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Identification of synthetic vaccine candidates against SARS CoV infection

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Abstract

Three peptides, D1 (amino acid residues 175–201), D2 (a.a. 434–467), and TM (a.a. 1128–1159), corresponding to the spike protein (S) of severe acute respiratory syndrome corona virus (SARS CoV) were synthesized and their immunological functions were investigated in three different animals models (mice, guinea pigs, and rabbits). The peptides mixture formulated either with Freund's adjuvant or synthetic adjuvant Montanide ISA-51/oligodeoxy nucleotide CpG (ISA/CpG) could elicit antisera in immunized animals which were capable of inhibiting SARS/HIV pseudovirus entry into HepG2 cells. The neutralizing epitopes were identified using peptides to block the neutralizing effect of guinea pig antisera. The major neutralizing epitope was located on the D2 peptide, and the amino acid residue was fine mapped to 434–453. In BALB/c mice T-cell proliferation assay revealed that only D2 peptide contained T-cell epitope, the sequence of which corresponded to amino acid residue 434–448. The ISA/CpG formulation generated anti-D2 IgG titer comparable to those obtained from Freund's adjuvant formulation, but generated fewer antibodies against D1 or TM peptides. The highly immunogenic D2 peptide contains both neutralizing and Th cell epitopes. These results suggest that synthetic peptide D2 would be useful as a component of SARS vaccine candidates.

Keywords: SARS CoV; Epitopes; Vaccine; Spike protein; Pseudovirus; Peptide

Emerging infectious disease caused by the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) had significant economic impacts in countries affected by the disease outbreak in 2002–2004 [1]. Since there is no licensed SARS vaccine, the risk posed by a worldwide outbreak of SARS in the future is still exist. There is an urgent need to find a safe and effective SARS vaccine for future use. SARS vaccine candidates could be developed using inactivated virus, attenuated virus, recombinant or synthetic viral components. The latter approach is particularly attractive because it reduces the risks involved when working with potentially hazardous live organisms. Moreover, the fully synthetic peptides derived from the immunogenic region of viral components could provide a well-defined immunogen that may more easily comply with regulatory requirements. Appropriate selection of major reactive epitopes and corresponding peptides for vaccine design is crucial for success. Increasing knowledge of the viral entry mechanism and antigenic structure of viral proteins has been of great help to peptide-based vaccine development. Spike proteins have been developed as vaccine candidates to induce humoral and cellular immunity in mouse models [2,3]. The spike protein of SARS CoV comprised of 1255 amino acids plays an important role in recognition and binding to the host receptor, angiotensin converting enzyme 2 (ACE2). The receptor binding motif has been localized between amino acid residues 424–494 on the S protein [4]. It is believed that virus binding could induce conformational change of the spike protein to form the trimer-of-hairpins organization which

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is characterized by a fusion core structure [5]. The fusion core contains two highly conserved heptad repeat (HR) regions (HR1 and HR2) that are believed to facilitate virus–cell fusion [6]. The predicted HR1 and HR2 regions have been localized at amino acid residues 915–949 and 1150–1184, respectively [7,8]. We previously found an important neutralizing epitope (a.a. 1143–1157) located at the front of the HR2 region [9]. Zeng et al. [10] found that neither of the antibodies against Sl nor S2 alone could neutralize the virus, but antibodies generated against both regions of the spike protein could block host–cell viral entry. It is very important to include different functional domains to design an effective peptide-based vaccine.

This study analyzed the ability of synthetic peptides to serve as vaccine candidates by assessing their ability elicit antibody responses to inhibit the functions of essential domains of virus binding and entry. Because of their importance for induction of peptide-specific immune response, we also investigated the immunological function of two potential human used adjuvants (Montanide ISA-51 and Oligodeoxynucleotide CpG) [11]. The peptides we used in this study were derived from the N-terminal of the spike protein (D1, a.a. 175–205), the receptor-binding region of the spike protein (D2, a.a. 434–467) and a region which overlaps the HR2 region of the spike protein (TM, a.a. 1128–1159). Three peptides were mixed with adjuvant to study the immunogenicity in different animals. A previously developed pseudovirus (SARS/HIV) model was used to determine the neutralizing ability of the peptides-induced antisera [12–14]. Immunological dominant T-cell epitopes were identified in the mouse model.

Materials and methods

Peptides. SARS CoV spike protein peptides used for the mapping of neutralizing epitopes and T-cell epitopes were synthesized as 15-mers with 10 amino acid overlap of the entire cover spike protein sequence (Supplementary data).

Production of peptide-specific antisera. Mice were immunized subcutaneously with 30 μg of each peptide (D1, D2, and TM) emulsified in complete Freund's adjuvant (CFA). The same amount of synthetic peptides emulsified in incomplete Freund's adjuvant (IFA) was used to boost immunization on day 14 and 28 after the initial immunization. The guinea pigs and rabbits were immunized with 50 and 200 μg of each peptide formulated in the same adjuvant, respectively, and the same immunization protocol was used as described above. Moreover, an adjuvant Montanide ISA-51 and 5 μg of CpG nucleotide (TCG TCG TTT TGT CGT TTT GTC GTT TTG TCG TT) of the equivalent volume with 30 μg peptides mixture were subcutaneously injected in mice using the same immunization protocol. Antisera were collected for immunological and biological assay every two weeks after the second boost. The antibody titers were determined by ELISA.

T-cells proliferation assay. To determine whether peptide immunogens could induce specific T-cell lymphoproliferative responses, one-hundred microliters of 2×10^6 cells/ml splenocytes in complete RPMI-10 culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum) was added to each well in 96-well plates. The peptide was added to each well at a final concentration of 5 μg/ml. Cells in all of the wells were cultured in a total volume of 200 μl of medium. After 3 days in culture, cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 16–18 h. Cells were then harvested, and the incorporated radioactivity was determined by Top Count scintillation counter (Packard, USA).

IFN-γ secreting T-cells subsets staining. $CD4^+$ and $CD8^+$ T-cells were purified by immunomagnetic separation using the MiniMACS system. Briefly, the spleen cells were washed and resuspended in FACS buffer (2% BSA/PBS), incubated with anti-CD4 or anti-CD8 microbeads, and separated by an LS column according to the manufacturer's instructions. The $CD4^+$ and $CD8^+$ cells were mixed with same number of mitomycin C-treated naïve splenocytes as antigen presenting cells in the presence or absence of peptides (10 μg/ml). Two days later, cells were harvested and INF-γ secreting cells were identified using a Mouse IFN-γ Secretion Assay Detection Kit (Miltenyi Biotec., Germany).

Neutralization of SARS pseudovirus infection. SARS pseudovirus was prepared as previously described [13]. Supernatants containing pseudovirus bearing SARS CoV S protein were harvested 48-h post-transfection and used for single cycle infection of HepG2 cells. The infected cells were lysed and the expression of luciferase activity was determined by adding the substrate and then detecting the luminescence with a TopCount. The neutralization inhibition (%) was calculated by the equation: neutralization inhibition (%) = (units of luminescence with antiserum and peptide − units of luminescence with antiserum)/(units of luminescence without antiserum − units of luminescence with antiserum) × 100.

Statistical analysis. The statistical significance of differential findings between experimental groups of animals was determined by Student's t test. Differences were considered statistically significant if the value of p was $\leqslant 0.05$.

Results

Immunogenicity of mice, guinea pigs, and rabbits anti-peptide antisera

In order to test their immunogenecity, the three peptides (D1, D2, and TM) were mixed at equal amounts (10 μg each peptide) and formulated either with CFA/IFA or ISA/CpG. After three immunizations (30 μg of peptides per dose), the peptide-specific antibody responses were measured using peptide-specific ELISA, and the results are shown in Table 1. Among the three peptides, D2 seemed to be immunodominant. The antibody titers against D2 peptide were 5.3×10^{-5}. and 2.3×10^{-5} in the CFA/IFA and ISA/CpG groups, respectively. Low levels

Table 1
Reactivity of antisera against synthetic peptides from SARS CoV spike protein

Species	Adjuvant	Reactive titer[a]		
		D1	D2	TM
Mouse[b]	CFA/IFA	1.0×10^{-3}	5.3×10^{-5}	5.5×10^{-4}
	ISA/CpG	1.0×10^{-2}	2.3×10^{-5}	1.0×10^{-3}
Guinea pig[c]	CFA/IFA	3.3×10^{-5}	1.6×10^{-6}	4.1×10^{-5}
Rabbit[d]	CFA/IFA	7.0×10^{-4}	9.0×10^{-4}	2.2×10^{-4}

[a] Pre-immunization titer were subtracted from post-immunization titers.
[b] Eight mice per group were used for the immunogenicity studies, and reactive titers are expressed as the means of the titers obtained from eight mice.
[c] Four guinea pigs per group were used for the immunogenicity studies, and reactive titers are expressed as the means of the titers obtained from four guinea pigs.
[d] Four rabbits per group were used for the immunogenicity studies, and reactive titers are expressed as the means of the titers obtained from four rabbits.

of antibodies against D1 peptide were found in mice immunized with peptide mixtures formulated with adjuvant CFA/IFA or ISA/CpG, with antibody titers between 10^{-3} and 10^{-2}, respectively. Moderate levels of anti-TM titer between 5.5×10^{-4} and 1×10^{-3} were found in the CFA/IFA and ISA/CpG groups, respectively.

Antisera from rabbits showed similar antibody titers against D1, D2, and TM (7×10^{-4}, 9×10^{-4} and 2.2×10^{-4}, respectively). Antisera from guinea pigs, showed good antibody titers against D1, D2, and TM (3.3×10^{-5}, 1.6×10^{-6}, and 4.1×10^{-5}, respectively). These results showed that guinea pigs generated higher antibody titers against D1, D2, and TM compared to those obtained from rabbits and mice.

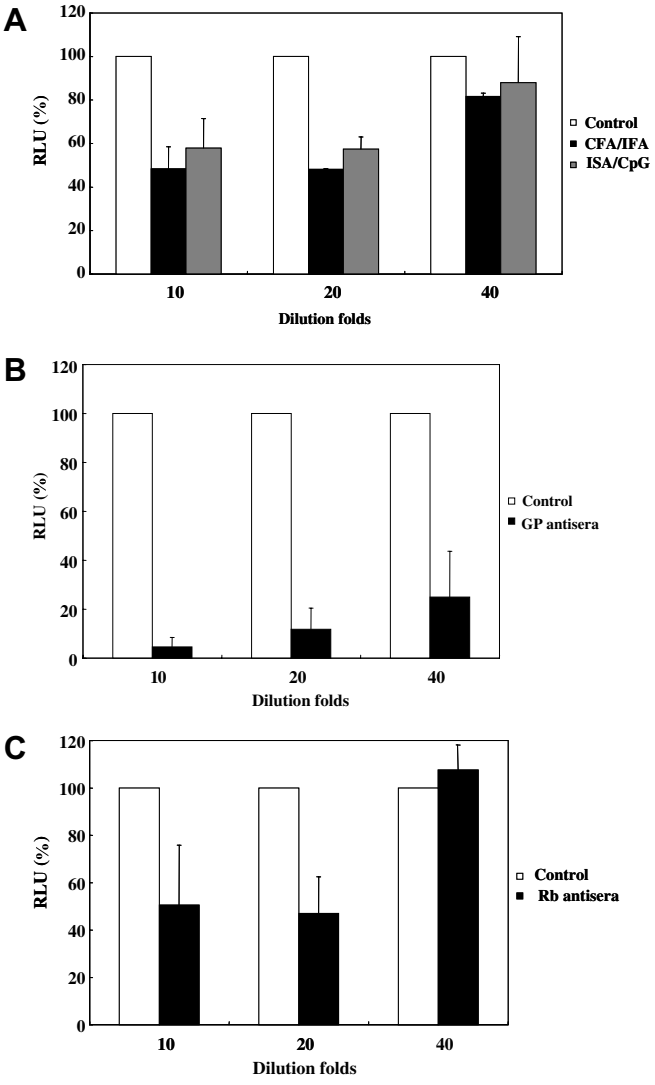

Fig. 1. Antisera from immunized animals can inhibit SARS/HIV pseudovirus entry. SARS/HIV pseudovirus was pre-incubated with each antisera dilution (10, 20, and 40) for 60 min at 37 °C, then used to infect HepG2 (2×10^5) cells. Luciferase activity in cell lysates was measured 3 days after infection. The tested sera are from (A) mice; (B) guinea pigs; (C) rabbits. Results are presented relative to luciferase activity units (RLUs) in the control group (pre-immune serum). The data shown are from three separate experiments.

Biological function of anti-peptide antisera

We used pseudotype-based neutralization assay to analyze the biological function of anti-peptide antisera obtained from animals immunized with the peptides [13]. As shown in Fig. 1A, the neutralizing antibodies were induced in mice immunizations with the CFA/IFA or ISA/CpG formulation. The 40-fold dilution of sera could inhibit ~20% of the luciferase activity in either the CFA/IFA group or the ISA/CpG group. A ten-fold dilution of anti peptide sera could inhibit 50% of luciferase activity. Guinea pig antisera at 40-fold dilution could inhibit ~80% of luciferase activity (Fig. 1B), but the antisera from rabbits could inhibit 50% of luciferase activity at 10- to 20-fold dilution (Fig. 1C). These results indicated that antibody elicited by peptide-based immunization was able to block virus entry.

Identification of neutralizing epitopes

The neutralization effects of anti-peptides sera were strongly inhibited in the presence of D2 peptide, moderately inhibited in the presence of TM and mildly inhibited in the presence of D1 peptides (Fig. 2A). D2 peptide could disrupt the neutralizing effect of antisera by ~67%, indicat-

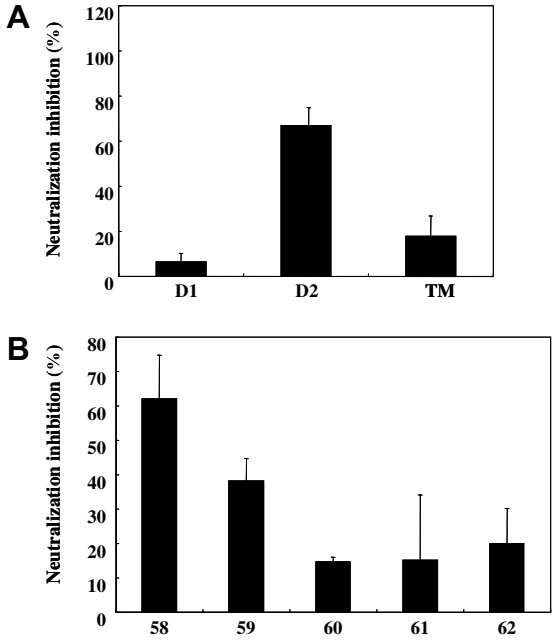

Fig. 2. Identification of neutralizing epitopes. Antisera from immunized guinea pigs were used to identify neutralizing epitopes. The 20-fold diluted serum was used as the standard neutralization. The 20-fold diluted serum was incubated with 8 μg of D1, D2, and TM peptide for 1 h at 37 °C before adding to the HepG2 cells with pseudovirus in a 12-well plates (A). The 15-mer overlapping peptide 58–62 was incubated with 20-fold diluted antiserum before adding to the Hep G2 cells with pseudovirus (B). The neutralization inhibition was calculated as: neutralization inhibition (%) = (units of luminescence with antiserum and peptide − units of luminescence with antiserum)/(units of luminescence without antiserum − units of luminescence with antiserum) × 100.

ing that the major neutralizing epitopes were most likely located on the D2 peptide region. In order to map the major neutralizing epitopes, we used 15-mer peptides 58–62 which corresponded to the D2 peptide. As shown in Fig. 2B, we found that peptide 58 and 59 could inhibit the neutralizing effect of antisera (60.5% and 52.3%, respectively). These results indicate that amino acid residue 434–453 contains major linear neutralizing epitopes.

Identification of immunodominant T-cell epitopes in BALB/c mice

T-cell responses of splenocytes were analyzed at 7 days after the last immunization. Two adjuvant formulations (CFA/IFA and ISA/CpG) were compared in BALB/c mice. Each individual peptide was tested using a [^{3}H]thymidine incorporation assay. There were no significant responses in the D1 and TM stimulation groups. In contrast, D2 peptide could significantly stimulate T-cell proliferation in the CFA/IFA and the ISA/CpG formulation (Fig. 3A). To identify T-cell epitopes located on the immunized peptides. Splenocytes from immunized mice were re-stimulated in vitro using the individual 15-mer peptides listed in Supplementary data 1. The significance of the proliferation was compared with the SI value of the respective peptide by *t*-test ($p < 0.05$). The results suggested the presence of one T-cell epitope (peptide 58) located at the amino acid residue 434–448 (Fig. 3B). Analysis of the supernatant of stimulated cells by IFN-γ ELISA yielded consistent results (Fig. 4A). The IFN-γ concentration in the ISA/CpG group was comparable to that of the CFA/IFA group (176 ± 32 vs. 192 ± 61).

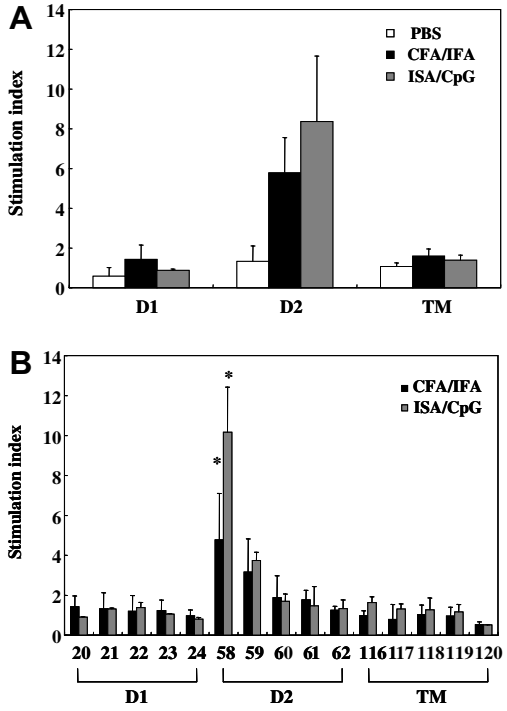

Fig. 3. T-cell proliferation generated by D1, D2, and TM peptides in BALB/c mice. (A) Splenocytes were cultured with 10 μg/ml of D1, D2, and TM for 3 days, then pulsed with [^{3}H]thymidine (1 μCi/well) for 16–18 h. The stimulation index was calculated as the mean counts per minute of the stimulated wells divided by the mean counts per minute of the control well. (B) A panel of 15-mer overlapping peptides that covered the entire sequence of immunized peptides was used to stimulate splenocytes. The significance of differences in the data between groups was analyzed by Student's *t*-test. $^*p \leqslant 0.05$

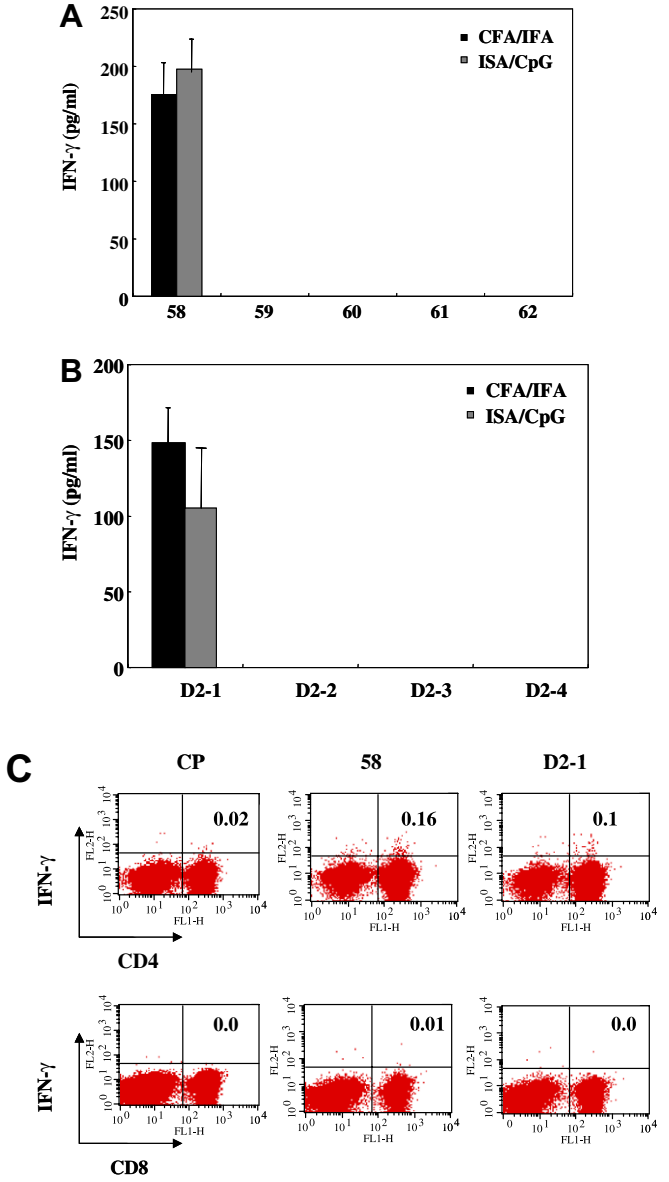

Fig. 4. CD4$^+$ and CD8$^+$ T-cells reactive peptide derived from mice immunized with D1, D2, and TM peptides. (A) Five 15-mer overlapping peptides that covered the entire sequence of D2 peptides were used to stimulate splenocytes. Splenocytes were cultured with 10 μg/ml of peptides for 3 days, and the supernatants were then harvested. The IFN-γ released in the supernatant was measured by ELISA. (B) Four 9-mer peptides from D2 peptides were used to stimulate splenocytes. Selected peptides are listed in Supplementary data 2. Splenocytes were cultured with 10 μg/ml of peptides for 3 days, and the supernatants were then harvested. The IFN-γ released in the supernatant was measured by ELISA. (C) The significance of differences in the data between groups was analyzed by Student's *t*-test.

Alternatively, the stimulation effect of the peptide could be due to the antigen processed to generate a $CD8^+$ reactive epitope. In order to determine the $CD8^+$ T-cell reactive epitopes, four 9-mer peptides on D2 were synthesized based on the website computer program SYFPEITHI (Supplementary data 2) [15]. These peptides were predicted to be recognized by the H-$2K^d$ MHC molecule. All four peptides were cultured with splenocytes, and the supernatants were then collected and analyzed by IFN-γ ELISA. The results showed that only peptide D2-1 could induce IFN-γ secretion (Fig. 4B). There was no significant difference in IFN-γ secretion between the CFA/IFA and ISA/CpG group (147 ± 25 vs. 105 ± 43). To further confirm that D2-1 was a MHC class I-restricted peptide, we used purified $CD4^+$ or $CD8^+$ T-cells to identify the responding T-cell subsets. The $CD4^+$ and $CD8^+$ T-cells were separated using magnetic beads, which yielded a purity of the enriched T-cell subsets of greater than 90% (data not shown). Mitomycin C-treated naïve splenocytes were added as antigen presenting cells. IFN-δδ-secreting T-cells were analyzed by flowcytometry. The results showed that only peptide 58 (0.16%) or D2-1 peptide (0.1%) could stimulate $CD4^+$ T-cells to secrete IFN-γ (Fig. 4C), indicating that 9-mer peptide D2-1 also stimulated proliferation of $CD4^+$ T-cells but not $CD8^+$ T-cells. The Th epitope is located at amino acid residue 434–448.

Discussion

SARS CoV spike protein plays very important functional roles during viral infection. Our approach to vaccine development in this study was to design peptides derived from different domains of the spike protein. This strategy is more efficient than using a single neutralizing epitope, since a peptide cocktail could potentially provide Th epitopes which enhance immune response. We used the peptide cocktail to immunize mice, guinea pigs and rabbits. In the mouse model we also used adjuvant ISA/CpG, which could potentially be used in a human vaccine, to study the immunogenicity of peptide vaccine candidates. In this study, we also identified a synthetic peptide D2 located at the receptor-binding region that contains both B cell and T-cell epitope in mice.

The D1 peptide (a.a. 175–205) is the highly hydrophilic region of the N-terminal domain of the SARS CoV spike protein. Unexpectedly, we found that this region has low antigenicity in mice, but moderate antigenicity in guinea pigs and rabbits. The D1 peptide may therefore not be a good candidate for vaccine development. The ACE2 receptor-binding domain has been identified on the_SARS CoV spike protein (a.a. 318–510) [16]. Furthermore, based on the crystal structure the minimum ACE2 receptor-binding motif on the spike protein has been suggested to be located at amino acid residue 424–494 [4]. The D2 peptide (434–467) is within the binding motif, and we found it to be both highly antigenic and immunogenic in mice, guinea pigs and rabbits. A neutralizing epitope located at amino acid residue 434–453 was identified using a pseudovirus entry assay. Furthermore, our previous collaboration with Shih et al. also found a monoclonal antibody which could block pseudovirus entry has a binding epitope located in the same region (a.a. 434–448) [14]. The importance of this region has been previously described by He et al. [17] [18] and Yi et al. [19]. These results indicate that D2 peptide could be a very good vaccine candidate.

In T-cell epitopes mapping, we used overlapping peptides to identify a reactive epitope on peptide 58 which can stimulate T-cell proliferation in [^{3}H]thymidine incorporation assay. We also found that peptide 58 can stimulate $CD4^+$ T-cells to secrete IFN-γ. These results demonstrated that amino acid residue 434–448 contains both neutralizing B cells and Th cell epitope. Further study of the B and T-cells response in HLA transgenic mice to investigate their potential for use in human vaccine is needed.

In order to facilitate their clinical applicability, the peptides synthesized in this study were formulated with two adjuvants (ISA-51 and CpG) with potential for use in humans. We found that the ISA/CpG formulation showed an adjuvant effect in B and T-cells response which was comparable with the IFA/CFA formulation (Fig. 3). These results indicated that the ISA/CpG formulation could be used as an adjuvant in developing peptide-based vaccines. Our previous study found that the N protein from SARS CoV formulated with ISA and CpG could enhance immunogenicity, and the immune response was biased towards Th1 immunity [20]. In this study, we did not observe a similar immune response when peptides were formulated with ISA and CpG (data not shown). This may have been due to the need for a different CpG dose than for the N protein for the peptide to bias toward immune response. Further optimization of the CpG dose formulation in these peptide-based vaccines is needed.

In conclusion, our results indicated that peptide cocktails could elicit efficient immune responses in mice, guinea pigs and rabbits. The increased antibody levels resulting from these immunizations could block pseudovirus entry. The preliminary information obtained with these peptide-based vaccines in this study suggested the possible neutralizing epitopes on the viral protein. It also could be possible to use the peptides synthesized in this study as drugs that mimic the viral protein interaction with the cell membrane. Further animal immunization study by including more neutralizing epitopes in our peptide cocktail to form a basis for SARS vaccine development in humans is needed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.164.

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