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Assessment of Immunoreactive Synthetic Peptides from the Structural Proteins of Severe Acute Respiratory Syndrome Coronavirus

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Background: **The widespread threat of severe acute respiratory syndrome (SARS) to human life has spawned challenges to develop fast and accurate analytical methods for its early diagnosis and to create a safe antiviral vaccine for preventive use. Consequently, we thoroughly investigated the immunoreactivities with patient sera of a series of synthesized peptides from SARS-coronavirus structural proteins.**

Methods: **We synthesized 41 peptides ranging in size from 16 to 25 amino acid residues of relatively high hydrophilicity. The immunoreactivities of the peptides with SARS patient sera were determined by ELISA.**

Results: **Four epitopic sites, S599, M137, N66, and N371-404, located in the SARS-coronavirus S, M, and N proteins, respectively, were detected by screening synthesized peptides. Notably, N371 and N385, located at the COOH terminus of the N protein, inhibited binding of antibodies to SARS-coronavirus lysate and bound to antibodies in >94% of samples from SARS study patients. N385 had the highest affinity for forming peptide-antibody complexes with SARS serum.**

Conclusions: **Five peptides from SARS structural proteins, especially two from the COOH terminus of the N protein, appear to be highly immunogenic and may be useful for serologic assays. The identification of these antigenic peptides contributes to the understanding of the immunogenicity and persistence of SARS coronavirus.**

The worldwide threat of severe acute respiratory syndrome (SARS)[6] becoming an epidemic creates urgent challenges for the scientific community. Several laboratories have unraveled the genetic information of the SARS virus *(1–4)*. The genome size of the SARS coronavirus is ~29 kb and has 11 open reading frames, composed of a stable region encoding an RNA-dependent RNA polymerase with 2 open reading frames, a variable region representing 4 coding sequences for viral structural genes [spike (S protein), envelope (E protein), membrane (M protein), and nucleocapsid (N protein)], and 5 putative uncharacterized proteins. Its gene order is similar to that of other known coronaviruses; however, phylogenetic analyses and sequence comparisons indicate that this virus does not closely resemble any of the previously characterized coronaviruses.

The incubation period of SARS is usually 2–7 days, but could be as long as 10 days. The disease progresses with unusual severity within a short time once a patient exhibits obvious clinical symptoms. Therefore, an urgent

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[6] Nonstandard abbreviations: SARS, severe acute respiratory syndrome; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and PBS, phosphate-buffered saline.

task is to develop accurate and sensitive diagnostic tools for identifying SARS, specifically for early diagnosis. A noninvasive diagnostic test for SARS coronavirus has been reported recently that uses quantitative reverse transcription-PCR with detection of SYBR green fluorescence *(5)*. The PCR primer design was based on a unique region within the RNA-dependent RNA polymerase-encoding sequence of the virus. The amplification was very specific and showed no cross-reaction with two serogroups of human coronavirus, 229E and OC43. In a total of 29 SARS patients and 58 uninfected controls, the PCR assay showed positive identification for 79% of SARS cases and negative identification for 98% of controls. However, this technique is limited in its clinical use. The samples for PCR were collected from nasopharyngeal aspirates of SARS patients. Because SARS is a respiratory infection, any attempt to obtain a sample from a patient's pharynx and larynx increases the risk of infection to the healthcare worker. Furthermore, 79% accuracy is below the level of acceptably when taking into account those in the early phase of infection, showing no symptoms, who were among the 20% excluded. This could certainly be cause for concern because these people could still pose a threat of transmitting the virus.

Serologic assays are used extensively for diagnosis of viral infection in a host *(6)*. In urgent situations, a common way to perform a serologic assay is to use complex viral lysates as antigens. The use of viral lysates, however, presents several disadvantages. Viral lysates consist of many viral antigens that can not be clearly purified and classified. The lysates are prepared from cells infected with the virus; thus, cellular proteins can contaminate the preparation. Moreover, SARS-coronavirus lysates present a considerable risk of infection to laboratory workers. To overcome these problems, the discovery of antigenic fragments in SARS-coronavirus proteins is expected to lead to the rapid development of new assays for the diagnosis of SARS infection. It has been shown in many cases that several epitopes located in viral proteins can be successfully mimicked with synthetic peptides *(7)*. To expedite epitope mapping of the SARS coronavirus, we have synthesized a group of peptides representing the most hydrophilic, as well as the most accessible, residue regions of the S, M, and N structural proteins of SARS coronavirus. Using sera from SARS patients, we probed these peptides by ELISA.

Materials and Methods

SERUM SPECIMENS

Sera from 31 SARS patients from eight different hospitals in Beijing and from 49 uninfected volunteers were collected for study. The clinical diagnostic criteria for SARS followed the clinical description of SARS released by WHO. Confirmation of SARS infection was evidenced by the presence of antibodies against SARS coronavirus in the serum. The control sera were divided into two groups: 24 samples obtained from healthy volunteers and 25 samples obtained from patients suffering from respiratory symptoms but not infected with the SARS coronavirus.

PEPTIDE DESIGN AND SYNTHESIS

On the basis of the published genome sequence of the SARS coronavirus, we downloaded structural proteins S, M, and N into the ProtScale program at the Swiss Institute of Bioinformatics to analyze the physical characteristics of the proteins, such as hydrophilicity, hydrophobicity, accessible residues, buried residues, molecular mass, and pI values. A total of 41 peptides ranging in size from 16 to 25 amino acid residues and in molecular mass from 2500 to 3000 Da were selected for synthesis (Table 1). All of the peptides were synthesized commercially by Chinese Peptide Co. The synthesized peptides were characterized by HPLC and mass spectrometry.

SARS CORONAVIRUS INFECTION AND PROTEIN EXTRACTION FROM INFECTED CELLS

The SARS coronavirus isolated from SARS case BJ01, whose genomic sequence was determined by the Beijing Genomics Institute, was used as a viral source and propagated in Vero-E6 cells as described previously *(8)*. After viral propagation, the cells were harvested and placed at 70 °C for 2 h to inactivate the virus.

The inactivated infected Vero-E6 cells were completely lysed by freeze–thaw cycles followed by centrifugation. After the cell pellet was removed, the supernatant was loaded on a Sephadex G-150 column for virus purification. The elution fractions were collected at 1 mL/min, and viral proteins in each fraction were detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Blue staining and confirmed by Western blot using SARS serum. Before ELISA the fractions containing virus were sonicated and concentrated with a Centricon-10 biological filter.

ELISA PROCEDURE

The reactivities of the various peptides with SARS sera were determined by ELISA. In brief, peptides (1 mg/L, 100 μL/well) were adsorbed to duplicate wells of 96-well microplates in 0.5 mol/L carbonate buffer (pH 12.5) and incubated at 4 °C for 12 h. After the wells were washed with phosphate-buffered saline (PBS), they were coated with 2 g/L bovine serum albumin at 37 °C for 2 h and then incubated at 37 °C for 30 min with 10 μL of sera in sample buffer (total volume of buffer, 100 μL). Each well was then washed and incubated with peroxidase-conjugated goat anti-human IgG at 37 °C for 20 min. Finally the wells were washed again with PBS containing 5 mL/L Tween 20. The peroxidase reaction was visualized by use of *o*-phenylenediamine solution as substrate. After 10 min of incubation at 37 °C, the reaction was stopped by the addition of 50 μL of 4 mol/L sulfuric acid, and the absorbance at 450 nm (reference wavelength, 630 nm) was measured by an automatic ELISA plate reader (Multiskan Microplate Photometer).

Table 1. Reactivity [absorbance/cutoff (*A*/CO)] of the synthesized peptides with sera from SARS patients in the ELISA.[a]

Peptides	Sequence	Position	Hydrophilicity	*A*/CO (n = 31)	*P*[b]	*x*/31[c]	*y*/24[c]
SARS-CoV				4.23 (1.85)		100	0
S-protein							
S67	TGFHTINHTFGNPVIPFKDGIYF	67–89	+	0.27 (0.35)	<0.05	2/31	0/24
S84	KDGIYFAATEKSNVVRGWVFGSTMN	84–108	+	0.90 (1.03)	<0.05	8/31	0/24
S101	WVFGSTMNNKSQSVIIINNSTN	101–122	+	0.77 (0.75)	<0.05	9/31	0/24
S118	NNSTNVVIRACNFELCDNPFFAVSK	118–142	−	1.60 (2.37)	<0.05	10/31	0/24
S265	TTFMLKYDENGTITDAVDCSQN	265–286	+	0.49 (0.49)	<0.05	2/31	1/24
S277	DCSQNPLAELKCSVKSFEIDKG	277–298	+	0.90 (1.17)	<0.05	7/31	0/24
S301	QTSNFRVVPSGDVVRFPNITNL	301–322	−	1.32 (1.91)	<0.05	11/31	0/24
S323	PFGEVFNATKFPSVYAWERKK	323–343	−	0.91 (1.56)	<0.05	8/31	3/24
S345	ISNCVADYSVLYNSTFFSTFKC	345–366	−	0.46 (0.40)	<0.05	3/31	0/24
S582	SVITPGTNASSEVAVLYQDVNCT	582–602	+	0.93 (1.16)	<0.05	6/31	0/24
S599	QDVNCTDVSTAIHADQLTPAWR	599–620	+	4.64 (3.48)	NS[d]	25/31	0/24
S624	TGNNVFQTQAGCLIGAEHVDTS	624–645	+	0.56 (0.45)	<0.05	6/31	0/24
S645	SYECDIPIGAGICASYHTVSLLR	645–667	+	1.16 (1.08)	<0.05	11/31	0/24
S886	YRFNGIGVTQNVLYENQKQIA	886–906	+	0.54 (0.75)	<0.05	3/31	0/24
S1130	FKEELDKYFKNHTSPDVD	1130–1147	−	0.74 (1.14)	<0.05	6/31	0/24
S1211	MVTILLCCMTSCCSCLKGACSC	1211–1232	−	0.41 (0.43)	<0.05	2/31	0/24
S1227	KGACSCGSCCKFDEDDSEPVLK	1227–1248	+	0.86 (0.77)	<0.05	11/31	0/24
S1234	SCCKFDEDDSEPVLKGVKLHYT	1234–1255	+	1.13 (0.92)	<0.05	16/31	1/24
M-protein							
M1	MADNGTITVEELKQLLEQWN	1–21	−	1.75 (1.99)	<0.05	16/31	0/24
M17	EQWNLVIGFLFLAWIMLLQFAYSNR	17–41	−	0.42 (0.51)	<0.05	2/31	0/24
M94	YFVASFRLFARTRSMWSFNPETN	94–116	−	2.55 (1.63)	<0.05	25/31	0/24
M103	ARTRSMWSFNPETNILLNVPLR	103–124	−	0.65 (0.98)	<0.05	3/31	0/24
M119	LNVPLRGTIVTRPLMESELVIG	119–140	−	0.34 (0.34)	<0.05	2/31	0/24
M137	LVIGAVIIRGHLRMAGHSLGR	137–158	+	3.09 (1.61)	0.1	25/31	0/24
M162	KDLPKEITVATSRTLSYYKLGASQR	162–186	+	0.69 (0.70)	<0.05	7/31	0/24
M189	DSGFAAYNRYRIGNYKLNTDHAG	189–211	+	1.10 (1.63)	<0.05	10/31	0/24
M206	NTDHAGSNDNIALLVQ	206–221	+	1.87 (2.22)	<0.05	14/31	0/24
N-protein							
N1	MSDNGPQSNQRSAPRITFGGPTD	1–23	+	1.18 (1.73)	<0.05	9/31	0/24
N21	PTDSTDNNQNGGRNGARPKQRR	21–42	+	2.03 (2.35)	<0.05	19/31	0/24
N35	GARPKQRRPQGLPNNTASWFTA	35–56	+	1.44 (1.52)	<0.05	14/31	0/24
N66	QLPQGTTLPKGFYAEGSRGGSQ	66–87	+	4.05 (4.14)	NS	18/31	1/24
N99	DGKMKELSPRWYFYYLGTGPEA	99–120	-	0.35 (0.48)	<0.05	3/31	0/24
N177	SRGGSQASSRSSSRSRGNSRNS	177–198	+	0.63 (0.97)	<0.05	6/31	0/24
N196	RNSTPGSSRGNSPARMASGGGE	196–217	+	0.48 (0.60)	<0.05	5/31	0/24
N215	GGETALALLLLDRLNQLESKVSGKG	215–239	−	2.10 (2.30)	<0.05	18/31	2/24
N245	QTVTKKSAAEASKKPRQKRTATKQ	245–268	+	2.05 (1.59)	<0.05	23/31	0/24
N258	KPRQKRTATKQYNVTQAFGRRG	258–279	+	0.78 (1.01)	<0.05	8/31	2/24
N355	NKHIDAYKTFPPTEPKKDKKKK	355–376	+	2.17 (1.93)	<0.05	19/31	0/24
N371	KDKKKKTDEAQPLPQRQKKQ	371–390	+	4.36 (2.61)	NS	30/31	1/24
N385	QRQKKQPTVTLLPAADMDDFSRQ	385–407	+	3.44 (2.56)	NS	29/31	0/24
N401	MDDFSRQLQNSMSGASADSTQA	401–422	+	1.36 (2.35)	<0.05	9/31	0/24

[a] ELISA reactivity was determined as described in *Materials and Methods*. A total of 31 sera were collected from SARS patients for the experiments. In the control group, the SARS-associated coronavirus preparation was used for detecting the antibodies in SARS patient's sera.

[b] The significance of the differences (*P*) in ELISA reactivities between coronavirus and the different synthesized peptides was analyzed by the Student *t*-test.

[c] *x*/31 and *y*/24 represent the ratios of true- and false-positive immunoreactivities, respectively, in the ELISA, in which each peptide was screened against 24 control sera and 31 SARS sera.

[d] NS, not significant.

To determine the background resulting from nonspecific binding of viral lysates or peptides to normal sera, the cutoff value was calibrated for each viral preparation or peptide by incubation with negative control serum. A panel of 24 sera from healthy individuals was examined routinely for the calibrations. The mean absorbance was considered as the cutoff value for a viral preparation or a peptide. The cutoff values were ~0.2.

WESTERN BLOT

For each sample, 50 μg of protein was subjected to 12% SDS-PAGE under reducing conditions and transferred to a Bio-Rad PVDF membrane. The membrane was blocked with PBS containing 50 g/L nonfat milk powder in Tris-buffered saline at 37 °C for 30 min before incubation with diluted SARS serum. Bound antibodies were detected by use of an appropriate alkaline phosphatase-coupled secondary antibody with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt added as the substrate for visualization.

ESTIMATION OF BINDING AFFINITIES OF SYNTHESIZED PEPTIDES TO SERA FROM SARS PATIENTS

To compare the affinities of the antigenic peptides to SARS-coronavirus antibodies, we estimated the binding constants by the Scatchard equation. Each antibody has a limited number of binding sites that become saturated as the concentration of antigen increases. This can be quantified by the following equation:

$$\alpha = n \times \frac{[P]}{(k_d + [P])}$$

where α is the proportion of bound antigens per antibody based on the ELISA measurement at 450 nm; n is the number of binding sites on the antibody; k_d is the dissociation constant of the antibody–antigen complex; and [P] is the antigen concentration.

STATISTICAL ANALYSIS

We used the Student *t*-test to calculate whether the reactivities of the synthetic peptides and SARS coronavirus preparations with the same serum in the ELISA were significantly different. *P* values <0.05 were considered statistically significant.

Results and Discussion

EPITOPE MAPPING OF THE S, M, AND N PEPTIDES

To effectively develop synthetic peptide immunogens from SARS-coronavirus structural proteins, we used several theoretical calculations for peptide design. For a potential epitopic peptide, we specifically looked for high local hydrophilicity, charged residues on the exposed protein surface, and accessible surface area. Statistical analysis based on the linear amino acid sequences of the three proteins suggests that, in contrast to the other two viral structural proteins, the N protein contains a high percentage of accessible residues and low hydrophobicity (See Figs. 1–3 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol49/issue12/). A total of 41 peptides spanning multiple possible epitopic sites along the S, M, and N proteins were designed and synthesized in a solid-phase peptide synthesizer. These peptides were purified by HPLC, incubated with panels of sera from both controls and SARS patients, and then subjected to ELISA for measurement of the immunoreactivities. The correlations between the length of amino acid sequence and immunoreactivity are shown in Fig. 1. The peptides representing the COOH terminus of the N protein, in particular N371 and N385, had high absorbance/cutoff value ratios with the highest positive detection rate and the lowest hydrophobicity score among all of the synthesized peptides (Fig. 1C, and Fig. 3 in the online Data Supplement). Peptide N66 is located in a hydrophilic region and reacts with SARS serum antibodies. However, compared with the peptide region N371–N404, which has a score of 9.4 for accessible residues and a hydrophobicity score of −3.5, peptide N66 has relatively low antigenicity because of a correspondingly lower score for accessible residues (6.2) and higher score for hydrophobicity (−1.8). These differences may partially explain the low number of true positives (58%) that we obtained when we used N66 as an antigen. According to the hydrophobicity analysis (Figs. 1 and 2 in the online Data Supplement), most regions in both the S and M proteins are very hydrophobic. Although many factors influence antigen–antibody interactions, two major factors, charge–charge interactions and hydrogen bonding caused by hydrophilicity, have been hypothesized to be crucial for an epitopic site *(9)*.

Of the 41 synthesized peptides, only 5 displayed significantly high immunoreactivity (Table 1). Interestingly, all five immunoreactive peptides are located in regions with a relatively high hydrophobicity score (Figs. 1–3 in the online Data Supplement). Moreover, the number of polar amino acids in each peptide correlates well with the percentage of true positive, e.g., N371 and N385 reacted with >94% of the samples from SARS patients and contain nine and seven polar amino acids, respectively, whereas S599, M137, and N66 reacted with ≤80% of the sera from SARS patients and have four, three, and three polar amino acids, respectively. Our findings thus support that both hydrophobicity and peptide charge are important in determining immunoreactive sites in SARS-coronavirus structural proteins.

The SARS coronavirus propagated in Vero-E6 was used as an antigen to test whether SARS-coronavirus antibodies were raised in sera. We collected sera from 24 healthy controls and measured their immunoreactivities against SARS coronavirus by ELISA. None of the control sera reacted with the SARS coronavirus. The same results were observed for 25 other control sera obtained from patients with respiratory diseases who did not have SARS. However, sera from all 31 SARS patients reacted with the SARS coronavirus, with high absorbance/cutoff value ratios [mean (SD), 4.23 (1.85)].

The results of the peptide screening are summarized in Table 1. There are two key parameters listed in Table 1, *P* values, which indicate whether there was a significant difference between the synthesized peptide and SARS coronavirus as antigen in the ELISA, and *x*/31, which represents the detection rate when particular peptides

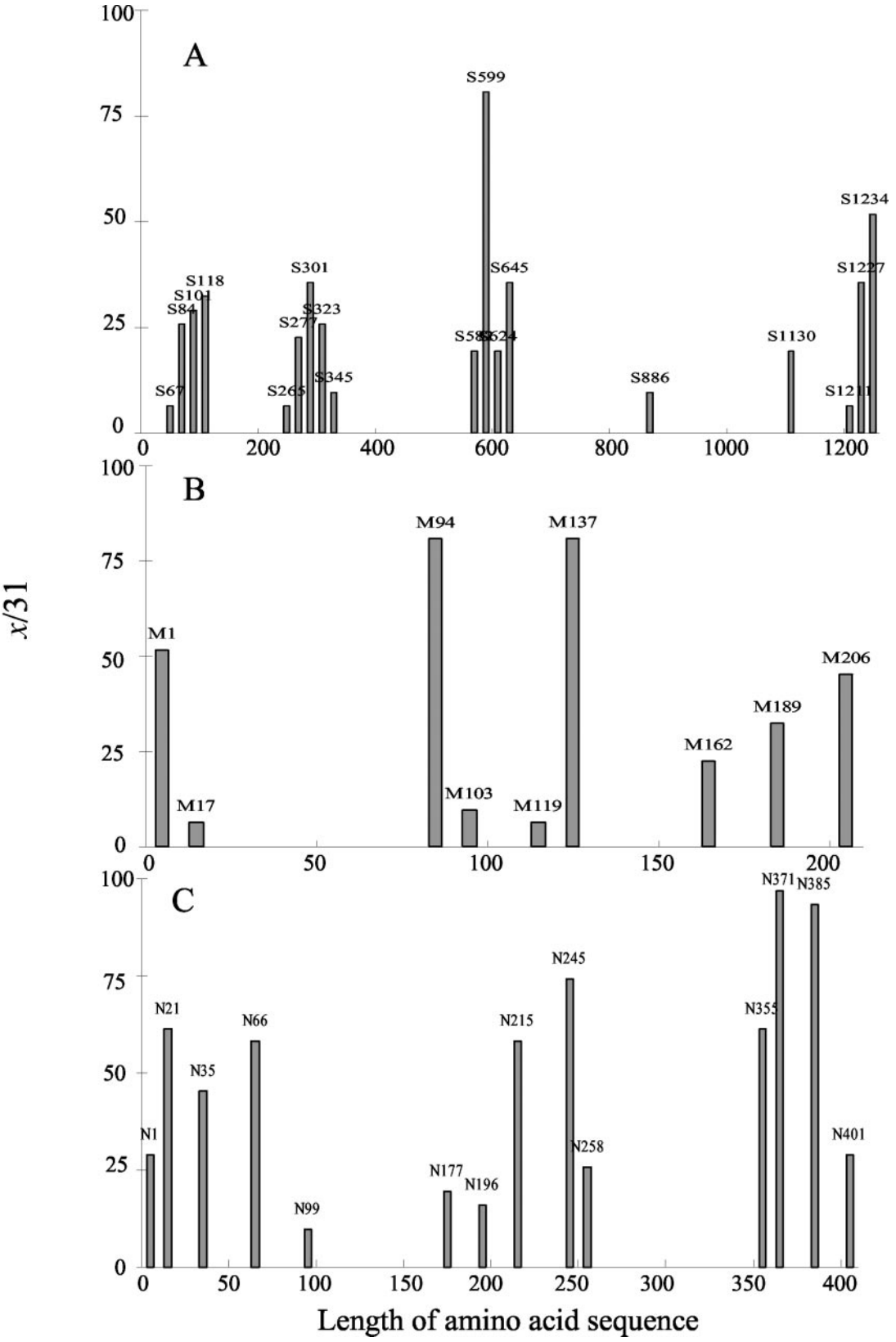

Fig. 1. Comparison of the detection rates of 41 synthesized peptides spanning different sequences of the SARS-coronavirus structural proteins.

Shown are peptides from SARS-coronavirus structural proteins S (*panel A*), M (*panel B*), and N (*panel C*). The *x*/31 axis represents the percentage of immunoreactivity of the synthesized peptide screened by sera from 31 SARS patients.

were used as antigen. For the S protein, 18 peptides spanning the protein sequence were synthesized. Only one peptide, S599, elicited a response in the ELISA that was not significantly different from the response elicited by the SARS coronavirus: ~80% (25 of 31) of the sera from SARS patients reacted with this peptide. The other 17 peptides reacted only slightly with the sera from SARS patients and gave low detection rates, suggesting that the regions of the S protein covered by these peptides have no epitopic site. For the M protein, a relatively small viral structural protein, nine peptides were synthesized. Compared with the SARS coronavirus, this peptide reacted with 80% (25 of 31) of the sera ($P = 0.1$), indicating that it may contain a weak epitopic site.

The highest immunoreactivities were for the peptides located within the N protein. Three of 14 synthesized N-protein peptides, N66, N371, and N385, showed reactivities comparable to that of the SARS coronavirus. Interestingly, N66 is located at the NH_2 terminus of the N protein, whereas N371 and N385 are neighboring fragments, both at the COOH terminus. We thus reasoned that N protein has at least two epitopic regions. These three peptides showed low $y/24$ values—0/24, 1/24, and 0/24, respectively—and high $x/31$ values: N371 and N385 reacted with 97% (30 of 31) and 94% (29 of 31) of the sera from SARS patients, respectively, whereas N66 reacted with ~58% (18 of 31). Taken together, these data suggest that the most immunoreactive epitopic site in the SARS coronavirus is located at the COOH terminus of the N protein.

Members of the coronavirus family all have three major structural proteins, S, M, and N, but the antigenicities and roles of these viral proteins in immunity remain unclear. Different viruses often cause different immunoresponses. For example, in infectious bronchitis virus *(10)*, the S glycoprotein is more antigenic than either the N or the M protein. Because S protein is exposed to the outer viral surface, it is often used as the antigenic group for serologic testing. The M protein, embedded in the viral envelope, has highly variable sequences and is considered to exhibit weak antigenicity, but glycosylation of this protein may elicit antibody production. One common phenomenon is that the N proteins have been shown to be strong immunogens in several coronaviruses, such as murine coronavirus *(11)*, turkey coronavirus *(12)*, and porcine reproductive and respiratory syndrome virus *(13)*. In these viruses, the N protein is a highly abundant and relatively conserved antigen. Importantly, the N protein may be involved in stimulating cytotoxic T lymphocytes. It has been reported that the N protein accumulates even before it is packaged in the mature virus *(14)*. A cellular immune response elicited early in infection by internal viral proteins could therefore be an important defense mechanism. On the basis of the genomic sequences published to date, most of the SARS-coronavirus N protein is highly conserved among strains. Thus, the N protein, which has high antigenicity, is likely to have a great impact on the development of diagnostic tests for SARS.

RECOGNITION OF SARS CORONAVIRUS S AND N PROTEINS BY SERA FROM SARS PATIENTS

The proteins extracted from Vero-E6 cells infected by SARS coronavirus were separated by SDS-PAGE (12% polyacrylamide), and the separated proteins were probed with antibodies in sera from SARS patients. As shown in Fig. 2, some immunostained bands appeared only on the membranes incubated with patient sera, suggesting that these proteins are associated with SARS. On the basis of genomic data, the structural proteins of the virus have molecular masses of 139 (S protein), 46 (N protein), 25 (M protein), and 8 (E protein) kDa *(4)*, respectively. In all of the Western blots performed with sera from three SARS patients as the primary antibody, no protein band <40 kDa was immunostained, suggesting that the M and E proteins either do not elicit antibody production or generate low antibody titers in SARS patients. Of the protein bands >40 kDa, most were located around two regions

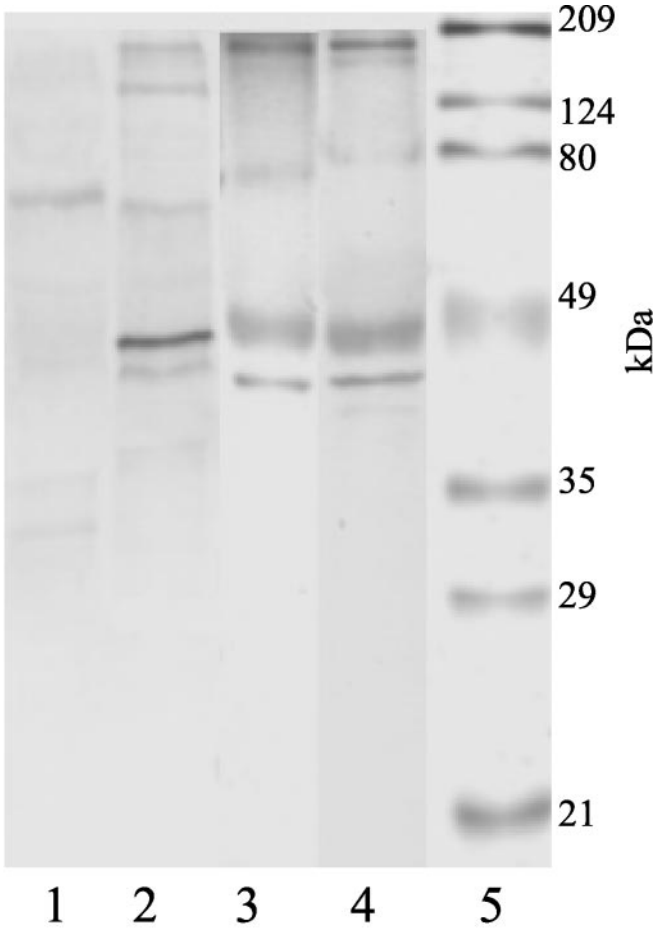

Fig. 2. Anti-SARS-coronavirus recognition patterns in Western blots.

Protein (50 μg) from extracts of Vero-E6 cells infected by SARS coronavirus was loaded in each lane. *Lane 1*, control (serum from a healthy donor as primary antibody); *lanes 2–4*, positive results for SARS (sera from SARS patients as primary antibodies); *lane 5*, protein markers.

(Fig. 2), 49 and 120–240 kDa. The protein band slightly smaller than 49 kDa reacted consistently with all SARS sera. Because its location is close to the theoretical molecular mass of the N protein, 46 kDa, we believe that this protein is the N protein. The immunostained bands around 120–240 kDa did not display a consistent pattern. Although the theoretical value of the S protein is 139 kDa, it has been reported to be glycosylated during infection of the host cells. It therefore is not surprising that SARS-coronavirus S proteins have significantly higher molecular masses than the theoretical value. We thus deduced that these immunostained proteins with high molecular masses are the S protein.

To further confirm the identities of these protein bands, we conducted competition experiments to determine whether the peptides from the S or N protein would inhibit the binding of the sera from SARS patients in the ELISA. The patient sera preincubated with 4 mg/L S599 or N385 gave a 25–30% lower response in the ELISA (data not shown), suggesting that the two peptides could compete with SARS coronavirus for binding to the antibodies in SARS serum. These observations suggest that the S and N proteins account for the antigenicity of the SARS-coronavirus structural proteins.

AFFINITY OF THE PEPTIDES LOCATED AT THE COOH TERMINUS OF N PROTEIN

ProtScale analysis suggested that the COOH terminus of the N protein has fewer buried residues and higher hydrophilicity than the other regions of this protein. We designed four peptides around the region, N355, N371, N385, and N401. Of these, N401, located at the end of the N protein, had low reactivity in the ELISA; conversely, the other three peptides were significantly more immunoreactive in the ELISA. To determine which peptides had higher affinities for antibodies from SARS sera, we quantitatively measured the binding of the peptides to the antibodies in the ELISA. In this experiment, the concentration of the antibodies was kept constant and the binding capacity was estimated as a function of peptide concentration (Fig. 3). Using the Scatchard equation, we calculated the constants n and k_d; the binding curves obtained for the three peptides gave similar n values but different k_d values. On the basis of the definitions of the two parameters in *Materials and Methods*, the fact that the curves give similar n numbers indicates that the peptides share similar epitopic sites, whereas the different k_d values indicate that the three peptides bind to the same epitope with different affinities. Of the three peptides, the k_d value for N385 was much lower than the k_d values for N355 and N371, approximately one-fourth of the k_d value for N371 and one-thirteenth of the k_d value for N355, suggesting that N385 is able to bind more strongly with antibodies in SARS sera. Thus, the region around N385 is most likely a strong epitopic site within the N protein.

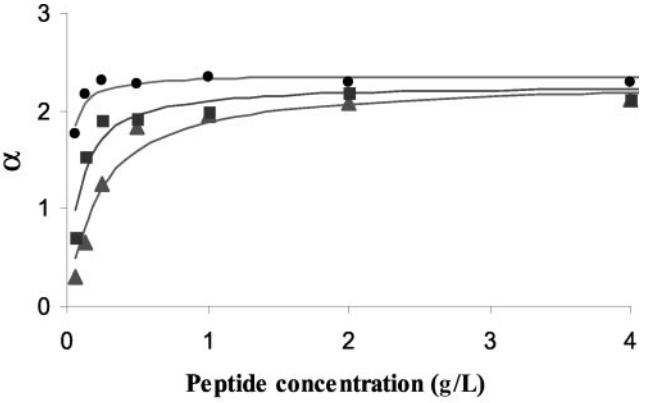

Fig. 3. Affinities of different peptides for antibodies from SARS serum.
x axis, peptide concentration (mg/L); *y axis*, proportion of bound peptides per antibody (α). ●, peptide N385; ■, peptide N371; ▲, peptide N355.

REACTIVITIES OF COMBINED PEPTIDES IN THE ELISA

Among the 31 SARS cases that were identified with use of the SARS coronavirus as antigen, 1 and 2 cases were not detected by peptides N371 and N385, respectively, although both were the most immunoreactive antigens of the 41 synthesized peptides. A possible explanation for this failure may relate to the specific recognition of the antibodies raised from a SARS case. In this case, the antibodies may not actively recognize the COOH terminus of the N protein. Hence, combining the peptides that represent different structural proteins could improve the detection coverage. We selected N371 and N385 to use in combination with peptides S599 and M137, respectively, and used these combinations as antigens for the ELISA. Contrary to our expectations, the use of peptide combinations did not improve the detection coverage, whereas single peptides displayed higher immunoreactivities in the ELISA (Table 1 in the Data Supplement). This unexpected behavior may be attributable to interactions between peptides N385 and S599 or between N371 and M137. If this hypothesis is correct, then generating a chimeric peptide by linking the two peptides could be a good solution for reducing interactions. We are currently pursuing this line of investigation.

In summary, the data presented indicate that (*a*) in three SARS-coronavirus structural proteins, a total of four epitopic sites, S599, M137, N66, and N371–404, were detected by screening with synthesized peptides; (*b*) of the five peptides with high reactivity against SARS sera, N371 and N385 gave significant results compared with the SARS coronavirus lysate in an ELISA as well as a positive detection rate, suggesting that the COOH terminus of the N protein could be extremely antigenic and useful in clinical diagnosis based on the ELISA; (*c*) of four peptides located at the COOH terminus of the N protein that were synthesized and tested for their binding with SARS serum antibodies, N385 was confirmed to have the highest affinity for forming the peptide–antibody complex. Taken together, these results show that synthesized

peptides could be important in exploring epitopes of immunogens, specifically in urgent situations such as that presented by the emergence of the SARS virus. The identification of these reactive peptides could aid in the development of diagnostic techniques for SARS.

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