Immunogenicity difference between the SARS coronavirus and the bat SARS-like coronavirus spike (S) proteins

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ABSTRACT

SARS-like coronavirus (SL-CoV) in bats have a similar genomic organization to the human SARS-CoV. Their cognate gene products are highly conserved with the exception of the N-terminal region of the S proteins, which have only 63–64% sequence identity. The N-terminal region of coronavirus S protein is responsible for virus–receptor interaction. In this study, the immunogenicity of the SL-CoV S protein (S_{SL}) was studied and compared with that of SARS-CoV (S_{SARS}). DNA immunization in mice with S_{SL} elicited a high titer of antibodies against HIV-pseudotyped S_{SL}. The sera had low cross-reactivity, but no neutralization activity, for the HIV-pseudotyped S_{SARS}. Studies using wild bat sera revealed that it is highly likely that the immunodominant epitopes overlap with the major neutralizing sites of the SL-CoV S protein. These results demonstrated that SL-CoV and SARS-CoV shared only a limited number of immunogenic epitopes in their S proteins and the major neutralization epitopes are substantially different. This work provides useful information for future development of differential serologic diagnosis and vaccines for coronaviruses with different S protein sequences.

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Searching for the origin of the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) in wild animals led to the discovery of several SARS-like CoVs (SL-CoVs) in several horseshoe bats of the genus *Rhinolophus* in China [1,2]. Our previous studies showed that the SL-CoV and SARS-CoV shared similar genomic organization and highly conserved gene products, with the exception of the spike protein (S protein), which had a low sequence identity, especially in the receptor binding domain (RBD) located at the N-terminal region of the S proteins. The difference of sequence in this region was responsible for the failure of the SL-CoV S protein (S_{SL}) to use angiotension converting enzyme 2 (ACE2), the known major receptor for SARS-CoV, as a functional receptor [3].

The SARS-CoV S protein (S_{SARS}) is responsible for virus entry and induction of neutralizing antibodies, mediated mainly by the RBD at aa 318–510 [4,5]. It has been demonstrated previously that bat sera from natural infection cross-reacted with, but failed to neutralize SARS-CoV [1]. Therefore, for the development of differential diagnosis and specific vaccines for these viruses, it is necessary and important to have a better understanding of the immunogenicity of S_{SL}, S_{SARS} and the difference between S_{SL} and S_{SARS}.

In this study, the immunogenicity and immunodominant region of S_{SL} was determined using sera generated from DNA immunization and naturally infected bats. The data presented here will be useful for future development of diagnostics and vaccines for SARS and SARS-like coronaviruses.

Material and methods

Cells. The human cell lines 293T and HeLa were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heated-inactivated fetal calf serum (Gibco, USA or Sijiqi, China). HeLa cell lines that stably expressing human ACE2 protein have been described in a previous report [6].

Preparation of pseudoviruses. The construction of a codon-optimized full-length S gene of SARS-CoV BJ01, bat SL-CoV Rp3, and chimeric S gene (designated $CS_{310–518}$), which has the Rp3-S gene containing aa 310–518 of BJ01 S in replacement of its corresponding region, has been described previously [3]. Briefly, 12 µg each of pHIV-Luc (pNL.4.3.Luc.E⁻R⁻) and plasmid pcDNA3.1 containing various S genes (or empty vector control) were co-transfected into 2×10^6 293T cells in 10 cm dishes by standard calcium phosphate method [6]. The pseudoviruses were purified by ultracentrifugation from cell culture supernatant through a 20% sucrose cushion (10 mL) at 55,000g for 60 min using a Ty70 rotor (Beckman). The pelleted pseudoviruses were dissolved in 100 µL of PBS and stored at −80 °C in aliquots until further use.

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Antisera. Six field bat sera showing positive (5 of 6) or negative (1 of 6) cross-reactivity with SARS-CoV [1] were used in this study. Hyperimmune mouse sera were generated by DNA immunizations with plasmids pcDNA 3.1(+) containing the codon-optimized full-length S gene of SARS-CoV BJ01, SL-CoV Rp3, and two chimeric plasmids $CS_{310-518}$ and $CS_{259-518}$ (see Fig. 1 for diagrams). The pcDNA 3.1(+) vector was used as a negative control. Five groups (five mice per group) of 6–8 week old female BALB/c mice were immunized with 30 μg of plasmid DNA in 30 μL PBS by *in vivo* electroporation according to the published method [7]. Mice were immunized three times at weeks 0, 3 and 5. Sera were collected at week 8. After injection into the right quadriceps muscle, a pair of electrode needles with 5 mm apart was inserted into the muscle to cover the DNA injection sites and electric pulses were delivered using an electric pulse generator (Electro Square Porator T830 M; BTX, San Diego, CA). Three pulses of 100 V each, followed by three pulses of the opposite polarity, were delivered to each injection site at a rate of one pulse per second. Each pulse lasted for 50 ms.

ELISA. All ELISAs were performed under stringent conditions to avoid nonspecific reactions. Briefly, 96-well microtiter plates were coated in 0.1 M carbonate buffer (pH 9.3) over night at 4 °C with purified pseudoviruses (50–200 ng/well). The plates were washed and blocked with 5% BSA in PBS-0.1% Tween 20, and then incubated with bat or mouse sera for 1 h at 37 °C. Bound antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse IgG (LingFei Tech.) or goat anti-bat IgG (Bethyl Laboratories, Inc.) (Dilution at 1:4000). Color development was conducted using 3,3,5,5,tetramethylbenzidine (TMB) and the absorbance at 450 nm was determined after the reaction was stopped with 2 M H_2SO_4. All washes were carried out using PBS-0.1% Tween for 5 washes (2 min/wash), and all antibodies were diluted using 0.5% BSA in PBS-0.1% Tween. Appropriate negative controls were included in every step.

Neutralization assays. A pseudovirus-based neutralization assay was used to determine the neutralization ability of immunized sera to pseudovirus HIV/BJ01-S, and HIV/$CS_{318-510}$. The neutralizing activity of heat-inactivated sera (56 °C, 30 min) was determined by mixing 10 ng of pseudovirus (in 30 μL) with diluted antisera (in 30 μL) at 37 °C for 1 h. Sera–pseudovirus complexes were then mixed with 16 ng polybrene (in 40 μL medium) before they were added to human ACE2 expressing HeLa cells. The infected cells were washed with PBS and lysed (Cell Culture Lysis Reagent; Promega) at 48 h post infection. The neutralization activity of each antiserum was monitored by measurement of luciferase activity.

Results and discussion

After the final boost, end-point dilution ELISA was used to detect the antibody response against BJ01 or Rp3-S protein in immunized
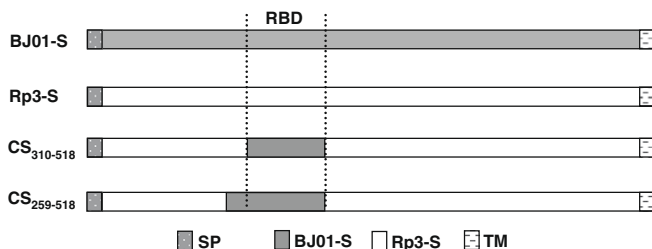
Fig. 1. Schematic diagram of DNA constructs for expression in HIV pseudoviruses. For the two chimeric S genes, the numbers given in subscripts indicate the aa location of the BJ01-S sequence used to replace the corresponding region in the Rp3-S gene (Ren et al. [3]). *Abbreviations used:* RBD, receptor binding domain; SP, signal peptide derived from tissue plasminogen activator; TM, transmembrane domain derived from the fusion protein of Sendai virus.

mice (Fig. 2). Other than the negative control, all immunized mice produced antibodies against HIV/Rp3-S or HIV/BJ01-S pseudovirus, albeit displaying different reactivity against the two pseudovirus antigens. When tested against HIV/Rp3-S pseudovirus, antisera generated to the full-length Rp3-S had a significantly higher titer than those from the other three groups (Fig. 2A). Also, the titers of sera from the two chimeric plasmids $CS_{310-518}$ and $CS_{259-518}$ were higher than that from the BJ01-S construct. On the other hand, when tested against HIV/BJ01-S pseudovirus, the antibody titer was lowest for the group immunized with plasmid expressing the full-length Rp3-S protein (Fig. 2B). There was little difference in titer among the other three groups.

All mouse sera generated using DNA immunization were tested for their ability to neutralize the HIV/BJ01-S or HIV/$CS_{318-510}$ pseudovirus. As shown in Fig. 3A, mouse sera from BJ01-S, $CS_{310-518}$ or $CS_{259-518}$ showed strong neutralizing activity to the pseudovirus HIV/BJ01-S, whereas the antiserum from Rp3-S showed a much weaker neutralizing activity to the same pseudovirus. Interestingly, all the sera, except the vector control group, had neutralizing activity to HIV/$CS_{318-510}$ (Fig. 3B). However, the Rp3-S serum showed neutralizing activity to HIV/$CS_{318-510}$ only at high concentrations, indicating the presence of some cross-neutralization epitopes located outside the BJ01-S RBD.

Similar to the results obtained above using mice sera, bat sera naturally infected by SL-CoV had a much stronger reactivity towards HIV/Rp3-S than the other three pseudoviruses (Fig. 4).

Knowledge of immunogenicity and immunodominant regions of major viral antigens is important for rational design of effective vaccines and diagnostic tests. SL-CoVs found in bats are very similar to human isolates of SARS-CoV in that they have almost identical genomic organization and their gene products share a high level of amino acid sequence identity. We have shown previously
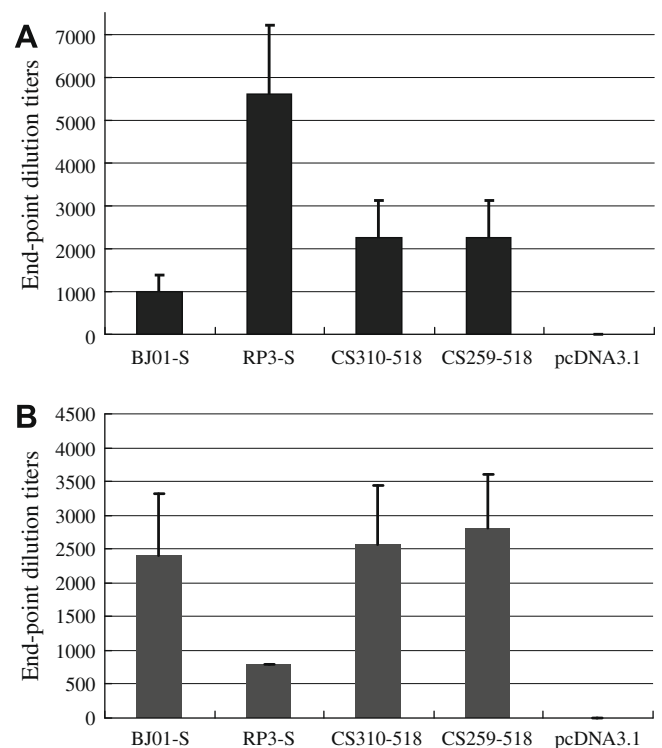
Fig. 2. Determination of anti-S antibody titers in mouse sera by ELISA. Antigens used were pseudoviruses HIV/Rp3-S (A) or HIV/BJ01-S (B), respectively. Sera from mice immunized with plasmids expressing BJ01-S, Rp3-S, and chimeric S ($CS_{310-518}$, $CS_{259-518}$) or empty plasmid pcDNA3.1 (negative control) were used. Error bars represent the standard deviation of the mean.
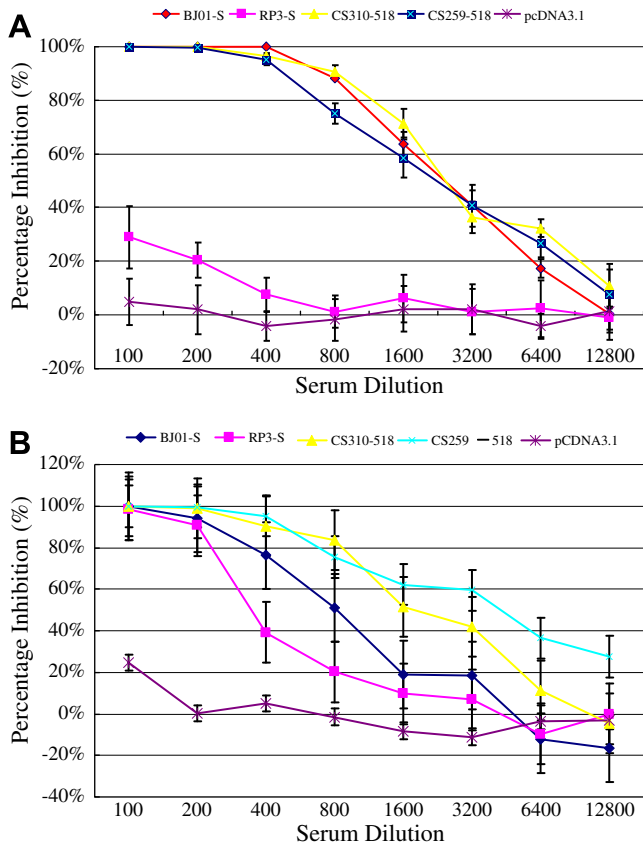
Fig. 3. Neutralization of pseudovirus HIV/BJ01-S or HIV/CS$_{318-510}$ by different mouse sera. Sera were diluted from 1:100 to 1:12800 and measured for the ability to neutralize HIV/BJ01-S (A) or HIV/CS$_{318-510}$ (B) (represented as percentage of inhibition). The mean percentage inhibition was plotted with the error bars indicating the standard deviation of the mean.
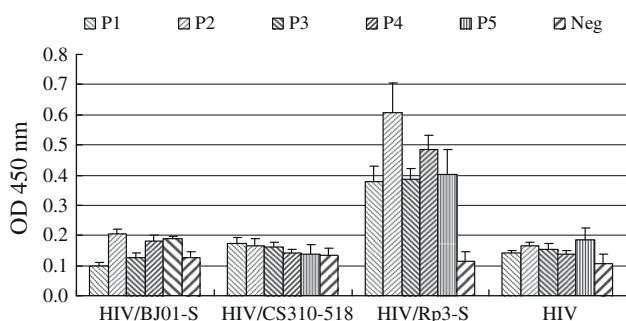

Fig. 4. Detection of anti-S antibodies in bat by ELISA. (A) Three S-expressing pseudoviruses (as indicated) and the negative control (HIV backbone alone) were used as ELISA antigens. Specific reactivity of five positive (P1–P5) and one negative bat sera (Neg) was determined against SARS-CoV in a previous study [1]. Data are presented as means ± SDs.

that despite substantial antigenic cross-reactivity between bat sera from naturally infected animals and SARS-CoV, there was no cross-neutralization detected [1]. This discrepancy could be explained by the low level of sequence identity shared at the N-terminal domain of the S proteins between the two classes of viruses [1,2]. The N-terminal region of coronavirus S proteins is known to be responsible for virus attachment to susceptible host cells, hence a major target of neutralizing antibodies [4].

In this study, DNA constructs expressing four different S proteins, SARS-CoV BJ01, SL-CoV Rp3 S and two chimeras, were used to generate hyperimmune sera in mice via DNA immunization.

The immunogenicity of these proteins was determined using ELISA against the S$_{SL}$ and S$_{SARS}$ proteins expressed in the form of HIV pseudoviruses. The neutralization activity of the mouse sera was further determined using the HIV/BJ01-S and HIV/CS$_{318-510}$ pseudovirus. The results obtained clearly demonstrated that mice immunized with BJ01-S, CS$_{310-518}$ or CS$_{259-518}$ generated antibodies with higher antigenic and neutralizing activity to HIV/BJ01-S and HIV/CS$_{318-510}$ than HIV/Rp3-S. Vice versa, mice immunized with Rp3-S displayed higher reactivity to HIV/Rp3-S than HIV/BJ01-S. These results demonstrated that SL-CoV and SARS-CoV shared only a limited number of immunogenic epitopes in their S proteins and the major neutralization epitopes are substantially different among these two viruses.

Since the RBD for the S$_{SL}$ protein is not known, we were not able to conclude whether the immunodominant regions overlap with the receptor attachment site. For the same reason, we have been unable to identify a suitable susceptible cell line to conduct neutralization tests for the HIV/Rp3-S pseudovirus. Until such an assay is available, it will be impossible to conduct a detailed comparative study of major neutralization epitopes between the two different viruses.

The study conducted with bat sera and four different pseudoviruses provided indirect evidence suggesting a substantial overlap of immunodominant and neutralizing epitopes for the S$_{SL}$ protein. This was best demonstrated by the reactivity of bat sera against pseudovirus HIV/Rp3-S and HIV/CS$_{310-518}$, respectively. The replacement of the sequence from aa 310–518 alone almost completely abolished the bat antibody reactivity towards the S$_{SL}$ protein.

A variety of SL-CoV and other CoVs found in bats demonstrate that bats are natural reservoir of diverse CoVs. The high density of bats in habitats provides ample opportunities for recombination, which will in turn increase virus diversity and the chance of spill over into other hosts including humans leading to zoonotic disease outbreaks [3,9]. Recently, it was shown that a synthetic SL-CoV containing a very small fragment of the SARS-CoV S gene was able to infect and cause disease in mice, further highlighting the potential emergence of novel viruses with subtle sequence difference in the S gene [10].

The results obtained from this work would suggest that the current diagnostic tools and candidate vaccines developed for SARS-CoV are not likely to be specific or effective enough to combat a disease outbreak caused by a SL-CoV variant. It is therefore necessary that we continue strategic research in this area to be able to rapidly response to disease outbreaks caused by coronaviruses with similar genetic and pathogenic features, but with different S gene sequences and receptor specificities.

Acknowledgments

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