Screening and Identification of Linear B-Cell Epitopes and Entry-Blocking Peptide of Severe Acute Respiratory Syndrome (SARS)-Associated Coronavirus Using Synthetic Overlapping Peptide Library

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A 10-mer overlapping peptide library has been synthesized for screening and identification of linear B-cell epitopes of severe acute respiratory syndrome associated coronavirus (SARS-CoV), which spanned the major structural proteins of SARS-CoV. One hundred and eleven candidate peptides were positive according to the result of PEPscan, which were assembled into 22 longer peptides. Five of these peptides showed high cross-immunoreactivities (~66.7 to 90.5%) to SARS convalescent patients' sera from the severest epidemic regions of the China mainland. Most interestingly, $S_{471-503}$, a peptide located at the receptor binding domain (RBD) of SARS-CoV, could specifically block the binding between the RBD and angiotensin-converting enzyme 2, resulting in the inhibition of SARS-CoV entrance into host cells in vitro. The study demonstrated that $S_{471-503}$ peptide was a potential immunoantigen for the development of peptide-based vaccine or a candidate for further drug evaluation against the SARS-CoV virus−cell fusion.

Introduction

Severe acute respiratory syndrome (SARS) is an emergent infectious disease that has spread globally from southern China since November 2002.[1] SARS has caused up to 8422 probable cases in 32 countries and areas, resulting in a cumulative 916 deaths from November 16, 2002, to August 7, 2003.[2] After the global outbreak, several new cases have been reported from the end of 2003 to early 2004 in China. These incidents indicated that the SARS epidemic retains a distinct possibility of resurgence, and we need to be prepared.

A novel coronavirus, SARS-associated coronavirus (SARS-CoV), has been identified as the causal agent of SARS.[3] SARS-CoV attaches to host cells via the specific receptor, angiotensin converting enzyme 2 (ACE2).[4] Other research showed that the first step of the virus−cell fusion course is mediated by spike glycoprotein association with ACE2.[5] Furthermore, the S1 subunit of the S protein is believed to play the crucial role in the virus−cell fusion, and the receptor binding domain (RBD) of SARS-CoV to ACE2 has been localized between amino acid residues 303 and 537 on the S protein.[6] The highly conserved heptad repeat (HR) regions (HR1 and HR2) in the S2 domain, on the other hand, is believed to facilitate the juxtaposition of the virus and cell membrane to induce the membrane fusion.[7] The information from research about other coronaviruses has suggested that nucleocapsid protein is also very important in the life cycle of the virus and is a major antigen interacting with the host's immune system.[8]

Vaccines are thought to be the most efficient weapons against infectious disease. The development of a vaccine is based on the identification of T-cell and B-cell epitopes. Vaccinal epitopes are composed of discontinuous epitopes

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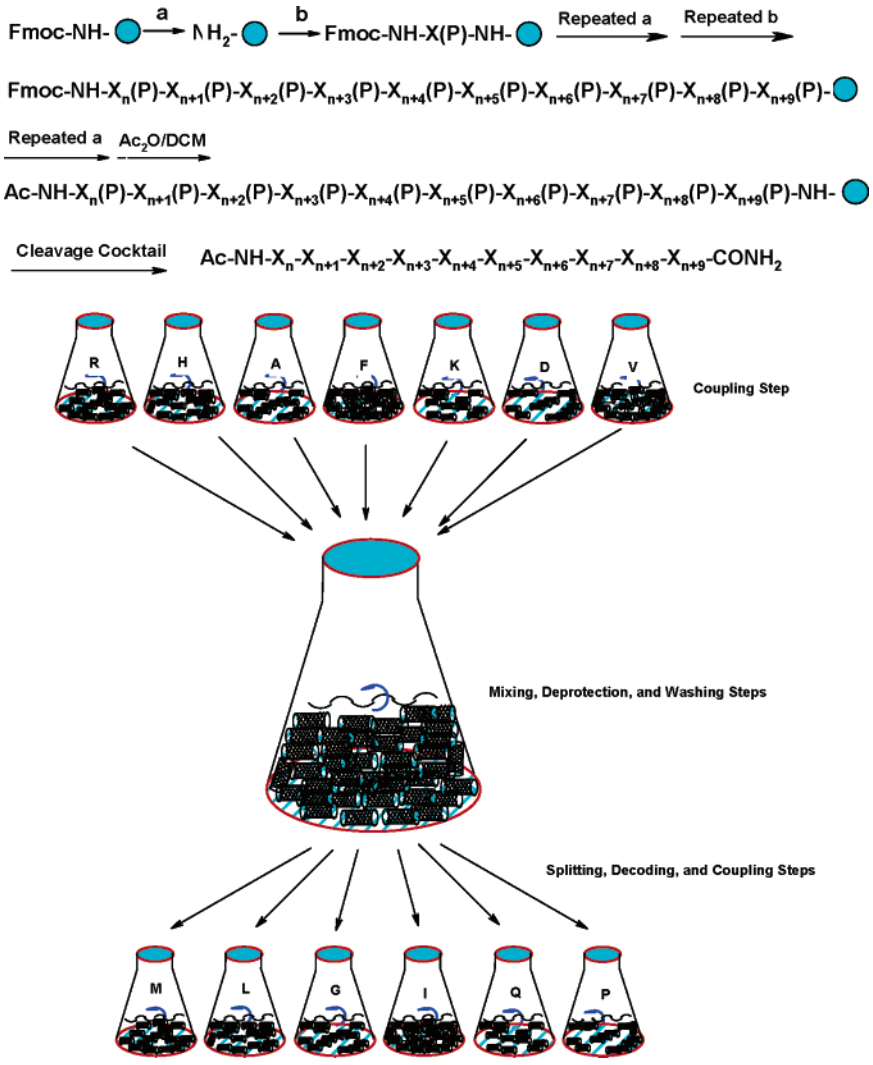

Figure 1. Solid-phase synthesis of 10-mer peptide library by Fmoc chemistry strategy (A) using a "mix and split" approach of IRORI physical coding method. (B) An acetyl group capped the N-terminus of 10 amino acids peptides. (a) 20% piperidine/DMF; (b) Fmoc-NH-X(P), BOP/HOBt/DMF. Cleavage cocktail: 81.5% TFA, 5% thioanisole, 5% phenol, 5% H_2O, 2.5% EDT, 1% TIS.

that are combinations of spatially binding sites of the protein as a conformational restriction and linear epitopes that could be screened and identified using various kinds of peptide libraries, for example, a biological expression of gene fragments, a phage display library, and a synthetic random or overlapping peptide library, etc. Several research groups have reported SARS-CoV specific T-cell epitopes.[9−11] In this study, we synthesized an overlapping peptide library to screen the linear peptides of SARS-CoV and explored an epitopic assembly approach for the identification of immunogenic multiple epitopes based on antigens of SARS-CoV. Thus, a 10-mer overlapped peptide library was synthesized by means of the IRORI sorting method.[12] The peptide library contained 1938 individual peptides that spanned all SARS-CoV BJ01 structural proteins, including the spike glycoprotein (S), membrane protein (M), envelope protein (E), and nucleocapsid protein (N). Enzyme-linked immunosorbent assay (ELISA) was employed for B-cell epitope screening and the sera of up to 42 SARS convalescent patients were used as primary antibodies. After assembly and redesign of the positive epitopes gained above, 22 longer peptides were resynthesized; 5 of these peptides ($S_{471-503}$, $S_{604-625}$, $S_{1164-1191}$, N_{67-76}, and $N_{367-389}$) were serologically highly cross-reactive with the sera of all tested SARS patients, indicating that they could be used as candidates of SARS-CoV diagnostic reagents. Competitive ELISA suggested that they were SARS-selective and immunoreactive. Most interestingly, peptide $S_{471-503}$ could specifically block the binding of ACE2 with the RBD of SARS-CoV and inhibit the SARS-CoV infection of Vero cells in vitro, indicating that peptide $S_{471-503}$ might serve as a potential immunoantigen for the development of a peptide-based vaccine or a valuable candidate for drug evaluation against the SARS-CoV virus−cell fusion.

Results

Synthesis of Peptide Library. The library scaffold is illustrated in Figure 1. X represents a natural amino acid which is located between the n or $n + 1$ and the $n + 9$ positions in all four of the SARS-CoV structural protein's sequences. Ten-mer overlapping peptides resulted in a 1938-member peptide library.

Peptide synthesis was carried out by a standard Fmoc chemistry[13] on Rink resin, as outlined in Figure 1. Each peptide sequence was coded by an Rf tag bearing 15 mg of resin (loading 0.8 mmol/g) of peptide synthetic scale.

Table 1. Results of SARS-CoV-Specific IgG Test and Source Distribution of Patients[a]

	number of cases			
sources of patients	total	positive	negative	OD value >1.0
Beijing	40	32	8	25
Guangdong	5	4	1	2
Shanxi	5	4	1	2
Inner Mongolia	5	2	3	1

[a] The OD value of negative controls was 0.05, and the cutoff value was 0.18.

Microkans were used as both the resin and the Rf tag container. The library was divided into three groups containing 600, 646, and 692 individual peptides and was synthesized. During the Fmoc deprotection and washing steps, all microkans were combined and treated together using 20% piperidine/ *N*,*N*-dimethylformamide (DMF) or thoroughly washed by various solvents. However, the microkans, which would couple with the same protected amino acid for the next coupling step, were grouped into the same reaction bottle after the sorting peptide sequence and then reacted with a 3-fold excess of Fmoc-protected amino acid together with a 3-fold excess of BOP and HOBt as coupling activator.

After all the amino acid assembly steps were finished, through mixing, deprotection of Fmoc, thoroughly washing and sorting, grouping, and coupling steps repeatedly, each peptide sequence was finally decoded and cleaved into single labeled tube. During the synthesis, the coupling efficacy of the control peptides in each group was monitored by the ninhydrin test.[14] Generally, the control peptides involved some "difficult" sequences with hydrophobic amino acid assembly. A total of 5% of the peptides were randomly analyzed by a reversed-phase auto liquid column-mass spectrometry/mass spectrometry (LC-MS/MS) system. The purity of peptides ranged from 30 to 95%.

Test of SARS-CoV-Specific IgG in SARS Patients' Sera. Sera from 55 SARS convalescent patients from the four severest SARS epidemic areas in China were collected in this study in order to avoid the missing viral mutants. They were from Beijing (37 cases), Guangdong (5 cases), Shanxi (5 case), and Inner Mongolia (5 cases) of China. The SARS-specific IgG was determined prior to the screening of epitopes using a commercially available SARS-CoV-specific antibody kit. In fact, 13 of the 55 sera from the SARS convalescent patients were identified as negative. The source distribution of patients and results of ELISA are shown in Table 1. The optical density (OD) values of 54.54% (30/55) of the antisera were above 1.0, indicating high titers (more than 1280) of SARS-CoV-specific IgG in those sera.

PEPscan (Peptide Screening) for the Linear B-Cell Epitopes of SARS-CoV. Initially, we mixed the SARS patients' sera in equal volumes to perform the peptide screening in order to increase the possibility of finding epitopes. After primary screening, 111 peptides were selected as potential epitopes whose OD values were 2-fold higher than the negative controls (the coating buffer as blank; the human sera from healthy donors as negative controls; the cutoff values were ~0.4). As shown in Figure 2, 99, 9, 2, and 1 peptide(s) were located in the S, N, M, and E proteins, respectively.

Twenty-two peptides (Table 2) containing one or two natural neighboring epitopes according to the structural protein sequences then were designed and synthesized to increase their antigenicity. The test of cross-immunoreactivities of these peptides was preformed on a larger scale of antisera (total 42 antisera from four SARS epidemic areas, Table 2). $S_{471-503}$, $S_{604-625}$ from the S1 fragment, $S_{1164-1191}$ from the S2 fragment, and $N_{367-389}$ peptides showed broad immunoreactivities, at 30/42 (71.4%), 30/42 (71.4%), 28/42 (66.7%), and 38/42 (90.5%), respectively. A short peptide,

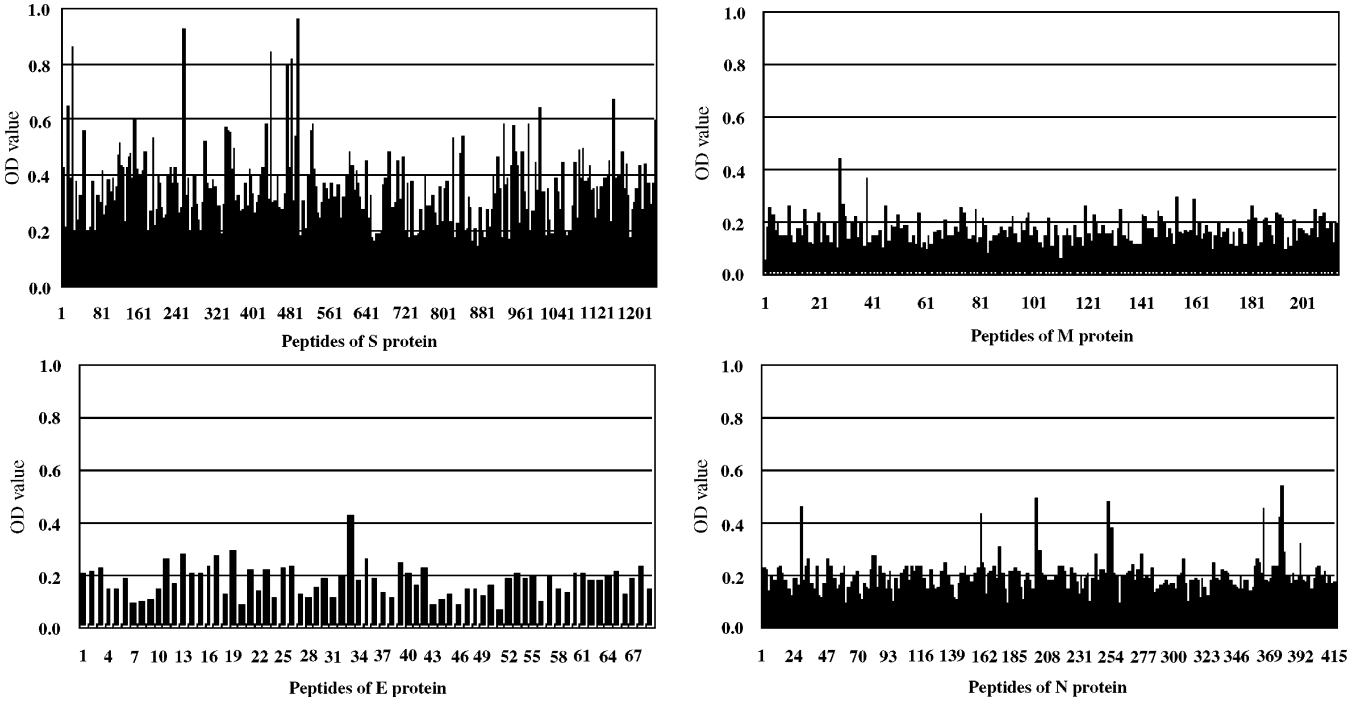

Figure 2. The results of peptide screening S, M, E, and N proteins.

Table 2. Designed Peptide Sequences and Locations of Assembled Peptides

Peptide	Structural Protein Location	Sequence*
S_{12-31}	S1	SGSDLDRCTTFDDVQAPNYT
$S_{141-163}$	S1	SKPMGTQTHTMIFDNAFNCTFEY
$S_{236-262}$	S1	TAFSPAQDTWGTSAAAYFVGYLKPTTF
$S_{298-312}$	S1	GIYQTSNFRVVPSGD
$S_{342-368}$	S1	RKKISNCVADYSVLYNSTFFSTFKCYG
$S_{420-445}$	S1	VLAWNTRNIDATSTGNYNYKYRYLRH
$S_{471-503}$	S1	ALNCYWPLNDYGFYTTTGIGYQPYRVVVLSFEL
$S_{524-537}$	S1	CVNFNFNGLTGTGV
$S_{604-625}$	S1	TDVSTAIHADQLTPAWRIYSTG
$S_{704-723}$	S2	ITTEVMPVSMAKTSVDCNMY
$S_{834-850}$	S2	AQKFNGLTVLPPLLTDD
$S_{945-963}$	S2	VKQLSSNFGAISSVLNDIL
$S_{965-986}$	S2	RLDKVEAEVQIDRLITGRLQSL
$S_{1003-1012}$	S2	SANLAATKMS
$S_{1073-1100}$	S2	REGVFVFNGTSWFITQRNFFSPQIITTD
$S_{1142-1166}$	S2	TSPDVDLGDISGINASVVNIQKEID
$S_{1164-1191}$	S2	EIDRLNEVAKNLNESLIDLQELGKYEQY
M_{29-48}	M	AWIMLLQFAYSNRNRFLYII
N_{32-41}	N	GRNGARPKQR
N_{67-76}	N	LPQGTTLPKG
$N_{203-212}$	N	SRGNSPARMA
$N_{254-266}$	N	EASKKPRQKRTAT
$N_{367-389}$	N	TEPKKDKKKKTDEAQPLPQRQKK

*Colorized sequences represent the epitopes that were assembled into immunoantigens.

N_{67-76} from the N-termiuns of the N protein, also showed high cross immunoreactivity (32/42, 76.2%) (Table 3). Three SARS patients' sera (BJ12, GD3, and SX3) were reactive to none of the peptides. One (BJ11) was reactive to only peptides from the S proteins. Two (BJ25, BJ30) were specific for the N protein. The epitopes from the S1 protein ($S_{471-503}$, $S_{604-625}$) were more antigenic than the one from the S2 protein ($S_{1164-1191}$). Most patients' sera, which were not reactive to N_{67-76}, serologically reacted with $N_{367-389}$, with the exception of one (BJ11) that did not respond to either of them.

Identification of Specificities and Immunoreactivities of Peptides. To determine the specificity of those five peptides, we preformed a competitive assay against viral lysates of SARS-CoV. In this experiment, the individual peptide or the mixture of five peptides was premixed with the patient's sera and incubated for 1 h. The residual ability of the patients' sera to bind to lysates of SARS-CoV then was tested. As illustrated in Figure 3, all five peptides decreased the binding capability of the patients' sera to the SARS-CoV lysates in a dose-dependent manner, whereas the two control peptides did not. Obviously, the mixture of peptides (cocktail) showed the strongest blockage when compared with a single peptide. This result indicated that SARS patients' sera did immunorespond against these five peptides. However, five mixed peptides were not completely additive in the competitive experiment, implying that there might be other conformation-restriction immunoantigens which could not be identified by this approach.

$S_{471-503}$ Could Inhibit the Binding of ACE2 and RBD. Among the five candidate peptides we obtained, $S_{471-503}$ from the S1 fragment was located on the RBD. Because the RBD was recently proved to be essential for the virus−cell fusion and will induce highly potent neutralizing antibodies,[6,15] we decided to investigate whether $S_{471-503}$ could interfere with the binding between the RBD and ACE2. A binding assay then was developed in which the RBD protein fused with the constant fragment of human antibody (RBD-Fc) was coated onto the 96-well polystyrene plate. After that, ACE2 was captured by an RBD-Fc protein, and mouse monoclonal antibody (mAb) of ACE2 was used to link the binding of RBD and ACE2 with mouse HRP-anti-IgG, which served as the specific reporter. It was demonstrated clearly that $S_{471-503}$ blocked the interaction between the RBD and ACE2 (Figure 4), while $S_{602-625}$ has no blocking effect, although $S_{602-625}$ is closed to the C-terminus of the RBD.

An additional experiment was carried out to examine the ability of $S_{471-503}$ to block SARS-CoV infection in Vero cells. As shown in Figure 5, $S_{471-503}$ could remarkably inhibit the plaque formation of SARS-CoV in Vero cells, with an EC_{50} value of 41.6 μM. The $S_{604-625}$, an epitope located outside the RBD, did not show the inhibitory activity. It is also noteworthy that peptide $S_{471-503}$ has no effect on inhibiting the polio or infuenza A viruses' infectivity in cellular models (data not shown), suggesting the inhibitory effects of $S_{471-503}$ are specific for SARS-CoV.

Discussion

B-cell epitopes are generally linear or have a conformational restriction, also known as discontinuous epitopes. In practice, only linear epitopes can be identified by the epitope scan method because conformational epitopes are composed of a combination of spatial binding sites of the protein antigen. One way to perform a linear epitope scan is to synthesize a peptide library derived from the antigenic protein and subsequently detect peptides using immunological methods to determine where the epitopes are located.

Highly efficient methods for the identification of the epitopes are classified as biologically expressed peptide library[16] and synthetic peptide libraries.[17] There are several combinatorial approaches to chemically construct peptide libraries, such as the mimotopes parallel approach,[18] "one-bead one-compound" methodologies[19] with the "mix and split" synthetic approach,[20] the "tea-bag" peptide pool method,[21] or spotter-assisted microarray peptide synthesis.[22] Overlapping peptide libraries are generally synthesized by a parallel approach. Herein, we successfully explored the "mix and split" approach for overlapping peptide library synthesis by means of IRORI sorting technology with a physical radio frequency coding tag.[23]

Since the outbreak of SARS, scientists have done tremendous work to investigate the nature of this emerging disease,

Table 3. Cross-Immunoreactivities of Peptides and Amino Acid Mutations under Immunopressure

serum source[a]	$S_{471-503}$[b] (30)[c]	$S_{604-625}$[b] (30)[c]	$S_{1164-1191}$[b] (28)[c]	N_{67-76}[b] (32)[c]	$N_{367-389}$[b] (38)[c]	SARS-CoV lysates[d]
BJ01	3	2	2	3	1	1.252
BJ02	3	1	2	2	1	1.143
BJ03	3	1	2	1	3	1.344
BJ04	3	2	1	1	1	1.215
BJ05	3	2	2	1	4	1.095
BJ06	3	3	1	1	2	1.258
BJ07	3	2	2	3	2	1.392
BJ08	3	1	2	1	2	1.067
BJ09	3	3	2	3	4	1.094
BJ10	3	1	3	1	3	1.347
BJ11	3	1	1	0	0	1.17
BJ12	0	0	0	0	0	0.569
BJ13	2	2	2	2	2	1.009
BJ14	2	1	1	2	2	1.112
BJ15	2	1	0	1	2	1.039
BJ16	0	0	1	1	3	1.072
BJ17	2	1	1	1	2	1.093
BJ18	0	1	0	3	1	0.663
BJ19	2	2	2	1	2	1.145
BJ20	0	1	0	0	1	0.487
BJ21	1	0	1	0	1	0.333
BJ22	0	1	0	3	2	1.224
BJ23	2	0	2	2	3	1.34
BJ24	1	1	2	1	2	1.027
BJ25	0	0	0	1	1	0.627
BJ26	0	2	0	1	2	1.117
BJ27	2	0	2	2	2	0.724
BJ28	2	2	0	0	1	1.233
BJ29	1	1	1	2	2	1.112
BJ30	0	0	0	0	1	0.423
BJ31	2	3	1	2	3	1.317
BJ32	2	1	2	2	3	1.16
GD01	0	0	0	0	2	0.592
GD02	2	2	1	1	2	1.435
GD03	0	0	0	0	0	0.465
GD04	2	2	2	2	1	1.131
SX01	2	1	0	2	2	0.972
SX02	2	0	1	2	2	1.191
SX03	0	0	0	0	0	0.593
SX04	2	2	1	2	1	1.435
IM01	0	2	0	0	2	0.669
IM02	2	0	1	2	2	1.023

Mutations in SARS Coronavirus[e]

$F_{501} \rightarrow Y_{501}$ in SARS coronavirus GD01 (AAP51227.1)	$A_{610} \rightarrow L_{610}$ in SARS coronavirus GD03T0013 (AAS10463.1)			
$L_{472} \rightarrow P_{472}$ in SARS coronavirus GD03T0013 (AAS10463.1)	$D_{614} \rightarrow E_{614}$ in SARS coronavirus GD03T0013			
$D_{480} \rightarrow G_{480}$ in SARS coronavirus GD03T0013	$D_{605} \rightarrow N_{605}$ in SARS coronavirus Shanghai QXC1(AAR86788.1) and ShanghaiQXC2 (AAR86775.1)	no mutation found in other SARS coronavirus strains (total analyzed 52 strains)	no mutation found in other SARS coronavirus strains (total analyzed 54 strains)	no mutation found in other SARS coronavirus strains (total analyzed 52 strains)
$T_{487} \rightarrow S_{487}$ in SARS coronavirus GD03T0013 (total analyzed 48 strains)	$T_{605} \rightarrow A_{605}$ in SARS coronavirus Shanghai QXC1 and ShanghaiQXC2 (total analyzed 47 strains)			

[a] BJ, GD, SX, and IM abbreviate for Beijing, Guangdong, Shanxi, and Inner Mongolia, respectively. [b] 1, 2, 3, and 4 in the table represent the OD absorptions are ranged at ~0.4 to 0.7, ~0.7 to 1.0, ~1.0 to 2.0, and >2.0, respectively. The negative control is less than 0.2. [c] Represents the total positive cases. [d] This column presents the OD absorptions data from a commercially available ELISA kit using SARS-CoV lysates as antigen. [e] A peptide ($S_{803-828}$) reported by Deng et al.[38] was found amino acid mutation ($L_{804} \rightarrow P_{804}$ in SARS coronavirus Shanghai LY(AY322207)).

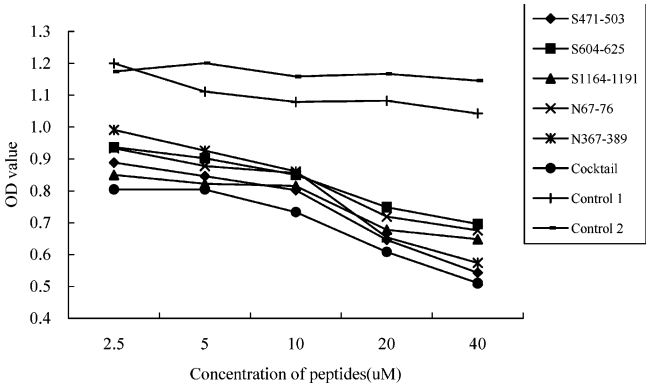

Figure 3. The results of competitive ELISA (lysates of SARS-CoV were coated).

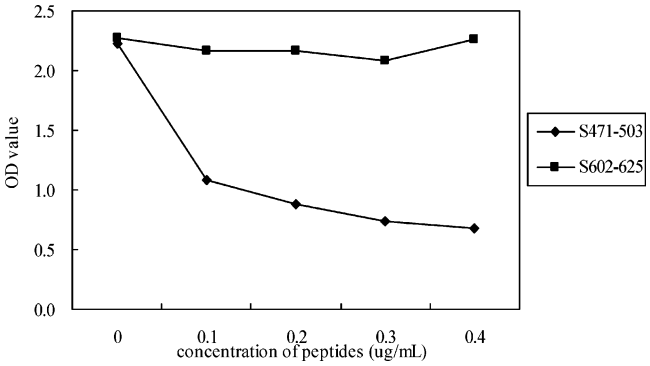

Figure 4. The blocking capability of $S_{471-503}$ or $S_{602-625}$ to the binding of hte RBD and ACE2. The corresponding peptide was preincubated with ACE2.

and many achievements have been achieved. Many aspects of the pathogenesis of SARS, however, have not yet been defined. Due to the lack of effective and specific drugs to treat SARS, the development of an effective and safe vaccine is urgently needed. Several reports illuminated the potential and feasibility of protective humoral immunity and vaccine development for prevention of SARS.[24−28] Until now, several kinds of vaccines, such as inactivated virus-based vaccines, DNA vaccines, and attenuated vaccine, which have been proved to induce a CTL response or neutralizing protection response in animals, have been under more extensive preclinical studies.[25−28] Some immunodominant antigens in the virions could trigger the humoral and cellular immune response, but as for SARS patients, the lung injury might be caused by a harmful inflammatory and immune response.[29] This means that the antibodies without neutralizing capability against the virus might actually enhance the inflammation. On the other hand, the excessive cellular immune and inflammatory responses are the potential causes of SARS pathogenesis.[30,31] Enserink reported that the ferrets immunized by virus-based SARS vaccine before a virus challenge suffered more serious liver inflammation and damage.[31] This observation indicated that the currently evaluated vaccine candidates might also cause the same safety problem. Thus, the development of a subunit-based vaccine for SARS-CoV is an alternative approach. To reach this goal, it is critical to systematically identify the B-cell epitopes.

In the present study, we explored a new epitope assembling approach to discover the linear B-cell immuno-cross-reactive epitopes of SARS-CoV. A 10-mer overlapped peptide library (Figure 1) was synthesized and estimated using peptides ~30 to 90% in purity. ELISA was employed for peptidic B-cell epitope screening, and 111 epitopic candidates were selected because their OD values were 2-fold higher than the healthy donor after the first screening. The peptides were then assembled and synthesized according to their locations in the original proteins, and only those peptides that lie close to each other were assembled into longer peptides to increase their antigenicity and immunogenicity. Among those assembled peptides, five ($S_{471-503}$, $S_{604-625}$, $S_{1164-1191}$, N_{67-76}, and $N_{367-389}$, Table 2) showed the highest immuno-crossreactive capabilities to the sera of 42 SARS patients. However, none of them showed the complete crossreaction with all tested SARS patients' sera.

Particularly, three sera could not be detected by all the peptides. One possibility is that the titers of antibody for these epitopes might be too low to be detected. Another possibility is that they might be false positive, as determined by the commercial diagnostic kit, because they had not been investigated by other methods, such as PCR or immunofluorescence. Lacking the clinical information of those patients, we had no idea of the difference between the viruses infecting those patients or whether they come from the same strain. However, four mutations were found for either $S_{471-503}$ or $S_{604-625}$ (Table 3). Such high variability of $S_{471-503}$ and $S_{604-625}$ implies that this region was recognized by the human immune system and escaped from the host immunopressure. Mutations of other peptides in this study were not observed.

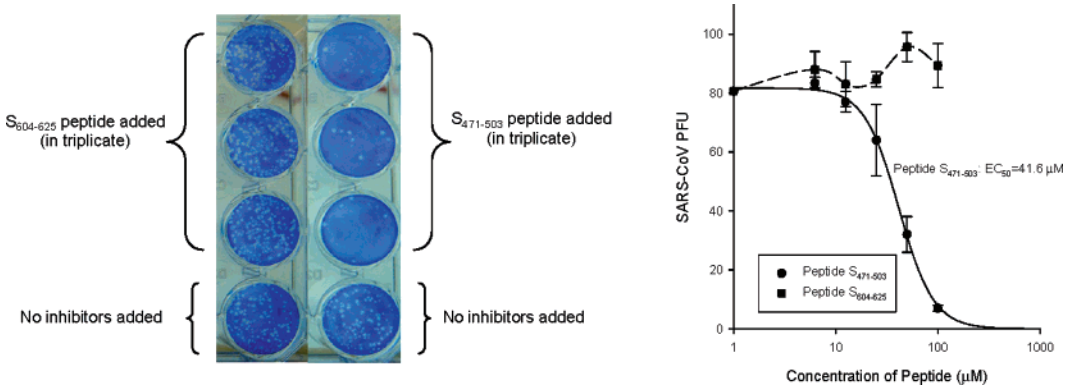

Figure 5. (A) $S_{471-503}$ peptide inhibits SARS-CoV plaque formation in Vero cells (B) The dose-dependent inhibition activities of $S_{471-503}$ against SARS-CoV in Vero cell.

This critically shows that the vaccine immunoantigens must contain the$S_{471-503}$ or $S_{604-625}$ regions.

Recently, several articles have reported major B-cell epitopes and B-cell immunodominant sites.[32−36] In Wang's research,[32] 41 peptides ranging in size from 16 to 25 amino acid residues were synthesized and evaluated by the ELISA method. The peptide possessing the highest binding affinity to the antisera with highly hydrophilic property was also proved by this study to be the $N_{367-389}$ peptide. This epitope could not be used as an immunoantigen to elicit neutralizing antibodies because the N protein of the coronavirus locates inside of the viral membrane. However, a large amount of the N protein was expressed in the infected host cells and, therefore, plays a very important role in the life cycle of the coronavirus.[37] In the course of the lysis of an infected cell, many N proteins are released and induce strong humoral and cellular responses in monkeys during the viral replication.[26] The antibodies against this protein should be a good indicator for diagnosis of SARS-CoV infection.[35] Our results showed that the epitopes of the N protein were more immunoreactive than those of the S protein. In other article, Jiang et al.[36] reported recently another peptide $S_{603-634}$ in S1 fragment. They concluded that $S_{603-634}$ was a potential diagnostic antigen, with 100% cross-reaction to the tested 42 antisera isolated from a clinic. This peptide had 71.4% alignments with our $S_{604-625}$ peptide. The difference in cross-reactivity of this immunodominance between our result and Jiang's report might be caused by the antisera from different epidemic areas. Deng[38] reported an epitope (Leu 803 to Ala 828) with one observed amino acid mutation from the S2 fragment with a 100% cross-reaction (Table 3). This peptide is different from ours ($S_{1164-1191}$) in this S2 protein subunit; however, they did not report the results tested with different sera from epidemic areas.

It was believed that the S1 subunit of spike glycoprotein played the key role in the virus−cell fusion.[39] The virus using the RBD of the S1 subunit first bound to the receptor of the host target cell (ACE2) and then changed conformations by the hepted 1 and hepted 2 interruptions and, finally, fused and entered the host cell.[40] Consequently, the RBD must extrude from the protein, so it would be an ideal candidate for the investigation of immunoantigens and drug candidates against the virus−cell binding and entry. The binding site of ACE2 on the SARS-CoV spike glycoprotein is localized between amino acid residues 303 and 537 on the S1 subunit protein, which was based on the analysis of the amino acid sequences and structural characteristics.[39] The whole RBD was recently demonstrated as a potent neutralizing immunoantigen.[41] A 193-amino acid fragment (318−510) of the S protein could efficiently bind ACE2 and block the S protein-mediated infection.[6] In this research, we clarified that the $S_{471-503}$ in the RBD could block the binding of RBD to ACE2 and, thus, the virus's binding and entry into the target cell. On the basis of the results we obtained, we believe that the $S_{471-503}$ peptide that locatesis located on the RBD of ACE2 plays a crucial role in the interaction of the RBD and ACE2. Subsequent investigation of its immunogenicity will be done. We have established a system using the recombinant RBD-Fc protein and ACE2 to simulate the binding of SARS-CoV and host cells. The results we obtained using this system were consistent with the SARS-CoV plaque reduction assay, so it could be used to scan epitopes and inhibitors specific for the RBD and ACE2.

In summary, we have determined five B-cell epitopes of SARS-CoV that were highly immunoreactive, antigenic, and immunogenic. Among them, a peptide, $S_{471-503}$ in the RBD, has been proved a candidate antigen for further evaluation for the peptide-base vaccine development or a candidate for drug development as a viral fusion inhibitor.

Conclusion

A 10-mer overlapped peptide library was synthesized by means of the IRORI sorting method.[12] The peptide library contained 1938 individual peptides that spanned all structural proteins of SARS-CoV BJ01. ELISA was employed for B-cell epitope screening, and up to 42 SARS convalescent patients' sera were used as primary antibodies. One hundred and eleven peptides were gained as positive candidate epitopes from the first screening. After assembly and redesign of those positive epitopes, 22 longer peptides were synthesized, and 5 of these peptides ($S_{471-503}$, $S_{604-625}$, $S_{1164-1191}$, N_{67-76}, and $N_{367-389}$) were serologically highly cross-reactive with all tested SARS patients' sera, indicating that they could be used as candidates for SARS-CoV diagnostic reagents. Competitive ELISA suggested that they were SARS-selective and immunoreactive. Most interestingly, peptide $S_{471-503}$ could specifically block the binding of ACE2 with RBD of SARS-CoV and inhibit the SARS-CoV infection of Vero cells in vitro, indicating that peptide $S_{471-503}$ might serve as a potential immunoantigen for the development of a peptide-based vaccine or as a valuable candidate for drug evaluation against the SARS-CoV virus−cell fusion.

Experimental Section

Design and Synthesis of Peptide Library. Materials and Methods. All of the L-α-Fmoc-protected amino acids and TES were obtained from GL Biochem (Shanghai, China). Rink amide resin (loading 0.8 mmol/g, 1% DVB, 100−200 mesh) was purchased from Hecheng, Co. (Tianjin, China). Coupling reagents (HBTU, BOP, and DIC) and HOBt were obtained from GL Biotechem Ltd. (Shanghai, China). Other reagents were obtained commercially and were of analytical grade. Unless otherwise stated, all solvents were of analytical grade and used without further purification. DMF was redistilled under reduced pressure after dried over 4A molecular sieves overnight.

Synthesis of Overlapping Peptide Library. The peptide library was synthesized in mix-and-split approach using the IRORI sorting system. An Rf tag and 15 mg of Rink amide resin were put into a MicroKan. The tag was coded before the first coupling and decoded before the next coupling step. Fmoc-based synthesis protocol was adopted. The resin-bound Fmoc-protected peptide was treated twice with 20% piperidine in DMF for 15 min for deprotection. The coupling procedure involved the appropriate Fmoc-amino acids (3 equiv), HOBt (3 equiv), and coupling reagent (HBTU, BOP or DIC, 3 equiv) dissolved in DMF (20−40 mL) containing 6.0 equiv of DIPEA. Coupling reactions were allowed to

proceed for different times, depending on the particular sequence. In case of insufficient coupling, double coupling was performed until the reaction was complete. The N-terminus was acetylated by 15% acetic anhydride in DCM for 30 min.

The library synthesis was finished in three times, with 600, 646 and 692 peptides per time, respectively. The weights and volumes of the different reagents were calculated according to a suitable formula using Microsoft Excel 2000 Software. Several difficult sequences were selected as controls during the course of the coupling reaction and were monitored by Kaiser test.

TFA Cleavage Procedure. Crude peptides without Cys, Met, Arg, and Trp residues were cleaved using a one-step TFA cleavage method under the following conditions: TFA (1.9 mL), deionized water (50 μL), and triethylsilane (50 μL). For those peptides containing Cys, Met, Arg, or Trp residues, the following conditions were used: TFA (1.63 mL), thioanisole (0.1 mL), phenol (0.1 g), deionized water (0.1 mL), EDT (50 μL), and triethylsilane (20 μL). The TFA cleavage mixture was added to a tube containing a peptide resin, and the mixture was shaken for 2 h at room temperature. Upon completion of the cleavage reaction, the "peptide + resin" mixture was filtered, and the tube was rinsed with 2−3 mL of cleavage mixture.

The solution was parallel-dried in a slow flow of nitrogen gas and then treated with ice-cooled methyl *tert*-butyl ether/petroleum ether (3:1) to precipitate the peptide. The crude peptides were washed twice with ice-cooled methyl *tert*-butyl ether/petroleum ether (3:1). Several representative crude peptides were redissolved in 60% CH_3CN-H_2O and analyzed by HPLC and ESI-MS. On the basis of the result of the first screening, 22 designed peptides containing one or two neighboring or overlapped epitopes to increase their immunogenicity were synthesized and purified by HPLC (Gilson 322 pump, Gilson UV/vis-152 detector, and Gilson 215 liquid handler). These peptides then were dissolved in $NaHCO_3-Na_2CO_3$ buffer ($NaHCO_3$, 0.07M; Na_2CO_3, 0.03M; pH 9.4) for the ELISA screening.

Test of SARS-CoV-Specific IgG in Patients' Sera. A commercially available ELISA kit (Beijing Genomics Institute) was used to detect the titers of SARS-specific antibody (IgG) of all patients' sera. The protocol provided in the kit was followed. The positive sera were used as the first antibody to screen the B-cell epitopes. The formula for calculating of the cutoff value is

$$V_c = V_n + 0.13$$

where V_c is the OD value of the cutoff, and V_n is the mean OD value of the negative controls. Above is positive and vice versa.

Peptide-Pin ELISA (PEPscan). The series of peptides were coated on the ELISA plates as candidates of the antigen and were incubated at 37 °C for 2 h, then blocked by phosphate-buffered saline (PBS) with 3% BSA (Sigma) for 2 h. Sera specimens were pooled and diluted in PBS plus 0.05% Tween 20 (PBST) with 1% BSA. The samples were added to the wells (100 μL/well) and incubated for 60 min at 37 °C. Goat antibody anti-human IgG conjugated to horseradish peroxidase (HRP) (Southern Biotechnology Associates) diluted in PBS with 1% BSA was added to the well and incubated for 30 min at 37 °C. The substrate of HRP (Roche) was added to the wells. The optical density was read at 450 and 630 nm in an optical density reader (Labsystem). The cutoff value was determined as twice the average value of the negative controls. All of the samples whose optical density values were above the cutoff were positive.

Competitive ELISA Test. The serum was mixed with various concentrations (ranging from 2.5 to 40.0 M) of the relevant synthetic peptide and incubated for 1 h at 37 °C. The sequence of AEFPSHTHSTVVHJTDWIGWQAI was calculated by randomization as control 1. The SARS-CoV (BJ01) sequence located at $S_{\sim 945-963}$ (VKQLSSNFGAISS-VLNDIL) was negative control 2, which has been determined without antigenicity. ELISA as described above assayed the residual activity of serum binding to peptides coated on wells. Percent inhibition of peptide binding to SARS-CoV by free peptides was calculated for each serum by comparing the assay values with those for a control serum incubated with irrelevant peptides. Each assay was run in duplicate, and three cases of sera were used.

Assay for Blocking Capability of ACE2 and RBD Domain. Fusion RBD of the S protein was gift from Dr. Chengyu Jiang. This protein contained 205 amino acid residuals (residues 303−507 of the S protein), and the N terminal was fused with an Fc fragment of human IgG, which was convenient for purification through an affinitive chromatogram. It was coated in an ELISA plate (0.02 g/well) and then blocked by PBS with 3% BSA for 2 h at 37 °C. The $S_{471-503}$ peptide whose concentrations were from 0.02 g/mL to 0.1 g/mL was added and incubated 2 h at 37 °C. Then, the ACE2 (0.01 g/well, R&D) was added to the plate and incubated at 4 °C overnight. The mAb anti-ACE2 was added to determine the ACE2 binding to the RBD. The following steps were similar to the ELISA method above.

Virus Plaque Reduction Assay. Twenty-four-well tissue culture plates (TPP, Switzerland) with a confluent monolayer of Vero cells (1 × 105 cells/well) in EMEM with 1% FBS were prepared. One hundred plaque forming units (PFU) of SARS-CoV were added to each well with or without the addition of peptides. After being incubated for 2 h at 37 °C with 5% CO_2, cell culture media and unbound viral particles were aspirated, and 1 mL of overlay (1% low-melting agarose in EMEM with 1% FBS and appropriate concentrations of inhibitors) was added to each well immediately. Plates were further incubated for 48 h under identical conditions. Cells were fixed by adding 1 mL of 10% formaldehyde. The agarose plugs were removed subsequently, the cells were stained with 0.5% crystal violet in 70% methanol, and the viral plaques counted. Experiments were carried out in triplicate and repeated twice. The mean value is shown with standard deviation (SD). Dose−response data are best fit to the logistic equation.

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