Brief communication

Assessment of synthetic peptides of severe acute respiratory syndrome coronavirus recognized by long-lasting immunity

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Abstract: In order to determine highly immunogenic severe acute respiratory syndrome coronavirus (SARS-CoV) epitope peptides capable of inducing long-lasting immunity, we first screened immunoglobulin-G (IgG) antibodies reactive to 197 different overlapping 15-mers from the SARS-CoV proteins in the sera of three infected patients. Forty-two peptides among them were reactive to the sera from all three patients. Consequently, we tested for the reactivity of these 42 peptides to patients' sera ($n = 45$) at 6-month post-infection. The significantly higher levels of IgG antibodies specific to three (S791, M207 and N161) of 42 peptides were detectable in the post-infection sera from 23 (51%), 27 (60%) and 19 (42%) of 45 patients, respectively. These three peptides, recognized by their long-lasting immunity, may provide a better understanding of the immunogenicity of SARS-CoV.

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A novel coronavirus (SARS-CoV) was discovered in association with the cases of life-threatening severe acute respiratory syndrome (SARS) that occurred in March of 2003 (1, 2). The genome of the SARS-CoV is 29,727 nucleotides in length and has 11 open reading frames, and its genome organization is similar to that of other coronaviruses (3). Since March of 2003, studies to determine immunogenic epitopes have been performed at a fast pace, within a short period of time, because of the urgent need to develop both therapeutic and diagnostic modalities for the SARS-CoV (4–8). The results of these studies indicate that both the spike (S) and nucleocapsid (N) proteins of the SARS-CoV contain immunogenic regions. However, further studies are needed in order to identify the hot spots, for which diagnostic and therapeutic tools can be developed. In order to determine highly immunogenic regions, an investigation was performed of SARS-CoV epitope peptides capable of inducing long-lasting immunity, and the three candidate peptides have been reported in this study.

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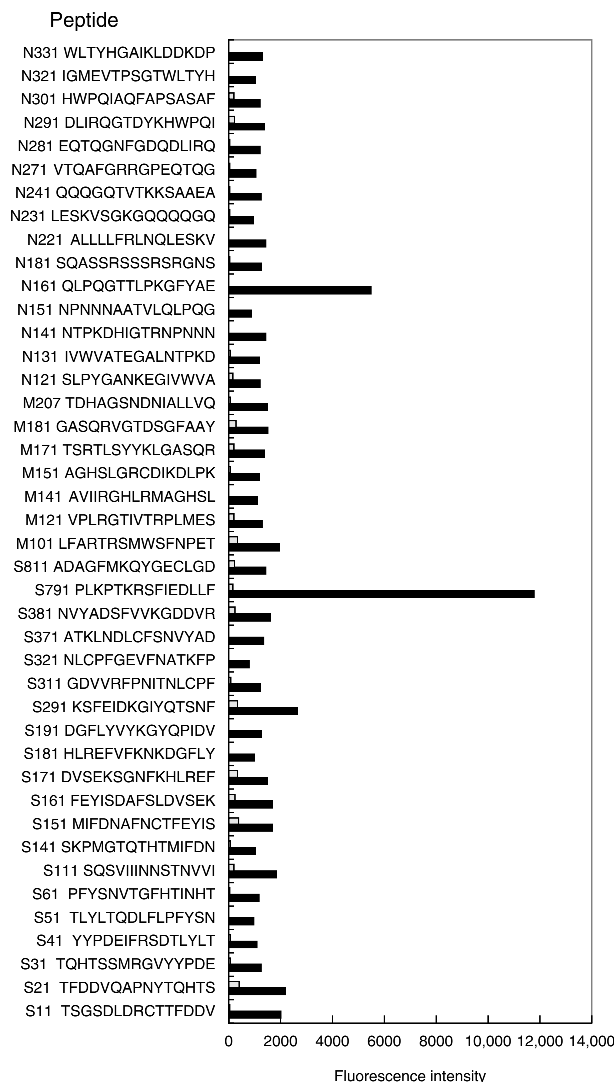

Fig. 1. **Screening of peptides.** A total of 197 different 15-mer peptides (>70%) possessing 5-amino acid overlap sequences based on the full genomic sequences of the severe acute respiratory syndrome coronavirus (SARS-CoV), including 125 spike (S), 43 nucleocapsid (N), 22 membrane (M) and seven envelope (E) proteins (3), were purchased from American Peptide Company, Inc. (Vista, CA). Each peptide was dissolved in dimethylsulfoxide (DMSO) and was then stored at −20°C until use. These peptides were tested for their reactivity to the sera of early stages of three Taiwanese SARS-CoV-infected patients by using flowmetry analysis with Luminex™ (Luminex Corp., Austin, TX) (9). The sera were collected from Jen-Ai Municipal Hospital, SaAn District, Taipei, Taiwan. The patients' sera showed significantly higher levels of immunoglobulin-G (IgG) ($P < 0.05$) activities reactive to 42 of 197 peptides tested, including 20 spike (S)-, seven membrane (M)- and 15 nucleocapsid (N)-derived peptides, when the means of the scores of fluorescence intensity (FI) from the sera (1000-fold dilution) of the three patients (closed bar) were compared to those of the three healthy donors (HD) (open bar). The peptides were coupled to colour-coded beads, according to the modified manufacturer's instructions (Luminex Corp.). In brief, 100 µl of colour-coded beads were mixed with 100 µl of peptide (1 mg/ml in 0.1 M morpholinoehanesulfonic acid (MES) buffer, pH 4.5). The peptide-loaded beads were then incubated with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide 2-(**N**-Morpholino)ethanesulfonic acid (EDC) (1 mg ml^{-1}) at room temperature for 30 min in darkness, and the beads were washed with Tween-20 phosphate-buffered saline (PBS). The beads were treated with 2-aminoethanol for 10 min at room temperature in darkness, washed twice and then re-suspended with 1 ml of 0.05% Block Ace (Snow Brand Milk Products Co., Ltd, Hokkaido, Japan) in Tween-20 PBS. Two microlitres of serum at dilutions of 100–10,000 times was incubated with 25 µl of the peptide-coupled colour-coded beads for 2 h at room temperature on a plate shaker in a 96-well filter plate (MultiScreen™-BV, Millipore Co., Bedford, MA). After incubation, the plate was washed by using a vacuum manifold apparatus and was incubated with 100 µl of biotinylated goat anti-human IgG (gamma-chain-specific: Vector Laboratory Inc., Burlingame, CA) for 1 h at room temperature on a plate shaker. The plate was then washed, and 100 µl of streptavidin-PE (Molecular Probes, Eugene, OR) was added into wells, followed by incubation for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 µl of Tween-20 PBS into each well, and the plate was placed for 3 min on a plate shaker. Fifty microlitres of sample was analysed by using the Luminex™ system with the help of the method reported previously (13, 9, 15).

We first measured the levels of immunoglobulin-G (IgG) antibodies reactive to each of the 197 peptides in the sera of the Taiwanese SARS-CoV-infected patients ($n = 3$) and the Japanese healthy donors (HD) ($n = 3$) as negative controls by means of the flowmetry analysis with Luminex™ (Luminex Corp., Austin, TX), a new method that has the great advantage of allowing users to measure a large number of serum samples against a large number of peptide antigens at relatively low cost, time and labour intensity, as recently reported by us (9). The patients' sera showed significantly higher levels of anti-peptide activities ($P < 0.05$) against 42 of 197 peptides tested, including 20 spike (S), seven membrane (M) and 15 nucleocapsid (N)-derived peptides (Fig. 1). The scores, for instance, for the fluorescence intensity (FI) of the anti-SARS-CoV spike protein at positions 791–805 (termed anti-S791) were highest among the peptides tested, and were 1813, 22,964 and 11,240 in the sera of the three patients, whereas those of the controls were 207, 58 and 210, respectively. The scores for the FI of the anti-SARS-CoV nucleocapsid protein at positions 161–175 (termed anti-N161) were 697, 815 and 14,084, whereas those of the controls were 0, 11 and 129, respectively. The results of the dose-dependent curves were obtained in all of the 42 peptides for all three cases. The representative results of the anti-S791 and anti-N161 antibodies have been presented in Fig. 2.

The 42 peptides shown in Fig. 1 were tested for their reactivity to the post-infection (6th month) sera from patients with the Vietnamese SARS-CoV infection ($n = 45$). As negative controls, sera of both Vietnamese HD ($n = 50$) and the contact persons ($n = 230$), who were free from illness but worked in the same hospitals, were simultaneously tested at a serum dilution of 1 : 100. Both the mean and the median of the FI of sera from Vietnamese HD, contact persons and the patients reactive to each of the 42 peptides have been presented in Table 1. The levels of anti-S791, anti-M207 and anti-N161 activities in the sera of the SARS-CoV patients were significantly ($P < 0.005$) higher than those of both Vietnamese HD and the contact persons as evaluated by means of both Student's t-test and the Mann–Whitney test (Table 1). In contrast, there were no significant differences in the reactivity against any of 42 peptides between the HD and the contact persons.

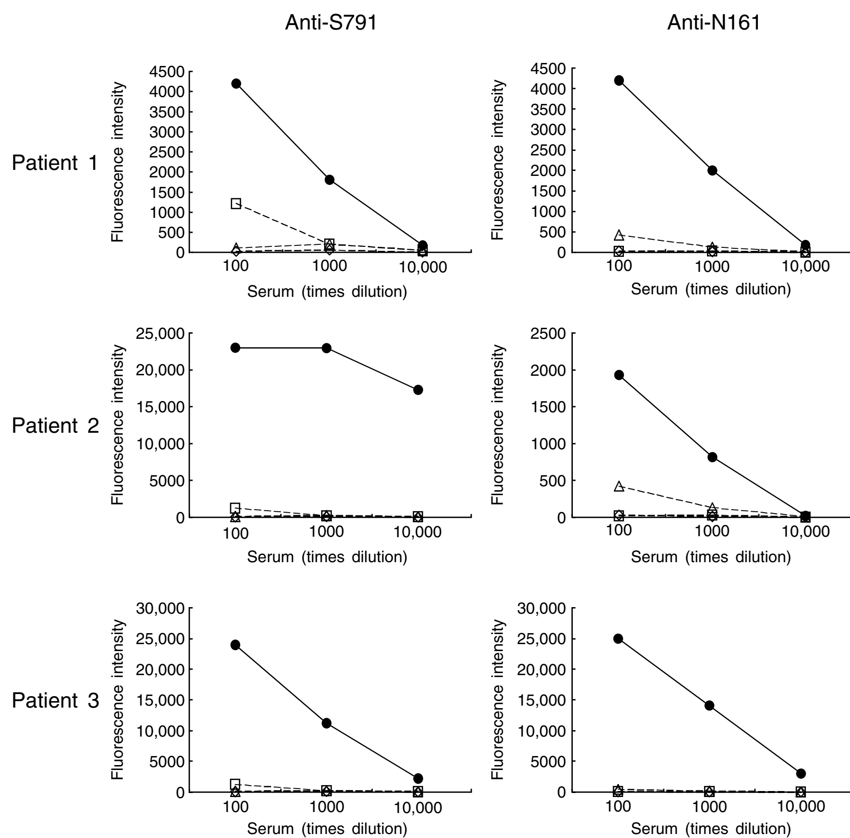

Fig. 2. **Dose dependency.** The dose dependency of the anti-peptide activities was observed in all of the 42 peptides at three different serum dilutions (100-, 1,000- and 10,000-fold). Representative results of the dose-dependent curves of the anti-S791 and anti-N161 activities have been shown in this figure. The levels of immunoglobulin-G (IgG) have been presented as closed circles (patients), and as an open circle, open square and open triangle (three healthy donors), respectively.

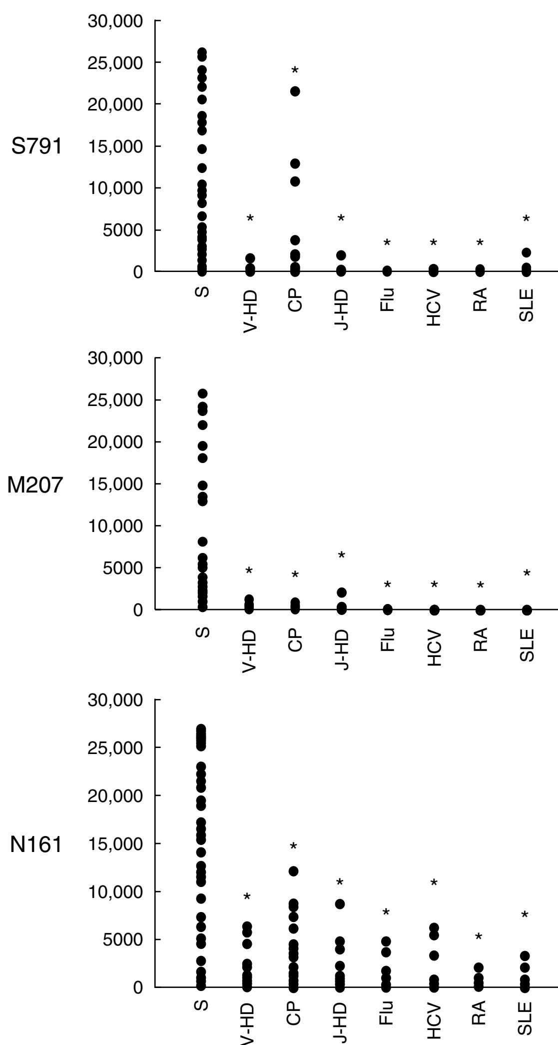

Fig. 3. **Anti-peptide immunoglobulin-G (IgG) at post-infection.** The 42 peptides shown in Fig. 1 were tested for their reactivity to sera from post-infection (6th month) patients with the Vietnamese SARS-CoV infection ($n = 45$). As negative controls, the sera of both Vietnamese healthy donors (V-HD) ($n = 50$) and the contact persons (CP), who were free from illness of SARS-CoV infection but worked in the same hospitals, were tested. Sera from Japanese patients with hepatitis-C virus (HCV), influenza (Flu), rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), along with Japanese HD (J-HD), were also tested at the same times at a serum dilution of 1 : 100. Representative results of the fluorescence intensity (FI) scores at a serum dilution of 100 : 1 of anti-S791, anti-M207 and anti-N161 activities have been shown in this figure. The significance of the differences (*$P < 0.05$) in Luminex reactivities of SARS patients against all of other negative control groups, including V-HD, CP, J-HD, Flu, HCV, RA and SLE, was observed by means of Mann–Whitney test. Plasma from 78 Vietnamese post-infection (6th month, $n = 45$) SARS-CoV patients was collected at Hanoi French Hospital and Bach Mai Hospital in Vietnam. All cases met a modified World Health Organization (WHO) case definition of SARS (16). This study was approved by both the Japanese and Vietnamese Institutional Review Board. Written informed consent was obtained from each of the participants. Sera from Japanese patients with Flu ($n = 12$), HCV ($n = 12$), RA ($n = 15$) and SLE ($n = 10$) and Japanese healthy donors (J-HD, $n = 27$) were provided by Kurume University Hospital and Kurume Medical Center after informed consent was obtained. SARS-CoV, severe acute respiratory syndrome coronavirus.)

Each of the FI scores at a serum dilution of 1 : 100 of anti-S791, anti-M207 and anti-N161 activities has been plotted in Fig. 3. The cut-off value of the FI scores for anti-S791 peptide activity at a serum dilution of 1 : 100 was set at 970 (mean: 199 plus 2 SD, 386×2 of 50 HD). Under these circumstances, significant levels (>970 at a serum dilution of 1 : 100) of anti-S791 activity were detected in the sera from 23 of 45 patients (51%), 18 of 230 contact persons (7.8%) and four of 50 HD (8%). When the cut-off value for anti-M207 activity was set at 896 (mean: 356 plus 2 SD, 270×2 of 50 HD), significant levels (>896) of anti-M207 activity were detected in the sera from 27 of 45 patients (60%), 10 of 230 contact persons (4.3%) and three of 50 HD (6%). Similarly, when the cut-off value for anti-N161 activity was set at 2705 (mean: 525 plus 2 SD, 1090×2 of 50 HD), significant levels (>2705) of anti-N161 activity were detected in the sera from 19 of 45 patients (42%), 21 of 230 contact persons (9.1%) and two of 50 HD (4%) (Fig. 3).

The levels of anti-M181 activity in the sera of the SARS-CoV patients were also significantly higher than those of Vietnamese HD and contact persons as evaluated with the help of both Student's *t*-test and Mann–Whitney test (Table 1). However, the positive cases showing FI scores of greater than the mean plus 2SD were only six of 45 patients (13%). In contrast to these four peptides, significant levels of IgG reactive to the remaining 36 peptides were either scarcely or not detected in the patients (Table 1).

Wang et al. (4) reported four different epitope peptides recognized by the sera of SARS-CoV patients. One of them, the N66 (nucleocapsid protein at positions 161–182) peptide, was 7-amino acids longer at the C-terminal than the N161 (at positions 161–175) peptide reported in this study. These findings suggest that this region of nucleocapsid proteins is one of the most highly immunogenic epitopes of the SARS-CoV when peptides are used. However, the S791 peptide shown to be the other candidate of immunogenic peptides in the present study was not tested by Wang et al. (4), because they selected peptides with relatively high hydrophilicity. They also did not detect the M207 peptide as an immunogenic epitope, although they tested the M206 peptide, which is one N-terminal amino acid longer than the M207 peptide.

In order to determine the cross-reactivity of the patients' sera to the other infectious diseases or auto-immune diseases, sera from Japanese patients with hepatitis-C virus ($n = 12$), influenza virus ($n = 12$), rheumatoid arthritis ($n = 15$) and systemic lupus erythematosus (SLE) ($n = 10$) and the Japanese HD ($n = 27$) were also tested at the same times at a serum dilution of 1 : 100. However, anti-S791, anti-M207 or anti-N161 activity was not detectable in the

sera of any groups tested, including SLE patients, although the cross-reactivity between the SARS-CoV and SLE was suggested in the study by using an Enzyme-linked immunosorbent assay (ELISA) kit coated by non-purified antigen (10). These results indicate that anti-S791, anti-M207 and anti-N161 activities were largely restricted to the SARS-CoV infection, although sera of Vietnamese patients shall be provided as controls to confirm this issue.

Both the Luminex™ and ELISA were employed for the measurement of anti-peptide antibodies in order to ensure the reliability of the former new method. As expected, both the assays could detect anti-SARS peptide activity reactive to each of the three peptides with relatively higher sensitivity in the Luminex assay for the measurement of anti-M207 antibody. Representative results of the three cases have been presented in Fig. 4.

Reactivity of the synthesized 15-mer peptides with sera from post-infection (6th month) SARS patients with the help of flowmetric analysis by using Luminex™

Name of peptide	Peptide sequence	FI of HD[a] (mean ± SD) ($n = 50$)	FI of CP[b] (mean ± SD) ($n = 230$)	FI of S[c] (mean ± SD) ($n = 45$)	Percentage of positive S samples	FI of HD[a] median[d] (75, 25)	median (75, 25)	median (75, 25)
S11	TSGSDLDRCTTFDDV	767 ± 1505	735 ± 1496	469 ± 631	0	217 (107, 520)	200 (109, 581)	210 (131, 474)
S21	TFDDVQAPNYTQHTS	92 ± 396	113 ± 356	117 ± 382	4	18 (13, 38)	24 (12, 56)	18 (9, 30)
S31	TQHTSSMRGVYYPDE	423 ± 766	573 ± 962	493 ± 847	7	231 (158, 376)	257 (164, 478)	207 (162, 288)
S41	YYPDEIFRSDTLYLT	32 ± 103	44 ± 130	43 ± 79	4	8 (0, 27)	15 (0, 32)	23 (7, 37)
S51	TLYLTQDLFLPFYSN	14 ± 64	7 ± 28	7 ± 21	0	0 (0, 0)	0 (0, 0)	0 (0, 0) 0 (0, 3)
S61	PFYSNVTGFHTINHT	17 ± 46	13 ± 45	13 ± 29	2	0 (0, 12)	0 (0, 9)	0 (0, 19)
S111	SQSVIIINNSTNVVI	48 ± 158	34 ± 75	53 ± 159	4	13 (5, 26)	17 (8, 30)	17 (12, 28)
S141	SKPMGTQTHTMIFDN	265 ± 630	290 ± 599	320 ± 686	4	119 (52, 269)	106 (51, 224)	121 (67, 208)
S151	MIFDNAFNCTFEYIS	353 ± 651	806 ± 3057	423 ± 623	7	180 (95, 344)	247 (141, 512)	239 (151, 387)
S161	FEYISDAFSLDVSEK	128 ± 423	168 ± 371	143 ± 279	4	46 (29, 85)	63 (37, 112)	60 (36, 87)
S171	DVSEKSGNFKHLREF	327 ± 610	528 ± 1865	418 ± 697	4	178 (122, 366)	198 (126, 371)	213 (164, 359)
S181	HLREFVFKNKDGFLY	20 ± 54	12 ± 39	10 ± 13	0	0 (0, 15)	0 (0, 7)	4 (0, 16)
S191	DGFLYVYKGYQPIDV	22 ± 65	14 ± 32	39 ± 113	7	0 (0, 13)	0 (0, 11)	0 (0, 35)
S291	KSFEIDKGIYQTSNF	440 ± 1088	604 ± 1321	564 ± 1054	4	165 (109, 331)	207 (121, 428)	229 (149, 371)
S311	GDVVRFPNITNLCPF	171 ± 338	180 ± 345	180 ± 315	2	73 (46, 135)	82 (52, 165)	125 (65, 191)
S321	NLCPFGEVFNATKFP	627 ± 1008	835 ± 2051	508 ± 631	2	322 (175, 596)	341 (202, 664)	333 (212, 481)
S371	ATKLNDLCFSNVYAD	495 ± 1056	875 ± 2035	719 ± 1405	7	231 (157, 474)	289 (189, 607)	237 (179, 521)
S381	NVYADSFVVKGDDVR	376 ± 1047	488 ± 1047	498 ± 1037	7	130 (72, 317)	158 (84, 339)	124 (83, 244)
S791	PLKPTKRSFIEDLLF	199 ± 386	489 ± 2027	3374 ± 5981[e,g,h]	51	39 (6, 214)	37 (9, 124)	1057 (189, 3160)[f,g,h]
S811	ADAGFMKQYGECLGD	268 ± 711	399 ± 970	356 ± 775	4	90 (66, 160)	105 (66, 216)	108 (88, 232)
M101	LFARTRSMWSFNPET	86 ± 224	152 ± 367	151 ± 358	4	42 (18, 76)	48 (18, 101)	51 (32, 117)
M121	VPLRGTIVTRPLMES	69 ± 338	61 ± 207	109 ± 330	4	0 (0, 11)	0 (0, 14)	2 (0, 39)
M141	AVIIRGHLRMAGHSL	17 ± 58	12 ± 32	8 ± 13	0	0 (0, 10)	0 (0, 9)	0 (0, 15)
M151	AGHSLGRCDIKDLPK	781 ± 726	951 ± 1623	1714 ± 3558	16	586 (324, 1035)	562 (296, 951)	627 (368, 1314)
M171	TSRTLSYYKLGASQR	77 ± 372	86 ± 230	114 ± 329	4	8 (0, 27]	14 (0, 51)	18 (7, 39)
M181	GASQRVGTDSGFAAY	904 ± 1655	1470 ± 2614	2596 ± 3007[g]	20	499 (296, 869)	588 (369, 1300)	1306 (706, 3237)[g,h]
M207	TDHAGSNDNIALLVQ	356 ± 270	339 ± 254	2867 ± 4928[g,h]	60	271 (177, 438)	279 (178, 451)	1150 (612, 2224)[g,h]
N121	SLPYGANKEGIVWVA	61 ± 375	101 ± 809	71 ± 268	2	0 (0, 0)	0 (0, 0)	0 (0, 9)
N131	IVWVATEGALNTPKD	169 ± 576	208 ± 576	194 ± 513	7	50 (26, 116)	46 (20, 104)	41 (22, 90)
N141	NTPKDHIGTRNPNNN	276 ± 397	283 ± 393	254 ± 294	2	162 (79, 290)	157 (71, 341)	136 (65, 338)
N151	NPNNNAATVLQLPQG	71 ± 288	189 ± 976	119 ± 411	7	7 (0, 47)	12 (0, 44)	12 (0, 36)
N161	QLPQGTTLPKGFYAE	525 ± 1090	962 ± 2664	6079 ± 7604[g,h]	42	136 (61, 376)	201 (82, 678)	1250 (111, 12099)[g,h]

continued

Name of peptide	Peptide sequence	FI of HD[a] (mean ± SD) ($n = 50$)	FI of CP[b] (mean ± SD) ($n = 230$)	FI of S[c] (mean ± SD) ($n = 45$)	Percentage of positive S samples	FI of HD[a] median[d] (75, 25)	median (75, 25)	median (75, 25)
N181	SQASSRSSSRSRGNS	661 ± 2588	437 ± 1240	388 ± 794	0	111 (63, 207)	87 (44, 231)	79 (49, 245)
N221	ALLLLFRLNQLESKV	181 ± 490	226 ± 505	220 ± 557	4	79 (36, 128)	80 (30, 171)	75 (40, 137)
N231	LESKVSGKGQQQQGQ	314 ± 408	522 ± 1857	312 ± 430	4	150 (109, 431)	209 (100, 370)	181 (99, 362)
N241	QQQGQTVTKKSAAEA	263 ± 594	428 ± 1851	283 ± 496	4	156 (57, 262)	148 (79, 253)	100 (66, 278)
N271	VTQAFGRRGPEQTQG	329 ± 926	681 ± 2088	421 ± 731	4	141 (95, 254)	185 (107, 385)	140 (113, 288)
N281	EQTQGNFGDQDLIRQ	126 ± 344	340 ± 1687	269 ± 375	9	72 (34, 110)	71 (35, 147)	118 (69, 238)[g,h]
N291	DLIRQGTDYKHWPQI	390 ± 865	760 ± 2302	493 ± 949	4	188 (138, 323)	243 (148, 434)	235 (170, 332)
N301	HWPQIAQFAPSASAF	176 ± 397	249 ± 946	155 ± 292	4	34 (17, 146)	40 (18, 97)	56 (37, 131)[h]
N321	IGMEVTPSGTWLTYH	419 ± 2038	307 ± 1782	202 ± 465	0	29 (11, 95)	27 (10, 73)	55 (32, 121)[g,h]
N331	WLTYHGAIKLDDKDP	200 ± 330	225 ± 334	216 ± 292	4	142 (12, 228)	111 (50, 245)	113 (74, 214)

[a]IgG level was determined by using Luminex as described in the legend of Fig. 1. A total of 50, 230 and 45 sera were collected from [a]Vietnamese healthy donors (V-HD), [b]contact persons (CP) and [c]SARS patients (S), respectively, for the experiments.
[d]median (25, 75): the FI at 25 and 75 percentiles were shown.
The significance of the differences (P) in Luminex reactivities between HD and patients was analysed by means of [e]Student's t-test and [f]Mann–Whitney test.
[g]S *vs* HD.
[h]S *vs* CP, $P < 0.005$ (statistical analysis).
SARS, severe acute respiratory syndrome.
FI, fluorescence intensity.

Table 1

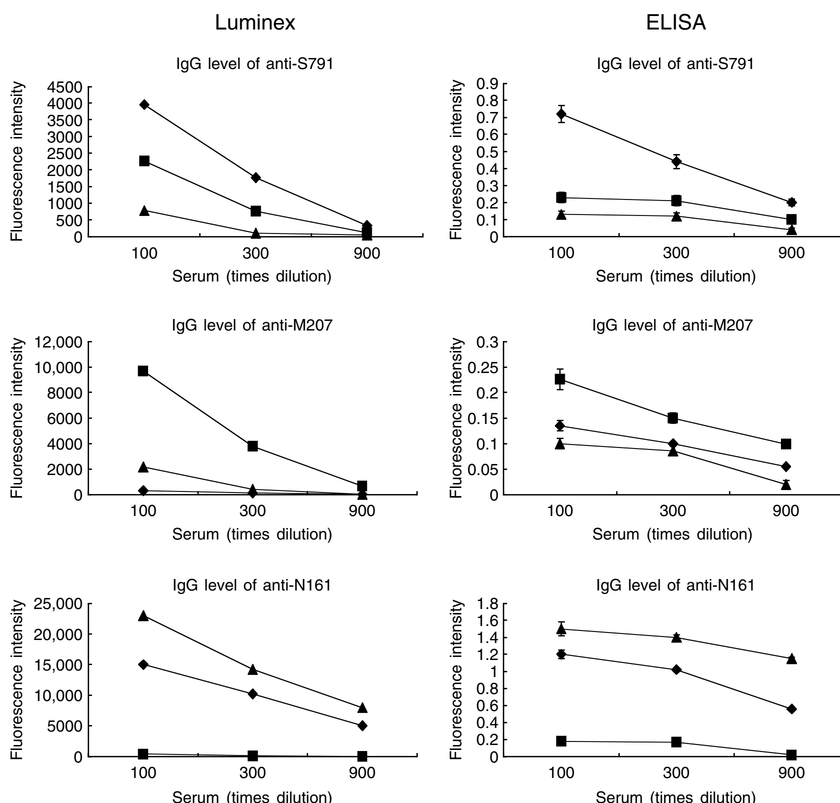

Fig. 4. **Assayed by conventional ELISA methods.** For the preparation of the peptide immobilized ELISA plate for the antibody absorption test, peptides were diluted in 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (Pierce, Rockford, IL), as reported previously (17). ELISA plates were coated overnight at 4°C with the target peptides at a dose of 200 μg/well. The wells were rinsed three times with 0.05% Tween-20 PBS (PBST). The plates were blocked overnight at 4°C with Block Ace. The representative results have been shown in this figure.

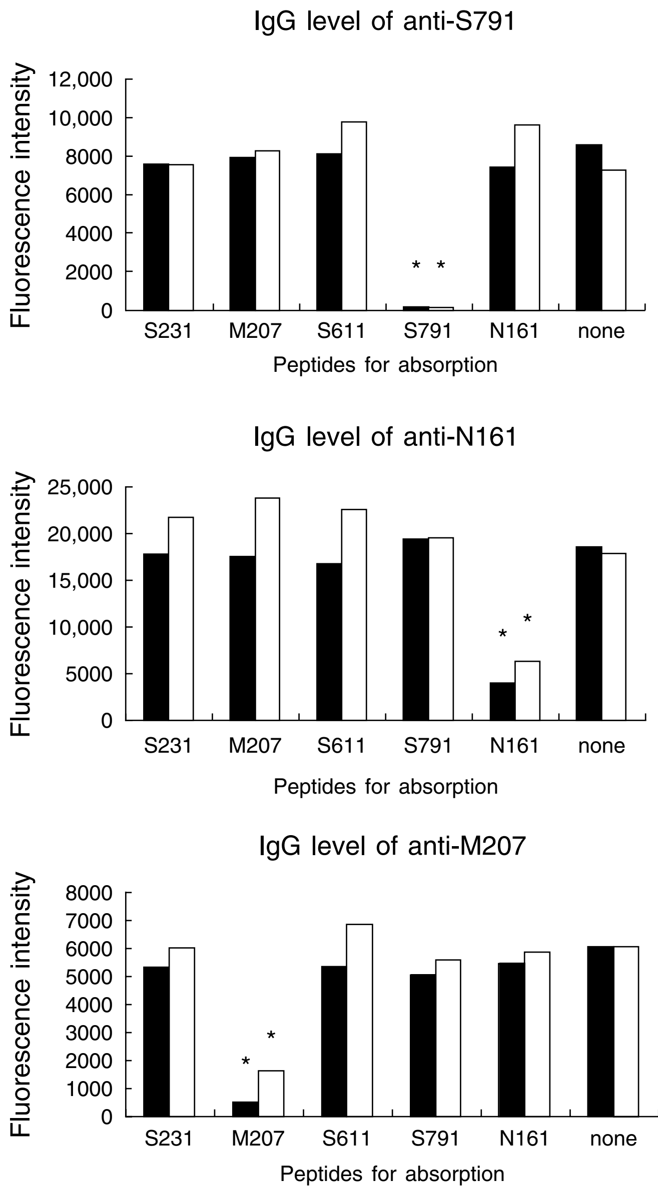

Fig. 5. **Absorption test.** The immunoglobulin-G (IgG) activity to each of the S791, M207 and N161 peptides was absorbed by using a triplicate assay with an immobilized corresponding peptide and each of the five different irrelevant peptides. The method for the preparation of immobilized peptides was the same as the method used for ELISA plate preparation, as described in the legend of Fig. 4. The results of the absorption test were analysed by means of a two-tailed Student's *t*-test. All tests of significance were two-sided. In order to test the specificity of anti-peptide IgG in the serum samples, 100 µl/well of serum samples (1 : 100 dilution with 0.05% PBST) was absorbed with the immobilized peptide (200 µg/well: closed bar or 40 µg/well: open bar, as final concentrations) in wells kept for 2 h at room temperature. The absorption was repeated three times, and then the level of peptide-specific IgG in the resultant supernatant was measured. PBST, Tween-20 PBS.

The specificity of anti-peptide activities was then confirmed by means of the absorption test with two different concentrations of peptides (200 and 40 µg/ml) for immobilization. As expected, anti-S791, anti-M207 or anti-N161 activities were significantly reduced by absorption with the corresponding peptide, but not with any of the irrelevant peptides tested in Fig. 5 The same results were obtained at the two different doses of peptides for plate immobilization, suggesting that the 200 µg/ml of peptide, a concentration usually used for immobilization, was excessive.

Kinetic studies showed that anti-nucleocapsid protein antibodies could be detected in <20%, 70–80% and >90% of probable SARS patients 1–7 days, 8–14 days and 15–61 days after the illness began, respectively (11). However, there was no information on sera obtained 6 months after the onset of the disease, at least in the literatures we read. Viral RNA may persist for some time in patients, who have humoral responses to it, whereas some patients may lack an antibody response to the SARS-CoV after the onset of illness (12, 13). Prolonged shedding of viral RNA in respiratory secretions (11 days after the onset of illness), plasma (up to 9 days) and stool specimens (25 days) has been documented (13). Serum antibody levels do not correlate with protection, although local antibody is believed to play an important role in protection (14). Rather, the upsurge of the IgG antibody to the SARS-CoV and its correlation with the progression of SARS were observed (13). Therefore, a comprehensive investigation of the timing and intensity of humoral responses and their association with clinical manifestations of the disease is needed to better understand Fig. 5. the pathogenesis of the SARS-CoV and to develop appropriate treatment modalities. In order to achieve the necessary level of understanding, we will perform kinetic studies for humoral responses to the three peptides reported in this study in the near future.

Gao et al. (6) reported that an adenovirus-based vaccine could induce strong SARS-CoV-specific immune responses in the monkey, and this research holds promise for the development of a protective vaccine against the SARS causal agent. However, in order to develop this type of protective vaccine, many hurdles in terms of the safety, efficacy, cost benefits and durability must be overcome by performing basic and clinical studies. One of the key basic studies for vaccine development could be to identify immunogenic regions capable of inducing long-lasting immunity, and thus the results shown in this study may provide new information that will help us determine suitable vaccine candidates.

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