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Synthetic Peptide Studies on the Severe Acute Respiratory Syndrome (SARS) Coronavirus Spike Glycoprotein: Perspective for SARS Vaccine Development

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Background: **The S (spike) protein of the etiologic coronavirus (CoV) agent of severe acute respiratory syndrome (SARS) plays a central role in mediating viral infection via receptor binding and membrane fusion between the virion and the host cell. We focused on using synthetic peptides for developing antibodies against SARS-CoV, which aimed to block viral invasion by eliciting an immune response specific to the native SARS-CoV S protein.**

Methods: **Six peptide sequences corresponding to the surface regions of SARS-CoV S protein were designed and investigated by use of combined bioinformatics and structural analysis. These synthetic peptides were used to immunize both rabbits and monkeys. Antisera collected 1 week after the second immunization were analyzed by ELISA and tested for antibody specificity against SARS-CoV by immunofluorescent confocal microscopy.**

Results: **Four of our six synthetic peptides (S2, S3, S5, and S6) elicited SARS-CoV-specific antibodies, of which S5 (residues 788–820) and S6 (residues 1002–1030) exhibited immunogenic responses similar to those found in a parallel investigation using truncated recombinant protein analogs of the SARS-CoV S protein. This suggested that our S5 and S6 peptides may represent two minimum biologically active sequences of the immunogenic regions of the SARS-CoV S protein.**

Conclusions: **Synthetic peptides can elicit specific antibodies to SARS-CoV. The study provides insights for the future development of SARS vaccine via the synthetic-peptide-based approach.**

Severe acute respiratory syndrome (SARS)[7] is a life-threatening infectious respiratory disease with symptoms including fever, dry cough, headache, dyspnea, and hypoxemia *(1)*. A novel type of human coronavirus (SARS-CoV) was identified to be the etiologic agent of SARS *(1–3)*. The coronaviruses belong to a diverse family of large, enveloped, single-stranded positive sense RNA viruses that replicate in the cytoplasm of animal host cells *(4)*. There are three groups of coronaviruses; mammalian viruses are found in groups 1 and 2, and avian viruses are found only in group 3 *(4)*. In the past, human coronaviruses (HCoVs) found in both group 1 (HCoV-229E) and group 2 (HCoV-OC43) were associated only with mild upper respiratory tract diseases *(5)*, whereas the novel SARS-CoV appears to be the first human coronavirus responsible for severe disease in humans. Phylogenetic analysis indicates that SARS-CoV does not belong to any of the above three groups, which contain all other known coronaviruses, including the human coronaviruses; this suggests that SARS-CoV did not arise by recombination or mutation of the known coronaviruses *(6, 7)*.

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[7] Nonstandard abbreviations: SARS, severe acute respiratory syndrome; CoV, coronavirus; ORF, open reading frame; KLH, keyhole limpet hemocyanin; and PBS, phosphate-buffered saline.

SARS-CoV contains a RNA genome of ~30 kb. Its overall genome organization is typical of other coronaviruses, with five major open reading frames (ORFs) encoding the replicase polyprotein, which comprises two-thirds of the genome, and the four major structural proteins. The nucleocapsid (N) protein and the membrane (M) protein interact to form a spherical core, whereas the spike (S) glycoprotein, and the envelope (E) protein constitute the viral envelope *(6–8)*. In addition to these proteins, the genome of SARS-CoV also encodes for other uncharacterized structural and nonstructural viral proteins.

The distinct crown appearance on the surface of SARS-CoV is attributed to the S protein. On coronavirus infection, the S protein recognizes and binds to species-specific host cell receptors, and the conformational changes induced in the S protein would then facilitate the fusion between the viral envelope and host cell membranes *(9–11)*. The potential receptor-binding site of SARS-CoV should lie within the surface region of S protein, and it would be a good target for the development of synthetic peptide-based vaccines against SARS-CoV. To date, there is no specific treatment for SARS, and the control of SARS-CoV infection by vaccination is not yet available. It is possible for SARS to recur in a seasonal pattern similar to other similar respiratory diseases; thus the development of an effective vaccine is necessary for sustained control of SARS. Although both attenuated and inactivated coronaviruses could be used for vaccine development, these types of SARS vaccines bear a potential risk of unpredictable SARS outbreak. In this study, we focused on the use of biologically active synthetic peptides for viral protein neutralization, which aimed to block the viral invasion by eliciting an immune response that could specifically recognize and neutralize the S protein of SARS-CoV. Our study could also provide insights into future vaccine development against SARS-CoV by the synthetic peptide-based approach and help to achieve better understanding of SARS to allow better preparation for possible recurrences of SARS.

Materials and Methods

PEPTIDE DESIGN

The genome sequence of SARS-CoV used for peptide design in this study was based on the SARS-CoV CUHK-Su10 complete genome sequence downloaded from GenBank (http://www.ncbi.nlm.nih.gov/entrez; accession number: AY282752). ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to determine the ORF of the SARS-CoV S glycoprotein, NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict potential N-glycosylation sites, and NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict potential phosphorylation sites. The secondary structure and hydrophobicity analyses on the sequence of the S protein were performed with DNASIS MAX (Ver. 2.0) software (MiraiBio), and peptide sequences that correspond to the potential surface regions of the S proteins were designed based on the combined data from the above protein analyses. The three-dimensional structures of these peptides in the solvated state were simulated on an Insight II molecular modeling platform (Accelrys) running on Silicon Graphics workstations.

PEPTIDE SYNTHESIS

The six designed peptides were synthesized by a solid-phase technique with an Applied Biosystems 433A Peptide Synthesizer on amide resins using standard Fmoc synthesis with HBTU/HOBt coupling. The NH_2 termini of peptides were acetylated on the resin with 150 g/L acetic anhydride in dimethyl formamide. The acetylated peptides were cleaved from the resins, and side-chain-protecting groups were removed by cleavage solution containing 25 g/L ethanedithiol and 50 g/L thioanisole in trifluoroacetic acid. The peptides were then precipitated with cold diethyl ether, washed, and lyophilized. The cleaved peptides were purified with Agilent Technologies 1100 Series analytical and preparative HPLC systems using linear gradients formed from the solvent systems A (50 mL/L trifluoroacetic acid in H_2O) and B (0.5 mL/L trifluoroacetic acid in acetonitrile) *(12)*. The identities of purified peptides were confirmed by mass spectrometry on an Ettan™ matrix-assisted laser desorption/ionization time-of-flight Pro Mass Spectrometer (Amersham Biosciences).

PEPTIDE CONJUGATION

Keyhole limpet hemocyanin (KLH) was dissolved in phosphate-buffered saline (PBS) at pH 7.0 to a final concentration of 5 g/L. Approximately 1 mg of synthetic peptide was added to 1 mL of KLH solution (i.e., ~200–300 mol peptides/mol of KLH). The mixture was sonicated in a water bath for 30 min; 5 mg of *N*-hydroxysuccinimide and 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were then added, and the mixture was stirred at room temperature for 30 min. The mixture was loaded on a HiTrap™ Desalting Column (Amersham Biosciences), and the eluate was collected as the KLH-conjugated peptide solution.

IMMUNIZATION IN RABBITS AND MONKEYS

Before the immunization of rabbits and monkeys, preimmune sera were collected and confirmed to be SARS-CoV negative by real-time quantitative reverse transcription-PCR *(13)*. In the primary injection, 0.5 mL of complete Freund's adjuvant was added to 0.5 mL of purified antigen with a peptide concentration of 1 g/L (either conjugate-free peptide or KLH-conjugated peptide) and emulsified. Each rabbit (male New Zealand White; weight, 2–3 kg) and monkey [male *Macaca fascicularis*; weight, 2–3 kg; the monkey strain *M. fascicularis* was chosen in this study because it has been demonstrated previously as a potent host of SARS-CoV that developed SARS symptoms after SARS-CoV infection *(14)*] was immunized with 1 mL of emulsion by subcutaneous injection at five different sites on day 0. The rabbits and

monkeys were then boosted by subcutaneous injection of an emulsion containing 0.5 mL of incomplete Freund's adjuvant and 0.5 mL of purified antigen on days 14 and 28. The first batch of rabbit and monkey antisera was collected 1 week after the second immunization.

ELISA analysis

Titrations of both the rabbit and monkey antisera against the corresponding peptide or peptide conjugate were determined in duplicate by ELISA as described previously *(15)*, and the results were averaged. The purified antigens of the six synthetic peptides (either conjugate-free peptide or KLH-conjugated peptide) were diluted to ~10 mg/L in PBS; 100 μL of the diluted antigen solution was added to the corresponding well of a 96-well microplate and incubated at 4 °C overnight. The wells were washed three times with 0.5 mL/L Tween 20 in PBS; 100 μL of PBS containing 50 mL/L skim milk and 0.5 mL/L Tween 20 was then added to each well and incubated at 37 °C for 2 h for blocking. The rabbit and monkey antisera against the synthetic peptides were diluted in a twofold series to 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200 in PBS containing 50 mL/L skimmed milk and 0.5 mL/L Tween 20, and 100 μL of diluted antiserum was added to each well. The plate was incubated at 37 °C for 1 h. After plates were washed three times with PBS containing 0.5 mL/L Tween 20, bound antibody was measured by use of either alkaline phosphatase-conjugated goat anti-rabbit IgG or alkaline phosphatase-conjugated goat anti-monkey IgGy (100 μL/well of 1:500 dilution of IgG in PBS containing 50 mL/L skimmed milk and 0.5 mL/L Tween 20). After incubation at 37 °C for 1 h and three washes with PBS containing 0.5 mL/L Tween 20, 100 μL of developing solution (5 mg of *p*-nitrophenyl phosphate added to 5 mL of substrate buffer containing 50 mL of diethanolamine, 50 mg of $MgCl_2 \cdot H_2O$, and 97.5 mg of NaN_3; adjusted to pH 9.7 with NaOH and brought to 500 mL with MilliQ H_2O) was added to each well. Absorbance was monitored by a microplate reader at 415 nm. Both the rabbit and monkey preimmune sera were used as negative controls in this assay.

Immunofluorescent confocal microscopy

The rabbit and monkey antisera against the synthetic peptides were diluted to 1:40-fold with PBS, and 10 μL of each diluted serum was added to a well of the slide that was coated with SARS-CoV-infected African green monkey kidney Vero cells and incubated at 37 °C for 1 h. The slide was washed three times with PBS and air dried. In each well of the slide, 10 μL of the diluted conjugate (1:40) and Evan Blue (1:400) were added, and the slide was incubated at 37 °C for 1 h. The slide was washed three times with PBS, air dried, and mounted. The mounted slide was observed with a confocal microscope. Noninfected African green monkey kidney Vero cells and the preimmune sera of both the rabbits and monkeys were used as negative controls in this assay.

Results

General features and structural analysis of the S protein

The ORF encoding for the S glycoprotein of SARS-CoV is 3768 nucleotides in length, comprising 12.67% of the total genome. The S protein is the largest structural viral protein in SARS-CoV and is composed of 1255 amino acids with an estimated molecular mass of 139.12 kDa; its isoelectric point (pI) is 5.48. There are 23 potential sites of N-linked glycosylation and 69 potential sites of phosphorylation, including 37 serine, 15 threonine, and 17 tyrosine residues. The S protein of SARS-CoV demonstrates <50% identity with the previous identified S proteins of other known coronaviruses. The secondary structure and the hydrophobicity of the S protein were predicted by use of DNASIS MAX (Ver. 2.0) software.

Peptide design and synthesis

Because the crystal structure of the SARS-CoV S protein is not yet available, we designed six peptide sequences (S1, S2, S3, S4, S5 and S6) ~20–30 amino acid residues in length (Table 1), which corresponded to the surface regions of S protein based on the combined data from protein analyses. Most of them were expected to adopt a helix-loop motif and were hydrophilic; they were relatively widespread throughout the sequence of the S protein. These six synthetic peptides were used as either conjugate-free or KLH-conjugated peptides for immunization of both the rabbits and monkeys. The three-dimensional structures of these six synthetic peptides in solvated states were simulated on an Insight II molecular modeling platform, and they were expected to adopt helix-loop motifs at their relatively stable energy states.

Table 1. Amino acid sequences of the six synthetic peptides.[a]

Peptide	Amino acid positions	Amino acid sequence	No. of amino acids	Molecular mass, Da	pI
S1	75–96	TFGNPVIPFKDGIYFAATEKSN	22	2416.6	6.74
S2	229–251	TNFRAILTAFSPAQDIWGTSAAA	23	2409.5	6.51
S3	573–593	ISPCSFGGVSVITPGTNASSE	21	2010.1	3.25
S4	1120–1140	YDPLQPELDSFKEELDKYFKN	21	2618.8	4.10
S5	788–820	LPDPLKPTKRSFIEDLLFNKVTLADAGFMKQYG	33	3754.2	9.53
S6	1002–1030	ASANLAATKMSECVLGQSKRVDFCGKGYH	29	3072.4	9.05

[a] Provisional patent application number 60/487,396 (filing date July 14, 2003).

ELISA analysis and immunofluorescent confocal microscopy

The first batch of the rabbit and monkey antisera against the six synthetic peptides was collected 1 week after the second immunization and was tested for antibody specificity against the corresponding antigen (either conjugate-free or KLH-conjugated peptide) by ELISA analysis. As shown in Figs. 1 and 2, all of these antisera were specific to their corresponding peptide antigens. Both the rabbit and monkey preimmune sera were used as negative controls in this assay.

These rabbit and monkey antisera were then tested for antibody specificity against SARS-CoV by immunofluorescent confocal microscopy. The antisera were incubated with African green monkey kidney Vero cells infected by SARS-CoV obtained from a SARS patient. The results of immunofluorescent confocal microscopy are shown in Fig. 3 and summarized in Table 2. For rabbit antisera, positive results were observed in samples R_S2_KLH, R_S5 R_S5_KLH, and R_S6_KLH, which were elicited by their corresponding antigens: S2-KLH conjugate, S5 peptide, S5-KLH conjugate, and S6-KLH conjugate, respectively. For monkey antisera, positive results were observed in samples M_S3, M_S6, M_S6_KLH, M_MIX, and M_MIX_KLH, which were elicited by their corresponding antigens: S3 peptide, S6 peptide, S6-KLH conjugate, MIX peptide, and MIX-KLH conjugate, respectively. Noninfected African green monkey kidney Vero cells and the preimmune sera of both the rabbits and monkeys were used as negative controls in this assay. The positive results indicated that most of the SARS-CoV was localized abundantly in the cytoplasm of the infected cells, which further confirmed the replication of SARS-CoV in the cytoplasm of animal host cells.

Discussion

In this study, we designed a total of six peptide sequences, S1–S6 (Table 1), that corresponded to the surface regions of SARS-CoV S protein based on the combined bioinformatics data from protein analyses. These six synthetic peptides were used to immunize both rabbits and monkeys. The results of immunofluorescent confocal microscopy indicated that four of the six synthetic peptides, S2, S3, S5, and S6, raised antibodies that could specifically

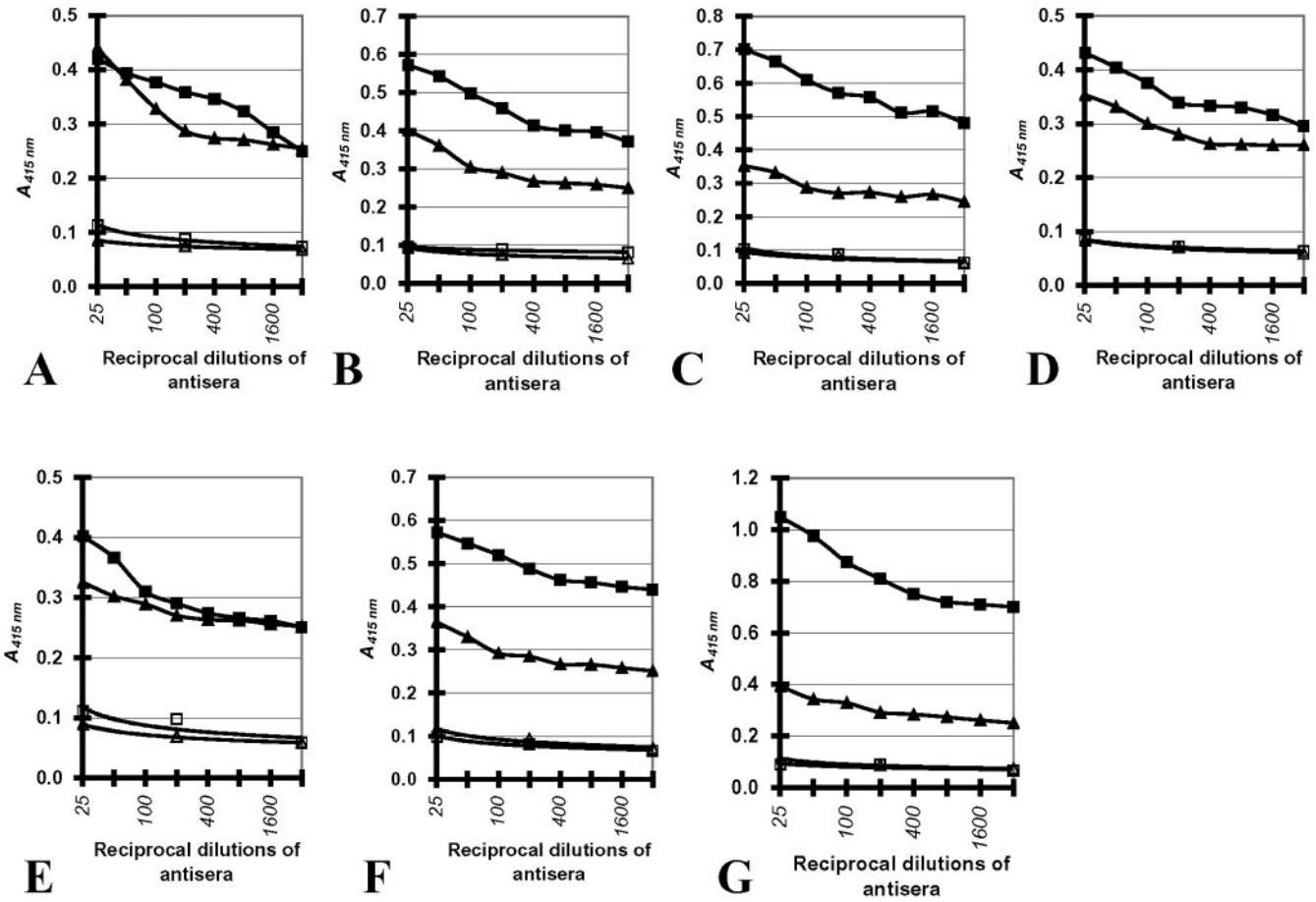

Fig. 1. Titration of rabbit serum samples diluted from 1:25 in a twofold series against constant antigen concentration of 10 mg/L.

Anti-peptide antiserum is indicated by ▲ with the corresponding preimmune serum, indicated by △, as negative control. Anti KLH-conjugated peptide antiserum is indicated by ■, and the corresponding preimmune serum is indicated by □ as negative control. (*A*), R_S1 antiserum (▲) and R_S1_KLH antiserum (■); (*B*), R_S2 antiserum (▲) and R_S2_KLH antiserum (■); (*C*), R_S3 antiserum (▲) and R_S3_KLH antiserum (■); (*D*), R_S4 antiserum (▲) and R_S4_KLH antiserum (■); (*E*), R_S5 antiserum (▲) and R_S5_KLH antiserum (■); (*F*), R_S6 antiserum (▲) and R_S6_KLH antiserum (■); (*G*), R_MIX antiserum (▲) and R_MIX_KLH antiserum (■).

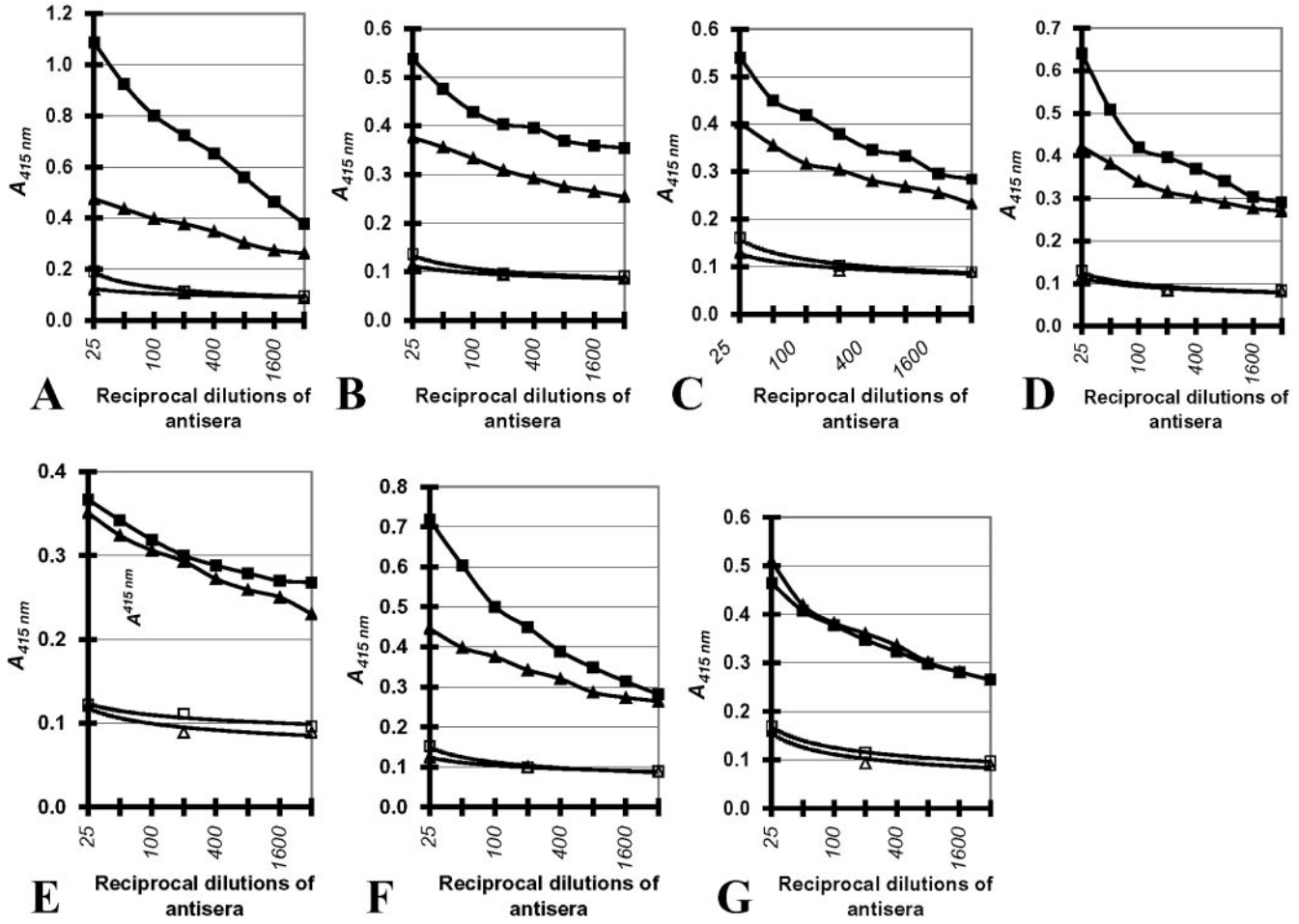

Fig. 2. Titration of monkey serum samples diluted from 1:25 in a twofold series against constant antigen concentration of 10 mg/L.

Anti-peptide antiserum is indicated by ▲ with the corresponding preimmune serum, indicated by △, as negative control. Anti KLH-conjugated peptide antiserum is indicated by ■, and the corresponding preimmune serum is indicated by □ as negative control. (*A*), M_S1 antiserum (▲) and M_S1_KLH antiserum (■); (*B*), M_S2 antiserum (▲) and M_S2_KLH antiserum (■); (*C*), M_S3 antiserum (▲) and M_S3_KLH antiserum (■); (*D*), M_S4 antiserum (▲) and M_S4_KLH antiserum (■); (*E*), M_S5 antiserum (▲) and M_S5_KLH antiserum (■); (*F*), M_S6 antiserum (▲) and M_S6_KLH antiserum (■); (*G*), M_MIX antiserum (▲) and M_MIX_KLH antiserum (■).

recognize SARS-CoV, which suggested the efficacy of using synthetic peptides as antigens to elicit specific immune responses.

In a parallel study carried out at Tsinghua University using recombinant protein approaches, similar results were obtained. Pang's team *(16)* at Tsinghua University has generated a series of truncated recombinant protein analogs (SG3, analog of residues 735–882; S10, analog of residues 730-1150; and S22, analog of residues 894-1192) of the SARS-CoV S protein to characterize the structural and functional properties of the S protein. Their immunoassays indicated that the SG3, S10, and S22 analogs of the S protein were recognized by the IgG in sera from recovered SARS patients. Their SG3 analog is suggested to be the most specific immunogenic region on the S protein. Our work clearly demonstrated that the immunogenic region of their SG3 analog (residues 735–882) could be further delineated to residues 788–820, which corresponds to our S5 peptide (Table 1). In addition, our S6 peptide (residues 1002–1030; Table 1) could be another minimum and significant immunogenic region of their S10 and S22 analogs of S protein. In addition, free S6 peptide is capable of inducing specific antibodies in monkeys.

There are several factors contributing to the ability of the four synthetic peptides, i.e., S2, S3, S5, and S6, to elicit the production of SARS-CoV-specific antibodies. The design of the peptide sequences was deliberately targeted to the surface region of the S protein. By use of sequence homology analysis, secondary structure, and hydrophobicity predictions, we could predict the potential surface region of the S protein. Most of the designed peptides were expected to adopt a helix motif to give a rigid structure to enhance antigenicity. To further enhance the antigenicity of these peptides, later work may include introducing several constraints to these peptides to increase structural rigidity, such as by adding linkers to cyclize the linear peptides or disulfide bonds for peptide stabilization. In addition, from the in silico energy minimization studies of our peptides, the predicted loop size

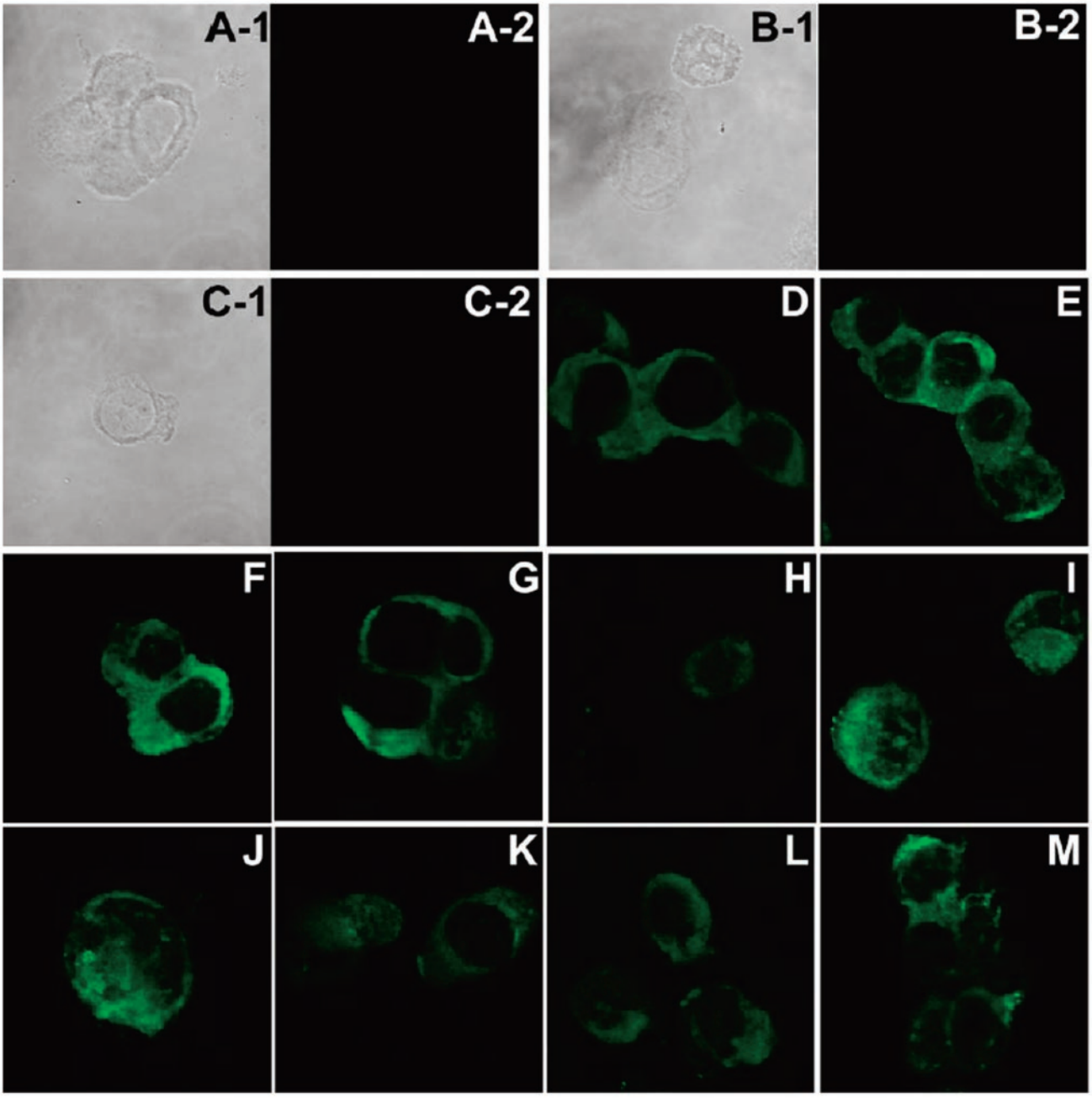

Fig. 3. Positive results in immunofluorescent confocal microscopy are indicated by the presence of *green* fluorescent signals in the cytoplasm of the African green monkey kidney Vero cells.

(*A*), negative control using rabbit R_S1 preimmune serum, observed under light microscope (*A-1*) and confocal microscope (*A-2*). Similar results were observed in all other rabbit preimmune sera. (*B*), negative control using monkey M_S1 preimmune serum, observed under light microscope (*B-1*) and confocal microscope (*B-2*). Similar results were observed in all other monkey preimmune sera. (*C*), negative control using SARS patient serum on noninfected Vero cells, observed by light microscope (*C-1*) and confocal microscope (*C-2*). (*D*), positive control using SARS patient serum. Positive results in confocal microscopy were shown for the following rabbit and monkey antiserum samples: R_S2_KLH antiserum (*E*); R_S5 antiserum (*F*); R_S5_KLH antiserum (*G*); R_S6_KLH antiserum (*H*); M_S3 antiserum (*I*); M_S6 antiserum (*J*); M_S6_KLH antiserum (*K*); M_MIX antiserum (*L*); and M_MIX_KLH antiserum (*M*).

carried on the peptide may reflect its antigenicity; thus, the loop structures of these peptides could be further adjusted and strengthened to optimize the corresponding antigenicity. In addition, our initial attempts at antibody production using the six synthetic peptides had deliberately avoided the regions rich in posttranslation modifications, such as glycosylation and phosphorylation, to simplify the situation for preliminary analysis.

On the basis of our studies, which demonstrated that four of the synthetic peptides could elicit the production of SARS-CoV-specific antibodies, future experiments will be carried out to address the protective nature of these

Table 2. Results of immunofluorescent confocal microscopy.

	Immunization in rabbits		Immunization in monkeys	
Antigen used for immunization	Antiserum	Result of confocal microscopy	Antiserum	Result of confocal microscopy
S1 peptide	R_S1	−	M_S1	−
S1-KLH conjugate	R_S1_KLH	−	M_S1_KLH	−
S2 peptide	R_S2	−	M_S2	−
S2-KLH conjugate	R_S2_KLH	+	M_S2_KLH	−
S3 peptide	R_S3	−	M_S3	+
S3-KLH conjugate	R_S3_KLH	−	M_S3_KLH	−
S4 peptide	R_S4	−	M_S4	−
S4-KLH conjugate	R_S4_KLH	−	M_S4_KLH	−
S5 peptide	R_S5	+	M_S5	−
S5-KLH conjugate	R_S5_KLH	+	M_S5_KLH	−
S6 peptide	R_S6	−	M_S6	+
S6-KLH conjugate	R_S6_KLH	+	M_S6_KLH	+
Mix peptide[a]	R_MIX	−	M_MIX	+
Mix-KLH conjugate[a]	R_MIX_KLH	−	M_MIX_KLH	+

[a] Mix peptide and Mix-KLH conjugate were prepared by mixing the six synthetic peptides (S1, S2, S3, S4, S5, and S6) in equal amounts.

antibody responses in monkeys. This can be done by vaccinating noninfected monkeys with these synthetic peptides. After exposure of these vaccinated monkeys to live SARS-CoV, their health status could be monitored to determine the efficacy of these synthetic peptides as potential SARS vaccines. The monkey strain *M. fascicularis* would be a good choice for this animal test because this strain has previously been demonstrated as a potent host of SARS-CoV that developed SARS symptoms after SARS-CoV infection *(14)*. Because of the high infectiousness of SARS-CoV, a biosafety P3/P4 level laboratory is required whenever performing any experiment using live SARS-CoV to prevent unpredictable outbreaks of SARS.

It is possible for SARS to recur in a seasonal pattern, as do other, similar respiratory diseases; thus the development of an effective vaccine is necessary for the sustained control of SARS. To date, vaccination against SARS is not available. A synthetic peptide-based approach could be a rapid initial step for providing useful information for SARS-related research. The results of synthetic peptide studies enable preliminary information about the possible antigenic sites on the native viral protein to be generated, which provides insights for generating a recombinant protein analog that could better mimic the conformation of the epitope regions. Moreover, the biophysical properties and binding kinetics of these synthetic peptide candidates could be systematically characterized to allow elucidation of the structural and functional relationships of different SARS-CoV proteins. Databases designed to contain future "novel" SARS-related RNA and protein sequences are being constructed, and updating of these databases will be an ongoing task to monitor "polymorphisms" in the viral RNA and protein sequences. The quality (biologically active sequences) and quantities of our peptide stocks can be easily adjusted to accommodate the ongoing investigation of polymorphisms in the SARS-CoV genome that produce changes in the primary sequences of the viral protein. Eventually, we will establish peptide databases that cover the motifs corresponding to the biologically active functional and structural regions of the SARS-CoV proteins. Our experimental data are informative and could advance the understanding of SARS, which is necessary if we are to be prepared for possible recurrences of this disease.

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