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Synthetic peptides derived from SARS coronavirus S protein with diagnostic and therapeutic potential

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Abstract The spike (S) protein of severe acute respiratory syndrome coronavirus (SARS-CoV) is an important viral structural protein. Based on bioinformatics analysis, 10 antigenic peptides derived from the S protein sequence were selected and synthesized. The antigenicity and immunoreactivity of all the peptides were tested in vivo and in vitro. Four peptides (P6, P8, P9 and P10) which contain B cell epitopes of the S protein were identified, and P8 peptide was confirmed in vivo to have a potential in serological diagnosis. By using a syncytia formation model, we tested the neutralization ability of all 10 peptides and their corresponding antibodies. It is interesting to find that P8 and P9 peptides inhibited syncytia formation, suggesting that the P8 and P9 spanning regions may provide a good target for anti-SARS-CoV drug design. Our data suggest that we have identified peptides derived from the S protein of SARS-CoV, which are useful for SARS treatment and diagnosis.

Keywords: Severe acute respiratory syndrome coronavirus; Spike protein; Synthetic peptide; Syncytia formation and disease prevention

1. Introduction

Severe acute respiratory syndrome (SARS) is a serious epidemic infectious disease that occurred from November 2002 to September 2003 and reappeared in parts of China in 2004. This devastating disease has a relatively high mortality [1].

The SARS-associated coronavirus, named SARS-CoV, is the pathogen of this disaster [2]. This new kind of coronavirus is an enveloped and positive-stranded RNA (+RNA) virus, with a single-stranded genome, which contains four structural proteins, spike (S) protein, membrane (M) protein, small envelope protein and nucleocapsid (N) protein [3].

The SARS-CoV spread quickly in China and many other countries. Since there is no effective method so far to prevent and treat this disease, an accurate diagnostic method and an effective clinical anti-viral drug or vaccine are urgently needed. The S protein of SARS-CoV is a good candidate for disease diagnosis and vaccine design.

The S protein (gi: 30173397) consists of 1255 amino acids and has several glycosylation sites. It is believed that the S protein has two domains, S1 and S2. S1 plays an important role in virus binding to virus-receptor (ACE2) while S2 mediates the fusion between the viral particle and its target cell [4,5]. According to our current work, we confirmed that S protein can be cleaved into two parts [5]. It is predicted that the S protein yields two fragments spanning residues from 1 to 667 (S1) and 668 to 1255 (S2). So 10 peptide sequences (with 18–30 residues in length) were selected from S protein (in which peptides P1-8 are located in the S1 part and peptides P9-10 are located in S2) based on a series of bioinformatics analysis [6,7].

The peptides then were used to immunize rabbits. The specificity of antisera induced by peptides was analyzed by Western blot with recombinant expressed S, S1 and S2 proteins as well as the lysates of SARS-CoV infected Vero E6 cells. SARS patients' sera were used to confirm the specific immunoreactivity of the selected peptides. The syncytia formation model was used to evaluate the neutralization ability of the peptides and their corresponding antibodies (Abs).

The data demonstrated that all 10 peptides showed high antigenicity and immunoreactivity. The antisera from peptides (P6, P8, P9, and P10) immunized rabbits showed good specificity and high affinity to S protein. It was confirmed that the P8 peptide is the best candidate for usage in SARS serological diagnosis. After some optimization, the peptide PL8 derived from P8, revealed a specific response to SARS specific IgG

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Abbreviations: SARS-CoV, severe acute respiratory syndrome-associated coronavirus; Abs, antibodies

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in SARS patients' sera, which gave a positive rate above 70% in all tested SARS patients.

Neutralization experiments using a syncytia formation model showed that the peptides P8 and P9 themselves can markedly inhibit syncytia formation, indicating that the corresponding regions spanning sequences (540–559) and (731–753) within the S protein may be new targets for efficient SARS drug development.

2. Materials and methods

2.1. Peptide design and synthesis

After analyzing the SARS spike protein (gi: 30173397) with software DNAstar, 10 peptides were selected from the whole sequence of the S protein based on their indices of antigenicity, hydrophilicity, surface probability, and flexible region. These peptides were synthesized using conventional solid-phase chemical methodology and purified in GL Bio-Chem (Shanghai) Ltd [8].

2.2. Immunization

Bovine serum albumin (BSA, Sigma) served as a carrier protein for conjugation with peptides. New Zealand white rabbits (Shanghai Laboratory Animal Center, Chinese Academy of Sciences) were immunized subcutaneously on the back of the rabbits with 200 μg of different BSA-conjugated peptides emulsified (1/1 v/v) with complete Freund's adjuvant (CFA). The same amount of peptides dissolved in incomplete Freund's adjuvant (IFA) was used to boost immunization in the rabbits on days 21 and 42 following immunization. The antisera were collected on day 49 following first immunization.

2.3. Prokaryotic S protein expression

The recombinant intact S protein, S1 and S2 fragments were expressed by *Escherichia coli* and they were purified by Model 422 Electro-Eluter (Bio-rad) and subjected to SDS–PAGE analysis [5].

2.4. Preparation of virus lysates

Vero E6 cells were infected with SARS-CoV for 24 h and collected as previously described [9]. Infected cells were lysed with a solution containing 40 mM Tris (pH 8.3) and 0.5% Nonident P-40 at 22 °C for 5 min. The virus lysate was centrifuged at 8000 rpm for 5 min, and then the supernatant was collected and boiled for 5 min. All experiments using virus were carried out in a biosafety level 3 laboratory.

2.5. Western blot

The expressed whole length S protein, S1 and S2 fragments as well as the native S proteins extracted from the lysate of SARS-CoV infected Vero E6 cells were used to detect their binding activities with peptide-induced antisera. Binding Abs were then detected by the second Ab (HRP-conjugated goat anti-rabbit IgG). ECL-detection reagents (Amersham Pharmacia Biotech) were used to develop the films.

2.6. Serum samples

58 sera of SARS patients and 40 sera of healthy individuals were used. All of the experiments using both of these sera were carried out in a biosafety level 3 laboratory. All the confirmed SARS patients' sera were IgG positive when analyzed by ELISA using whole virus lysates.

2.7. ELISA procedure

The reactivity of the peptides to the SARS patients' sera was also measured by ELISA. The 96-well plates were coated with the BSA–peptide conjugates(5 μg/ml, 50 μl/well) in coating buffer (pH 9.4) at 4 °C overnight. Afterwards the wells were washed with PBS, and were blocked with BSA at 37 °C for 2 h and then the sera or the purified IgG from SARS patients' sera was added as the first Ab. The sera dilution was 1:10 and the purified IgG dilution was 1:1000. Plates were stored at 37 °C for 30 min, then washed again with PBS. After that, HRP-coupled goat anti-human IgG (Bio-rad) was added, and OD values were measured with a microplate autoreader (Biotek) at 450 nm.

2.8. Syncytia inhibition assay

Syncytia formation was used to mimic the SARS-CoV infection procedure [10]. In our study, we modified this assay by introducing luciferase assay in the detecting system (Fig. 5A and B).

The cell fusion-dependent reporter gene (luciferase) activation assay was adapted to our studies of spike protein-mediated membrane fusion. In brief, effector cells were generated by cotransfection of HEK293 T cell monolayers (10^6 cells/60 mm dish) with plasmids pCDNA3.1-ACE2 and pUHD 15-1(SV40), while target cells (containing the luciferase reporter gene) were generated by cotransfection of HEK293 T cell with pCDNA3.1-spike-Ig and pUHD 10-3. Twenty-four hours after transfection, effector cells and target cells were trypsinized, resuspended in DMEM–10% FBS, then two kinds of cell suspensions were mixed (1:1 cell number) and repelleted.

All the antisera and peptides (20 μl of each antiserum and 125 μM of each peptide) were added into the cell suspension mixture to investigate their inhibitory capacity. Antiserum or peptide was incubated with cell suspensions at 23 °C for 1 h, and then re-plated into a 48-well culture plate at 37 °C for 24 h. Finally, media were removed and cells were lysed with 100 μl Passive Lysis Buffer (Promega). Luciferase intensity was measured in a luminometer TD-20/20.

3. Results

3.1. Bioinformatics analysis and peptide selection

We predicted that the cleavage site of the SARS-CoV spike protein containing 1255 residues is located between amino acid residues 667 and 668 [5]. The fragments of 1–667 and 668–1255 represent S1 and S2, respectively. All peptides are designated according to four characteristics: antigenicity, surface probability, hydrophilicity and flexible region. The peptides P1 to P8 were derived from the S1 fragment and the peptides P9 and P10 were from the S2 fragment (Fig. 1 and Table 1).

3.2. Antigenicities of synthesized peptides were evaluated by Western blot

In order to test the antigenicity of these synthetic peptides to immunize rabbits, all of them were coupled with BSA. The antisera with high titers were collected on day 49 following immunization. The binding activity and specificity of Abs were determined by Western blot with *E. coli* expressed S1 fragment, S2 fragment and full-length S proteins as well as the native S protein isolated from the lysates of SARS-CoV infected Vero-E6 cells. The data demonstrated that anti-P3, P5, P7 antisera bound to S1 and S2 fragments. They also showed weak and non-specific binding activities to the lysates of virus

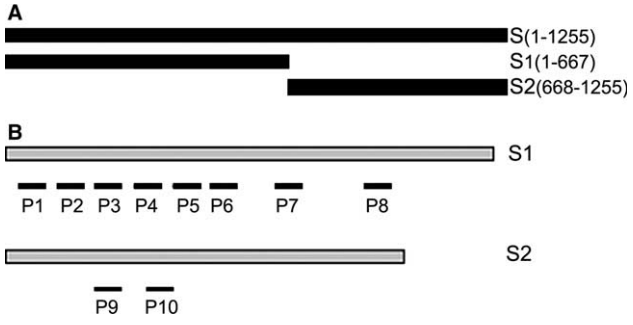

Fig. 1. Map of peptides used for immunization. The spike protein is predicted to be cleaved at amino acid 667 and 668 according to bioinformatics analysis. (A) Full-length S protein, S1 and S2 fragments were expressed by *E. coli* and served as target proteins for Western blot. (B) Eight peptide sequences were selected from the S1 fragment and two peptide sequences were selected from the S2 fragment.