus, one safety concern for a SARS CoV vaccine is that it may induce similar antibody- or cell-mediated immunopathologies. Although antibodies directed against SARS CoV were found to be protective and not to enhance viral infectivity in

* Corresponding author. Mailing address for Zhihong Guo: Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China. Phone: 852-2358-7352. Fax: 852-2358-1594. E-mail: chguo@ust.hk. Mailing address for Yong Xie: Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China. Phone: 852-2358-7340. Fax: 852-2358-1559. E-mail: boyxie@ust.hk.

the mouse model (1, 30, 42), their effects in humans remain unknown.

To avoid potential immunopathology, examination of the humoral and cellular immunity to the SARS CoV generated in convalescent SARS patients should provide the most relevant information for vaccine design. In this connection, studies have been directed towards mapping the T-cell epitopes in the cellular immune responses of patients who have recovered (34, 36). However, little is known about the precise viral targets of the convalescent-phase antibodies. Here we report the mapping of the viral components targeted by the serological antibodies from convalescent SARS patients, using a phage display dodecapeptide library. Such convalescent-phase antibodies were shown to be safe and in many cases to provide immune protection in passive immunization of infected patients in the last SARS outbreak in 2003 (39, 44). Identification of their viral targets should be able to define the viral components leading to safe and neutralizing antibodies for inclusion in a vaccine, thereby excluding potential ADE-inducing viral components.

MATERIALS AND METHODS

Preparation of serological samples. Serological samples were prepared within 1 month after discharge from 40 patients who had recovered from SARS, after 7 days of hospitalization from 2 patients who had a confirmed diagnosis of SARS but eventually recovered from the disease, after 15 days of hospitalization from 10 patients who had a confirmed diagnosis of SARS but later died of the infection, and from 10 patients who were confirmed not to be infected by SARS CoV. The patients, 20 to 65 years of age, were hospitalized in Princess Margaret Hospital, Hong Kong SAR, China. Collection and preparation of the serological samples were agreed to by patients through written consent and authorized by the Hospital Ethics Review Committee. After deactivation at 56°C for 45 min, the serological samples were stored at −20°C until used.

Phage panning. In a typical panning experiment, 10 μl of the Ph. D.-12 M13 phage-displayed dodecapeptide library (~2×10^{11} phage particles) (New England Biolabs) and 5.0 μl of plasma from a convalescent SARS patient were mixed and diluted to a final volume of 400 μl with TBST buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20 [pH 7.6]). After 30 min of incubation at room temperature, protein G-Sepharose Fastflow resin (Amersham Biosciences) preblocked in TBST buffer supplemented with 2% bovine serum albumin was used to capture immunoglobulin G (IgG)-binding phages, being washed 10 times with 1 ml of TBST buffer. The captured phages were then released in 1 ml of 0.2 M glycine-HCl solution (pH 2.2) and eluted into a microcentrifuge tube containing 150 μl of 1 M Tris-HCl (pH 9.1), and then the titers were determined and the phages were amplified in ER2738 cells (New England Biolabs). This panning process was repeated twice with the amplified phage sublibrary while the concentration of Tween 20 in the TBST buffer was increased from 0.1 to 0.2%. A total of 80 random phage clones after three rounds of panning were sequenced, and the sequences were aligned with Clustal X1.81.

Expression and purification of nucleocapsid and spike S1 and S2 proteins. The genes of the nucleocapsid and spike S1 and S2 proteins were amplified from cDNA clones of the SARS coronavirus (BJ01). The PCR products were digested with appropriate restriction enzymes, purified with a gel extraction kit (Qiagen), ligated to the digested vector pET22b (S1 and nucleocapsid) or pET28a(+) (S2) (Novagen), and transformed into *Escherichia coli*. Primers used for gene amplification were GGAATTCCATATGAGTGACCTTGACCGGTGC (S1 forward), CATGCTCGAGTGTATGGTAACTAGCAC (S1 reverse), CGCGGATCCTTAGGTGCTGATAGTTCAATTG (S2 forward), CCGCTCGAGTTATTAGAAGCCGAGCCAAACATACC (S2 reverse), GGAATTCCATATGTCTGATAATGGACCCCAATC (nucleocapsid forward), and CATGGGATCCGCCTGAGTTGAATCAGCAG (nucleocapsid reverse). All three genes were fused with a six-histidine tag at either the N or C terminus. Both the spike S1 and S2 genes were expressed as insoluble inclusions at 37°C in *E. coli* BL21(DE3) Codon Plus (Novagen), whereas the nucleocapsid gene was expressed as a soluble protein at 30°C in the same host. After cell lysis, precipitates containing the spike S1 and S2 proteins were solubilized by heating in buffer (20 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 20 mM dithiothreitol, 400 mM NaCl [pH 7.9]), desalted and purified by metal-chelating affinity column chromatography with a 5-ml HiTrap column (Amersham Biosciences) according to the manufacturer's instructions, and stored at −20°C in TBS buffer (20 mM Tris-HCl, 137 mM NaCl [pH 7.6]) supplemented with 1% SDS and 2 mM β-mercaptoethanol. The soluble nucleocapsid protein was similarly purified by metal-chelating affinity column chromatography with a 5-ml HiTrap column (Amersham Biosciences) according to the manufacturer's instructions and stored at −20°C in TBS buffer containing 2 mM β-mercaptoethanol. The proteins were >90% pure as determined by SDS-polyacrylamide gel electrophoresis.

Synthesis of peptides. Viral antigenic peptide fragments were synthesized by the 9-fluorenylmethoxy carbonyl method of solid-phase peptide synthesis with standard *N*,*N*′-diisopropylcarbodiimine/1-hydroxybenzotriazole chemistry. The antigenic fragments were divided into groups of 10 to 20 peptides, and each group was synthesized by a manual split-and-pool approach, using IRORI MicroKan reactors (ChemTech) with 30 mg of Wang resin (loading value, 1.3 mmol/g) for each peptide. After synthesis, the peptides were capped with an acetyl group by reaction with acetic anhydride and cleaved from the resin with a mixture of trifluoroacetic acid, phenol, triisopropylsilane, and water (88:5:2:5). Short peptides (7 to 15 residues) after cold ether precipitation were dissolved in dimethyl sulfoxide or H_2O and used directly in the immunoassays without further purification. Longer peptides corresponding to the epitope-rich viral protein fragments were purified by high-pressure liquid chromatography and obtained as white solids after lyophilization. High-pressure liquid chromatography purification was carried out with a Waters 600E system, using a reversed-phase column (Waters XTerra RP_{18}; 7 μm, 7.8 by 300 mm) and a linear gradient from 0 to 75% acetonitrile in 0.1% trifluoroacetic acid–water over 45 min.

Western dot blotting. Appropriate amount of peptides, proteins, or deactivated SARS CoV in 2-μl volumes in a suitable solvent were transferred onto the centers of polyvinylidene difluoride membrane circles (6 mm in diameter) cut from a Hybond membrane (Amersham Biosciences). The blots were blocked for 1 h in a blocking buffer (8% milk, 20 mM Tris-HCl, 137 mM NaCl, 0.2% Tween 20 [pH 7.6]), rinsed with TBST buffer (20 mM Tris-HCl, 137 mM NaCl, 0.2% Tween 20 [pH 7.6]), distributed into wells of a 48-well microtiter plate containing diluted serological samples from SARS patients or uninfected donors, and incubated at room temperature for 1 h. After extensive washing with TBST buffer, the blots were blocked one more time, incubated for 1 h with anti-human IgG-horseradish peroxidase conjugate (1:50,000; Sigma), and washed extensively again with TBST buffer. Finally, the blotting signal was detected with the ECL Plus Western blotting detection system (Amersham Biosciences). Dilution factors for the serological samples in blotting with small peptides, proteins, or the deactivated SARS CoV as the antigen were 1:200, 1:400, and 1:800, respectively. To block antibodies directed against certain peptide or protein antigens, the diluted serological samples in TBST were preincubated with the target antigen for 1 h before the addition of the blotted polyvinylidene difluoride membrane circles.

ELISA analysis of anti-SARS CoV antibodies. The diagnostic kit for antibody to SARS virus (enzyme-linked immunosorbent assay [ELISA]) from BeiJing DaJiBiAi Bio-Technology Co. was used in the ELISA analysis. The antigen was prepared from purified SARS coronaviruses in a phosphate-buffered saline suspension, which were irradiated under 254-nm UV light for 30 min, and viral proteins were extracted with 1% NP-40 solution in PBS. The extracted viral proteins were then applied to 96-well plates and UV irradiated again for another 30 min. The kits were used to determine the titers of antibodies against viral proteins in plasma samples from patients who had recovered from SARS in a series dilution of 1:20, 1:40, 1:80, and 1:160, according to the manufacturer's instructions. All assays were carried out in duplicate. To determine the anti-SARS CoV antibody levels after blocking with the identified antigens, the diluted plasma samples from convalescent SARS patients were incubated for 1 h at room temperature with the nucleocapsid protein and peptides containing the identified epitopes on the spike, 3a, and 9b proteins at appropriate concentrations, before addition to the supplied 96-well plate coated with deactivated SARS CoV.

SARS CoV neutralization. All experiments were carried out under biosafety level 3 conditions. Vero E6 cells were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. Viral infection was carried out by addition of 100 μl of medium containing 5×10^4 50% tissue culture infective doses ($TCID_{50}$) of SARS CoV (BJ01) to microtiter wells containing 4×10^4 host cells per well in a volume of 100 μl of medium and incubation for 48 h with 5% CO_2 at 37°C. For neutralization of the virus, plasma from convalescent patient J was diluted in 100 μl of medium in a twofold series to achieve dilution factors of from 1:10 to 1: 5120, incubated with the virus (5×10^4 $TCID_{50}$) at 37°C for 1 h, and added to microtiter wells containing the host cells (4×10^4 cells per well). To block the neutralizing effect of the plasma antibodies, diluted plasma solutions were preincubated with 4 μM peptide SL26

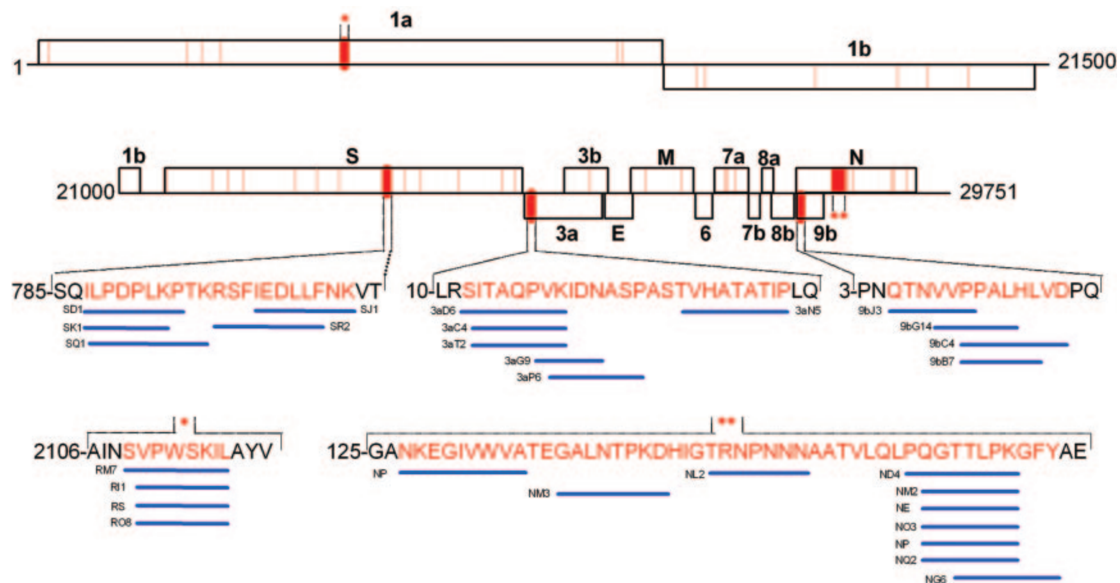

FIG. 1. Identified antigenic sites on the SARS coronavirus. The vertical red lines indicate confirmed epitopes, red shaded areas indicate convergent antigenic sites consisting of four or more identified epitopes within a consecutive viral protein sequence, and each horizontal blue bar stands for one epitope within a convergent antigenic site which was determined from a different convalescent-phase serum sample. Boxes indicate translated viral proteins. S, spike glycoprotein; E, small enveloped glycoprotein; M, matrix glycoprotein; N, nucleocapsid protein; 1a and 1b, replication polyproteins. Proteins 3a, 3b, 6, 7a, 7b, 8a, 8b, and 9b are unknown proteins.

before incubation with the virus and the host cells. All cell culture experiments were performed in duplicate on two separate 96-well microtiter plates. Cytopathic effect was observed and cell pictures were taken with a phase-contrast microscope after the cultures were inoculated with 5% CO_2 at 37°C for 48 h. Live cells were also stained with crystal violet for easy observation of viral infection.

RESULTS

Epitope mapping. A global approach was used to determine the immunodeterminants on the viral pathogen through biopanning of a random dodecapeptide M13 phage library of 1.9 $\times 10^9$ independent clones, directly using plasma samples from convalescent SARS patients. Probable epitope sequences were derived from alignment of the dodecapeptide inserts of the enriched phage clones and comparison of the resulting consensus sequences with the total proteins encoded by the SARS CoV genome (Tor 2 strain, GenBank accession number NC_004718) or the viral proteins deposited in the GenBank, using the BLAST program seeking short and nearly exact matches. Plasma samples from a total of 20 convalescent SARS patients (patients A to T) were subjected to the epitope mapping process, one at a time, and 299 distinct consensus sequences were obtained. A significant portion (92) of these consensus sequences matched a protein fragment of the SARS CoV which is defined as containing a probable SARS coronavirus epitope. Most of the remaining consensus sequences aligned well with proteins of other viruses that might have infected the patients during the course of the disease, such as human metapeumovirus or influenza viruses. Another small portion of the consensus sequences neither matched a SARS protein fragment nor mapped to sequences of other viruses. Under identical conditions, phage clones from biopanning with control sera also showed sequence convergence in the dodecapeptide inserts, but no consensus sequences matching protein fragments in SARS coronavirus were identified. These control sera were from two SARS patients before seroconversion (7 days after hospital admission) who eventually recovered from the disease and two patients after the projected seroconversion period (15 days after hospital admission) who eventually died of the infection.

Small viral protein fragments matching the consensus sequences from the biopanning were chemically synthesized and subjected to Western dot blotting to test the presence of complementary IgG antibodies in the plasma samples from which the probable epitopes were derived. Of the total of 97 probable epitopes (a few consensus sequences have more than one matching homologue on the viral proteins), 63 sequences were confirmed, while the other 34 were excluded because of the negative testing results. ELISAs with the short peptides alone or in conjugation with a carrier protein (bovine serum albumin) did not give consistent results because of high background signals. Interestingly, a significant portion (29) of the confirmed SARS epitopes converged to five short isolated fragments that belong to different viral proteins (Fig. 1). These convergent antigenic sites are located in the spike protein between amino acids 787 and 809, in the nucleocapsid protein between amino acids 127 and 173, in the unknown protein 3a between amino acids 12 and 27, in the unknown protein 9b between amino acids 5 and 17, and the replication polyprotein 1a between amino acids 2109 and 2116 (amino acids 1291 to 1298 of nsp 3). Every epitope within a convergent antigenic locus originated from a different convalescent SARS patient. The other 34 immunodeterminants dispersed outside the identified convergent loci without apparent colocalization. Notably, only one very short epitope-rich fragment on the S2 domain of the spike protein was identified in the entire viral envelope comprising the S, M, and E glycoproteins.

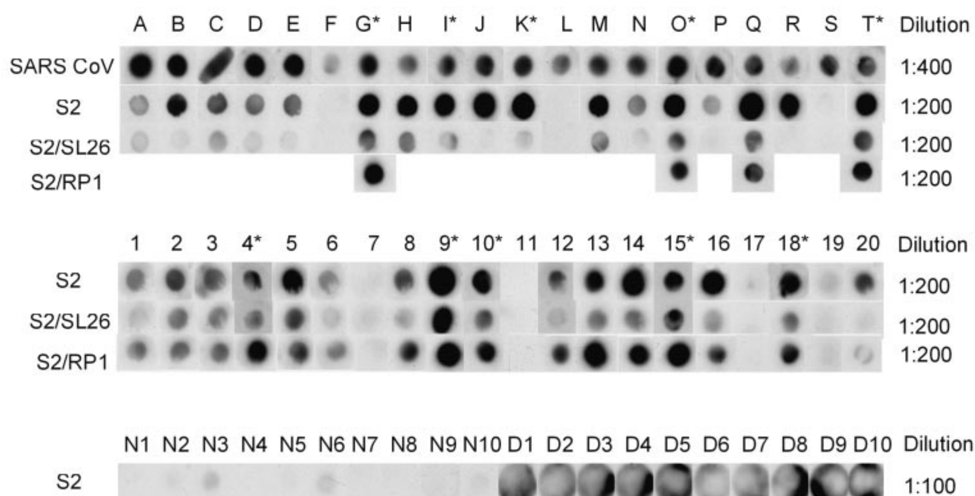

FIG. 2. Analysis of antibodies targeting the immunodominant site in the spike S2 protein in plasma samples from convalescent SARS patients (A to T and 1 to 20) through blocking with peptide SL26. SL26 is the epitope-rich fragment identified on the spike S2 protein and has the sequence QAc-QILPDPLKPTKRSFIEDLLFNKVTLA-OH. The spike S2 protein (375 ng/dot) was used as the antigen for the rows labeled S2, and heat-deactivated SARS coronavirus (50 ng/dot) was used for the SARS CoV rows. S2/SL26 and S2/RP1 indicate presaturation of plasma with peptide SL26 and an irrelevant peptide, RP1 (Ac-GPNLRNPVEQPLSVQA-OH), respectively, before blotting and use of the spike S2 protein (375 ng/dot) as the antigen. N1 to N10 are plasma samples from 10 uninfected patients, and D1 to D10 are the sera withdrawn after 15 days of hospitalization from 10 SARS patients who eventually died of the infection. SL26 and RP1 were at concentrations of 5 and 10 μM in the blocking of the plasma antibodies from patient A to H and 1 to 20, respectively; an asterisk indicates a concentration of 200 μM for both SL26 and RP1 in the blocking of the complementary antibodies.

Analysis of spike-specific antibodies by antigen-blocking assays. Colocalization of the epitopes to the short viral protein fragments indicates that these fragments are the immunodominant sites on the SARS coronavirus. Due to limitations of the phage panning method used, there may be more complementary antibodies than those detected that also target these identified convergent loci but escape detection in the biopanning because of low abundance or low affinity for short peptides in comparison to the cognate intact protein (32). To test this possibility, we analyzed the antibodies complementary to the convergent site identified on the S2 domain of the spike protein. The spike S1 (amino acids 1 to 685) and S2 (amino acids 681 to 1203) proteins without the transmembrane domain and the intraviral segment were expressed in fusion to an N-terminal histidine tag in *E. coli* as inclusion bodies. By using purified denatured protein as the antigen for Western dot blotting, S2-targeting antibodies were detected in most plasma samples (33 out of 40) (Fig. 2), whereas a much lower percentage of plasma samples (7 out of 40) tested positive for S1-specific antibodies. No spike-specific antibodies were found in plasma from uninfected donors (N1 to N10) or in sera (D1 to D10) from SARS patients who died of the viral infection (Fig. 2). In 23 of the 33 positive serological samples, most of the antibodies were significantly blocked with a low concentration (5 or 10 μM) of a peptide, SL26 (Ac-QILPDPLKPTKRSFIEDLLF NKVTLA-OH, where Ac is acetyl), encompassing the identified convergent antigenic region. Antibodies in 10 other plasma samples that were unaffected by low concentrations of peptide SL26 were also significantly blocked when the peptide concentration was increased to 200 μM, indicating that they were also complementary to the identified convergent antigenic region on the spike S2 protein but with a low affinity for the synthetic peptide. Indeed, these results show that the identified epitope-rich region on the spike S2 protein is a humoral immunodominant site that induces antibodies in a majority of patients who have recovered from SARS.

A shortcoming of the epitope-determining method used is its inability to find the conformational epitopes and carbohydrate epitopes of the surface spike, matrix, and small envelope glycoproteins of SARS CoV. By using the same dot blot assay, the nonsurface nucleocapsid protein of the SARS CoV was found to be highly antigenic and to contain significant amount of conformational epitopes. All plasma samples from the examined convalescent SARS patients contained nucleocapsid-specific antibodies that could not be blocked with synthetic peptides encompassing the identified linear epitopes (data not shown). Next, we used a similar antigen-blocking assay to examine the levels of the antibodies complementary to the identified epitope fragments from viral proteins except the nucleocapsid protein. The nondenatured SARS coronaviral proteins were used as the antigen to determine antibody levels in plasma samples from 10 recovered patients (patients A to J) by ELISA. Only the plasma sample from patient F tested negative in the assay (Fig. 3), whereas all 10 plasma samples from uninfected patients tested negative. The anti-SARS CoV antibodies in the remaining nine serological samples were found to be significantly diminished to very low levels after the plasma antibodies were presaturated with soluble recombinant nucleocapsid protein and peptides containing the identified epitopes of the spike, 3a, and 9b proteins (Fig. 3). These results show that the antibodies complementary to the identified linear epitopes are a significant portion of the antibodies generated in the humoral responses of patients who have recovered from SARS.

Neutralization of SARS CoV by antibodies targeting the epitope-rich S2 site. To determine the virus-neutralizing activ-

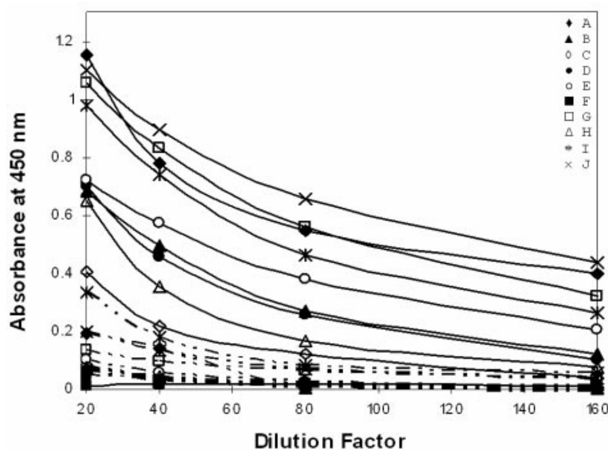

FIG. 3. Blocking of the SARS CoV-specific antibodies in plasma samples from convalescent patients A to J by the nucelocapsid protein and identified epitope peptides. Shown are ELISA results before (solid lines) and after (dotted lines) incubation with nucleocapsid protein and peptides encompassing the epitopes identified in the spike, 3a, and 9b proteins at concentrations of 26.8 and 100 μM, respectively. Other individual epitope peptides identified from the plasma samples were added at 20 μM. Under identical conditions, the readout for ELISA analysis of plasma samples from 10 uninfected donors was in the range of 0 to 0.15. Blocking of the readout signal for the plasma samples by an irrelevant nonviral protein and peptide RP1 at the same concentrations was found to be <10%.

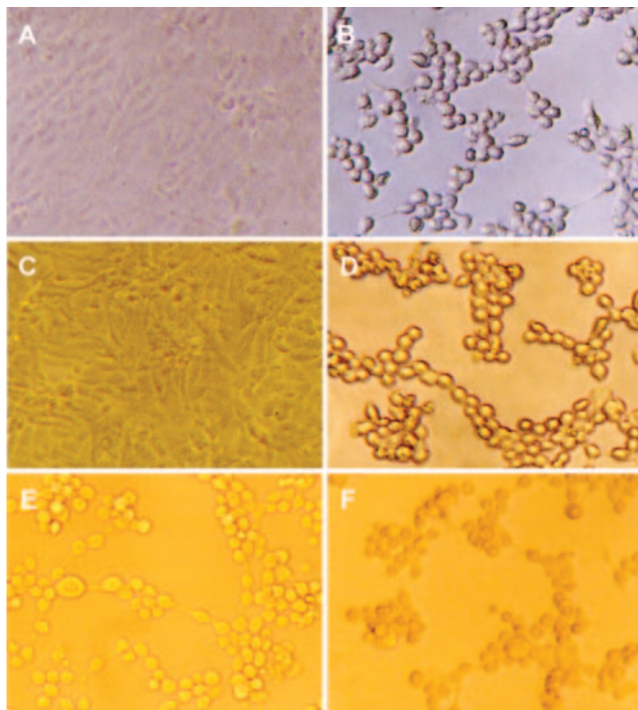

FIG. 4. Neutralization of SARS coronavirus by antibodies targeting the immunodominant site on the spike S2 protein. (A) Normal Vero E6 cells in the presence of 4 μM peptide SL26 encompassing the immunodominant region. (B) Typical cytopathic effect observed for Vero E6 cells inoculated with SARS coronavirus (BJ01). (C) Protection of Vero E6 cells from SARS CoV infection by plasma antibodies (1:40) from convalescent patient J. (D) Blocking of the neutralization effect seen in panel C by preincubation of the plasma antibodies with 4 μM peptide SL26. (E and F) No protection of Vero E6 cells from SARS CoV infection was observed for the plasma antibodies (1:10) from uninfected patient N1 (E) or the control plasma (1:10) together with 4 μM peptide SL26 (F). Cell pictures were taken with a phase-contrast microscope at a magnification of ×100 after 48 h of incubation at 37°C. Cell cultures started with 4×10^4 Vero E6 cells in a total volume of 300 μl, including 5×10^4 $TCID_{50}$ of SARS coronavirus (BJ01) where appropriate. Peptide SL26 is not cytotoxic to the host Vero E6 cells at concentrations of up to 1 mM.

ity of the antibodies complementary to the identified epitope-rich site on the spike S2 protein (Fig. 1), we tested the plasma sample from patient J, whose anti-S2 antibodies could be effectively blocked by the synthetic peptide SL26 (Fig. 2). Diluted plasma antibodies (1:40) from this patient were found to completely protect the host Vero E6 cells from infection by the SARS CoV (BJ01) (Fig. 4). However, this virus-neutralizing ability of the plasma was essentially nullified by preincubation of the plasma antibodies with 4 μM peptide SL26 (Fig. 4 and Table 1). Indeed, these results show that antibodies targeting the identified spike immunodominant site can effectively neutralize the infectivity of the viral pathogen. In addition, the efficient blocking of the virus-neutralizing antibodies by peptide SL26 alone indicates that a majority of the neutralizing antibodies from patient J are complementary to the epitope-rich S2 site, providing further support for the conclusion that the epitope-rich S2 site is an important immunodominant site of the SARS CoV.

DISCUSSION

The antigenic properties of the SARS coronavirus revealed in this study show both similarities to and differences from those of other coronaviruses. Among the many epitopes determined in the phage panning, very few were located in the viral envelope proteins M and E. In addition, the SARS CoV-specific antibodies from the recovered patients could be efficiently absorbed by a combination of the nucleocapsid protein and the identified epitope peptides. These results are consistent with the low antigenicities of the M and E proteins and the sugar moieties of the viral envelopes in other coronaviruses (5). On the other hand, the nonsurface nucleocapsid protein of the SARS CoV was found to be highly antigenic, containing many linear and conformational epitopes. This high antigenicity of nucleocapsid protein is consistent with results from other investigations (3, 15, 35) and similar to the properties of other coronaviral nucleocapsid proteins. However, in other coronaviruses, the S1 domain of the major envelope spike protein was the most antigenic; e.g., several antigenic sites were found in the N-terminal S1 domain of transmissible gastroenteritis virus (8). In contrast, the only identified surface immunodominant site in this study was found in the S2 domain of the spike protein in SARS CoV. Although a few epitopes were also found in the spike S1 domain, they were not colocalized. This antigenicity difference might result from the corticosteroids used to suppress the hyperinflammatory symptoms during the course of disease treatment (14, 24, 31), which might have altered the B-cell responses to the SARS CoV in the patients.

The short epitope-rich fragment of the spike S2 protein between amino acids 787 and 809 is an important antigen of the viral pathogen. It is located in the loop region between the

TABLE 1. Neutralization of the SARS coronavirus by the convalescent-phase plasma from patient J and its inhibition by peptide SL26[a]

Plasma dilution factor	Cytopathic effect[b]	
	Without SL26	With 4μM SL26
10	−	+++++
20	−	+++++
40	−	+++++
80	++	ND
160	++++	ND
320	+++++	ND
640	+++++	ND

[a] SL26 is the epitope-rich fragment identified on the spike S2 protein and has a sequence of Ac-QILPDPLKPTKRSFIEDLLFNKVTLA-OH.

[b] Cell cultures of Vero E6 cells (4×10^4) were infected with 5×10^4 $TCID_{50}$ of SARS coronavirus (BJ01) and incubated at 37°C for 48 h. Cytopathic effects were observed under a microscope and confirmed by crystal violet staining for live cells. +, 0 to 25% of cells infected; ++, 25 to 50% of cells infected; +++, 50 to 75% cells infected; ++++, 75 to 100% of cells infected; +++++, 100% of cells infected; ND, not determined.

predicted N and M helices of the spike protein (4) and is in close proximity to a glycosylation site identified at glutamine 783 (12), suggesting that this short peptide fragment is on the surface of the spike protein. Apparently, the glycosylation of the glutamine does not block the accessibility of the short antigenic peptide fragment, since its complementary antibodies are effective for virus neutralization. More important, a majority of convalescent patients (82.5%) (Fig. 2) developed complementary antibodies to this short antigenic spike S2 fragment, indicating that it is an immunodominant site through which the human humoral immune system recognizes and interacts with the SARS CoV. The neutralization activity of the complementary antibodies of this spike immunodominant site and the presence of such antibodies in the immune responses of most patients who have recovered from SARS seemed to suggest that antibodies targeting this site were a major contributor to the immune protection acquired by the patients, which helped them recover from the infection with the SARS CoV. However, seven of the examined convalescent-phase plasma samples (17.5%) (Fig. 2) were not found to contain any antibodies to the spike S2 domain, even though they all contained antibodies to other proteins such as the replicases of the SARS CoV. This indicated that immune protection from other sources was needed for these SARS patients.

One important source of additional immune protection from the SARS CoV came from the cellular immunity mediated by $CD8^+$ T cells, which play an important role in immune responses to all viral infections. In addition, immune protection might also come from the antibodies targeting the dispersed epitopes identified in the spike S1 domain or S2 domain of the SARS CoV, outside the immunodominant site. These antibodies should be present in more convalescent-phase plasma samples than the ones in which they were identified, like the antibodies complementary to the epitope-rich region in the spike S2 domain. Apparently not all antibodies complementary to the identified epitopes are virus neutralizing, e.g., the epitope identified in the intraviral C terminus as shown in Fig. 1. Nevertheless, at least some of the complementary antibodies are neutralizing and contribute to immune protection from the SARS CoV in some patients. This is supported by the fact that immunization of rhesus macaques with the spike S1 domain leads to virus-neutralizing antibodies (11). Moreover, antibodies complementary to the identified epitope-rich region in the unknown protein 3a (Fig. 1) might be another source of immune protection. They were found in plasma samples from a significant proportion of the convalescent SARS patients examined (40%) in a dot blot assay using a synthetic peptide with a sequence corresponding to amino acids 12 to 27 of the N terminus of the protein as the antigen (data not shown). While its exact functions remain unknown, protein 3a has been predicted to be a transmembrane protein with its N-terminal residues 1 to 35 exposed to the extracellular environment (16), and expression of its gene in infected cells has been demonstrated by transcriptional analysis (29). Generation of antibodies to this N-terminal domain should positively contribute to neutralization of the infected cells and thus augment the immune protection against the pathogen. These additional immune responses in the convalescent SARS patients are expected to complement the protective effects of the antibodies directed against the identified immunodominant site in the spike S2 domain, and they might even dominate the protective immunity in certain groups of recovered patients.

Coronaviruses are enveloped positive-stranded RNA viruses that may have an error-prone replication mechanism similar to that of human immunodeficiency virus (7), another RNA virus. Indeed, a number of mutations have been identified in the SARS genome (4, 28, 43), particularly in the gene coding the major antigenic envelope spike protein. Although molecular evolution studies of the novel coronavirus have found that its mutation rate is only one-third of that of HIV (4), escape mutations in response to immune pressure that will affect the efficacy of a vaccine are still highly possible. Fortunately, none of the mutations identified in the known genome sequences are within the antigenic sites in the spike protein and the unknown protein 3a, whose complementary antibodies most likely confer immune protection in the infected patients. This lack of mutagenicity in these antigenic regions makes them suitable for use as the immunogen in a vaccine to elicit humoral responses.

SARS is a life-threatening infectious disease with high morbidity and mortality. The lack of effective treatment and potential for reemergence make it extremely urgent to develop a vaccine against the viral pathogen. Due to the adverse effects of immune responses to previously known coronaviruses, and particularly the antibody-mediated enhancement found in vaccination of cats against feline infectious peritonitis virus, development of a SARS vaccine should be pursued with great caution. To minimize the risk, an epitope vaccine based on the immunodominant sites responsible for the beneficial humoral and cellular immune responses in convalescent SARS patients, such as the antigens determined in this study and the T-cell epitopes that have begun to be revealed, may be an attractive alternative to the whole killed vaccines, live attenuated vaccines, and recombinant vaccines currently under active development.

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