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Brief Report

Protection from Infection with Severe Acute Respiratory Syndrome Coronavirus in a Chinese Hamster Model by Equine Neutralizing $F(ab')_2$

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ABSTRACT

To warrant potential clinical testing, the equine anti-severe acute respiratory syndrome coronavirus (SARS-CoV) $F(ab')_2$ requires evaluation in as many animal models as possible. In this study, we established a new animal model, the Chinese hamster, susceptible to SARS-CoV infection. SARS-CoV could propagate effectively and sustain high levels for 1 wk in animal lungs. All animals were protected from SARS-CoV infection in preventive settings. Further, when used therapeutically this antibody led to an approximately 4-$\log_{10}$ decrease in viral burden in infected animal lungs. The pathological changes in lungs correlated closely with the dose of antibody administered. The excellent preventive and therapeutic roles of equine anti-SARS-CoV $F(ab')_2$ in several animal models, including the novel Chinese hamster model described in this study, have provided exciting data concerning its potential clinical study.

INTRODUCTION

THE PREVENTION AND TREATMENT of severe acute respiratory syndrome (SARS) includes several strategies, including vaccines currently under development (1–4), antiviral drugs, and passive transfer of antibodies. Some antiviral agents such as interferons, ribavirin, and human immunodeficiency virus (HIV) protease inhibitors have already shown promising results (5–7), although they were usually used empirically during the 2002–2003 SARS outbreak.

Passive immunity has long been used in the prevention and treatment of infectious diseases (8). The practice of administering polyclonal immunoglobulins from hyperimmune sera of animal or human origin has a century-old history of being effective against some viruses (9–13), pro-

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viding another candidate strategy for protection against SARS-CoV infection. Soo and colleagues found that infusion of convalescent plasma demonstrated beneficial clinical outcomes in SARS patients (14). Subbarao *et al*. verified that passive transfer of SARS-CoV-specific antisera reduces pulmonary viral titers in mice infected with SARS-CoV (15), indicating that hyperimmune sera against SARS-CoV could protect against this viral infection.

Equine antiserum has been applied as an antiviral regimen to control rabies (16), hepatitis B virus (HBV) (11,13), and HIV (9,12) infections. We have generated equine anti-SARS-CoV $F(ab')_2$ fragments, which were shown to neutralize effectively SARS-CoV *in vitro* and *in vivo* in a BALB/c mouse model (17). However, before any possible clinical applications, this antibody must be tested rigorously in as many animal models as possible to ensure its efficacy and safety.

To date, several animal models of SARS-CoV infection have been established, including mouse, ferret, golden Syrian hamster, rabbit, and monkey (1,3,4,18–20). However, the search for suitable animal models is still ongoing. Good models are needed to provide accurate evaluation of intervention strategies. In some cases, when efficacy evaluations cannot be conducted in humans, two or more animal models may be needed for licensure of a vaccine. Also, more animal models will help us to better understand the natural hosts of SARS-CoV and potential chains of transmission that occur in nature, which will enable us to take measures to intervene in this transmission pathway when necessary.

This study was designed to establish a Chinese hamster model of SARS-CoV and investigate the efficiency of the equine anti-SARS-CoV against this viral infection in this model.

MATERIALS AND METHODS

Virus and animals

SARS-CoV (strain BJ-01; GenBank accession number AY278488) was maintained in the Institute of Microbiology and Epidemiology (Academy of Military Medical Sciences, Beijing, China). The viral titer was 1.13×10^7 $TCID_{50}$ (50% tissue culture infective doses)/mL. All operations with SARS-CoV were performed in a bio-safety level 3 (BSL-3) laboratory.

All animals in this study were provided by the Animal Center of the Academy of Military Medical Sciences (Beijing, China). Six-week-old female Chinese hamsters, weighing 18 ± 2 g each, were housed four hamsters per cage. Approval for animal experiments was obtained from the institutional animal welfare committee.

To evaluate the susceptibility of Chinese hamsters to SARS-CoV infection, after light anesthetization with isoflurane, a 100-μL SARS-CoV particle suspension (1×10^4 $TCID_{50}$) was administered intranasally to the animals on day 0. Animals from each group were killed on days 1, 3, 5, 7, and 9 postinfection (p.i.). The lungs of experimental animals were removed and homogenized in a 10% (w/v) suspension of Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA). Viral titers and copies in the homogenates were then determined on the basis of cytopathic effect (CPE) and TaqMan real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays. The pathology and the location of SARS-CoV in the lungs of infected animals were determined by pathological observation and immunohistochemistry (IHC).

To investigate the preventive role of the antibody against SARS-CoV infection, animals were injected intraperitoneally with anti-SARS-CoV $F(ab')_2$ (2.5, 5, or 10 mg/kg body weight) or nonimmunized normal horse antibody (50 mg/kg body weight), as a negative control, on day -1, the day before viral infection. Twenty-four hours later (day 0), the Chinese hamsters were challenged intranasally with 1×10^4 $TCID_{50}$ of SARS-CoV, and were killed 2 d later (day 3 p.i.). The viral titer, copy and location, as well as the pathologic changes in the infected animal lung were then determined on the basis of CPE, qRT-PCR, IHC, and pathological observation.

To evaluate the therapeutic role of antibody, the animals received intraperitoneally 100 μL of SARS-CoV (1×10^4 $TCID_{50}$) on day 0, followed by intraperitoneal injection of the $F(ab')_2$ at one of the four doses (12.5, 25, 50, and 100 mg/kg body weight) or normal horse antibody (50 mg/kg) lacking neutralizing activity as a negative control on day 1 p.i. The viral titer, copy and localization, as well as the pathologic changes in the infected animal lung were determined on days 3 and 4 p.i., respectively.

Cytopathic effect and qRT-PCR assay

The real-time quantitative TaqMan PCR and CPE assays were conducted as described previously (17).

Histopathology and immunohistochemistry

Routine histology and the IHC assay were done as described by Subbarao *et al*. (15). For IHC, purified equine anti-SARS-CoV IgG (1:10,000) was used as the primary antibody.

Statistical analysis

Statistical analyses were performed by one-way analysis of variance (ANOVA) or/and multiple comparison (Scheffé) and Student *t* test. All graphs represent means $\pm$ SEM.

RESULTS

A new animal model for SARS-CoV infection

On the first day postinfection, only a small part of each animal lung was infected and no significant pathological changes could be observed (Fig. 1C and D), in contrast to normal Chinese hamster lung tissue (Fig. 1A and B). On day 3 p.i., overt mononuclear inflammatory cell infiltrates around the bronchioles and small vessels (arrow in Fig. 1E) and large collections of mixed inflammatory cells and necrotic debris of bronchiole epithelial cells appeared in the bronchiole passages (arrowhead in Fig. 1E). In the meantime, the alveolar pneumocytes identified by morphology showed extensive viral infection (Fig. 1F). On day 5 p.i., intraalveolar edema (arrows in Fig. 1G and H) and shedding of bronchiole epithelium cells into the bronchial lumen could also be observed (arrowhead in Fig. 1G). Many alveolar cells could be stained with anti-SARS-CoV antibody (Fig. 1H). On day 7 p.i., besides inflammatory cell infiltrations, focal consolidations of the lung were apparent, accompanied by focal necrosis (Fig. 1I and J). SARS-CoV located mainly in the local lung tissue undergoing consolidation (Fig. 1J). On day 9 p.i., progressive lung consolidation was observed all over the animal lung tissue (Fig. 1K and L) with much foam in the consolidation region (arrow in Fig. 1K), whereas the amount of virus decreased and in some animals the virus was undetectable by IHC (Fig. 1L).

CPE assays showed that viral titers increased rapidly on day 1 p.i. and peaked on day 3 p.i. (Fig. 2A). High viral titers persisted until day 7 p.i. The virus could be detected even on day 9 p.i., but the titer was remarkably decreased (Fig. 2A). These results matched the IHC results (Fig. 1), which showed that virus was most abundant before day 7 p.i. (Fig. 1). Concordantly, the qRT-PCR results showed that the virus amounted to about 10^{10} copies/g lung tissue during days 3 to 5 p.i. and sustained itself at 10^7 copies/g lung tissue until day 7 p.i. (Fig. 2B). The virus could still be detected as late as

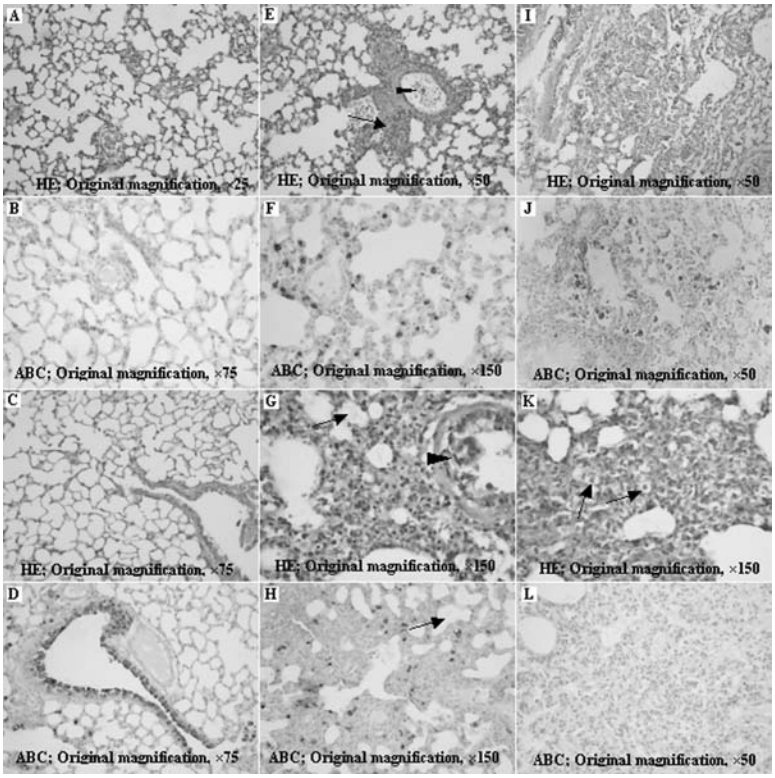

FIG. 1. Histopathological observation and IHC of the lung tissue of Chinese hamsters after SARS-CoV infection. Chinese hamsters were administered 1×10^4 $TCID_{50}$ of SARS-CoV on day 0. Animal lungs were fixed with formalin and then stained with hematoxylin–eosin (H&E) for pathological observation, or examined by IHC using anti-SARS-CoV F(ab′)$_2$ as the primary antibody (1:10,000) and DAB as chromogenic substrate. Lung tissues from normal hamster (**A**) and from the study animals on days 1 (**C**), 3 (**E**), 5 (**G**), 7 (**I**), and 9 (**K**) p.i. were examined by H&E staining. The distribution of SARS-CoV in affected animal lungs was determined by IHC in normal animal lung (**B**) and in animals inoculated with the virus on days 1 (**D**), 3 (**F**), 5 (**H**), 7 (**J**), and 9 (**L**) p.i. Images shown are representative of the four animals per group. For details, see text.

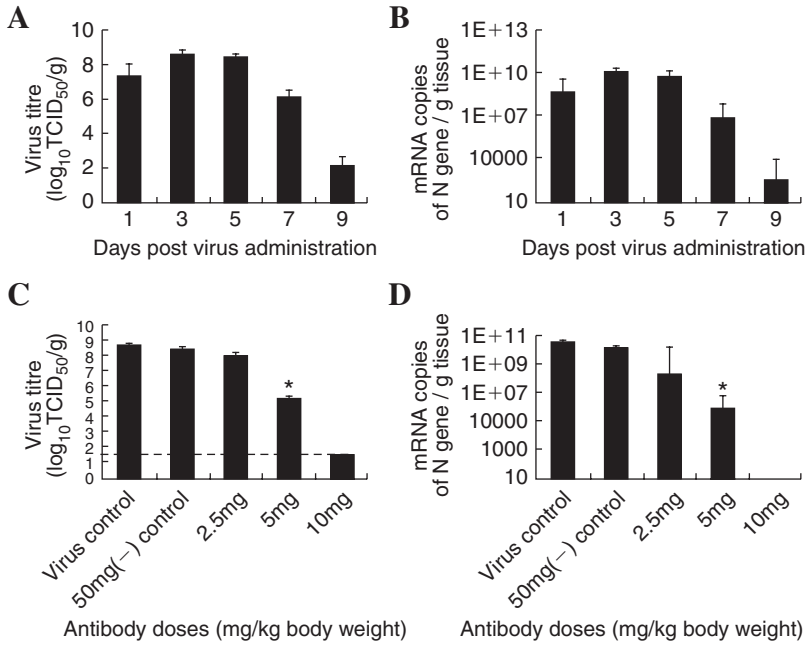

FIG. 2. Preventive role of equine anti-SARS $F(ab')_2$ fragments *in vivo*. (**A** and **B**) Replication of SARS-CoV in lung tissue of Chinese hamster model. Chinese hamsters were administered 1×10^4 $TCID_{50}$ of SARS-CoV on day 0. Viral titers and copies in lung homogenates were measured on the indicated days postinfection and are shown as mean values calculated from four hamsters per day. (**A**) CPE assays of viral titers in Chinese hamster lungs. (**B**) TaqMan real-time RT-PCR assays of viral copies in infected Chinese hamster lungs. (**C** and **D**) Prevention of SARS-CoV infection in Chinese hamsters. The indicated amounts of equine anti-SARS-CoV $F(ab')_2$ fragments were injected intraperitoneally in Chinese hamsters on day –1 (the day before inoculation of the virus) and the animals were administered 1×10^4 $TCID_{50}$ of SARS-CoV intranasally on day 0. Viral titers and copies in lung homogenates were measured on day 3 p.i. and are shown as mean values calculated from four hamsters per group. (**C**) CPE assays of viral titers in Chinese hamster lungs. (**D**) TaqMan real-time RT-PCR assays of viral copies in experimental animal lungs. Antibody-negative control hamsters received the indicated amount of nonimmune equine antibody. Virus control animals did not receive any antibody. Error bars indicate standard errors. Viral titers are expressed as $\log_{10}$ $TCID_{50}$ per gram of lung tissue. The lower limit of detection of virus in a 10% (w/v) suspension of lung homogenate is 1.5 $\log_{10}$ $TCID_{50}$ per gram (dotted line). Copies of N gene derived from SARS-CoV are expressed as absolute copies of N gene per gram of lung tissue. $^*p < 0.01$, compared with nonimmune equine antibody control (50 mg/kg).

day 9 p.i. in the lungs of some infected animals (two of four).

Protective effects of equine anti-SARS-CoV $F(ab')_2$ in inhibiting SARS-CoV infection in Chinese hamsters

Because viral titers in experimental animal lungs peaked during days 3–5 p.i., we investigated the preventive roles of anti-SARS-CoV $F(ab')_2$ on day 3 p.i. CPE assays showed that 10 mg/kg body weight of this antibody could completely neutralize the inoculated virus (Fig. 2C). Even half of this dose (5 mg/kg) could also provide about 50% protection, whereas the negative control could not provide any protection against SARS-CoV infection (Fig. 2C). Accordingly, qRT-PCR confirmed that anti-SARS-CoV $F(ab')_2$ at 10 mg/kg completely prevents viral infection of animal lungs, because N gene copies of SARS-CoV were undetectable in the lungs on day 3 p.i. (Fig. 2D). The pathological and IHC assays matched the CPE and qRT-PCR data. No virus and pathological changes in animal lungs that received anti-SARS-CoV $F(ab')_2$ at 10 mg/kg were observed, but mild to moderate pathological changes and a moderate amount of virus in the lungs of animals that received anti-SARS-CoV $F(ab')_2$ at 5 mg/kg could be detected (data not shown).

In the therapeutic settings, because the half-life of the $F(ab')_2$ fragments is about 60 h, we measured the therapeutic roles of the antibody at days 3 and 4 p.i., respectively. CPE assays indicated that $F(ab')_2$ at 50 mg/kg could decrease the viral load in animals by about 4 $\log_{10}$, compared with controls ($p < 0.01$; Fig. 3A). However, the 100-mg/kg dose of $F(ab')_2$ could not decrease the virus titers further (Fig. 3A). Results also showed that the therapeutic efficiency of the antibody on day 4 was sim-

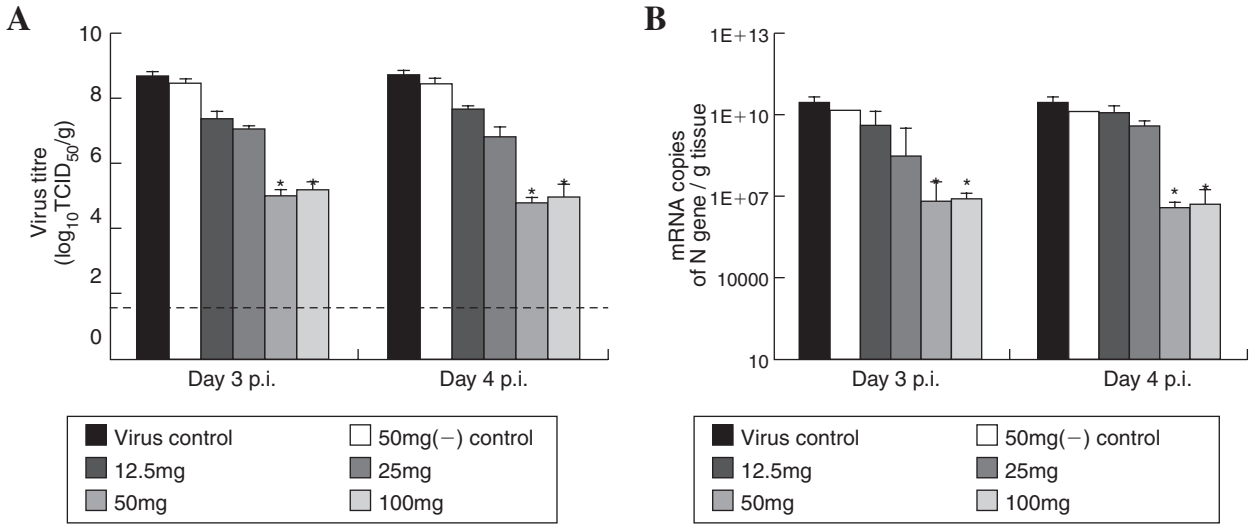

FIG. 3. Therapeutic role of equine anti-SARS-CoV F(ab′)$_2$ *in vivo*. (**A** and **B**) Therapeutic effect of equine anti-SARS-CoV F(ab′)$_2$ on SARS-CoV infection in Chinese hamsters. Animals were administered 1×10^4 TCID$_{50}$ of SARS-CoV on day 0 intranasally and protected by intraperitoneal injection of the indicated amount of anti-SARS-CoV F(ab′)$_2$ fragments on day 1 p.i. Viral titers and copies in lung homogenates were measured on the indicated days and are shown as mean values calculated from four hamsters per group. (**A**) CPE assays of viral titers in Chinese hamster lungs. (**B**) TaqMan real-time RT-PCR assays of viral copies in experimental animal lungs. Antibody-negative control hamsters received the indicated amount of nonimmune equine antibody. Virus control animals did not receive any antibody. Error bars indicate standard errors. Viral titers are expressed as $\log_{10}$ TCID$_{50}$ per gram of lung tissue. The lower limit of detection of virus in a 10% (w/v) suspension of lung homogenate is 1.5 $\log_{10}$ TCID$_{50}$ per gram (dotted line). Copies of N gene derived from SARS-CoV are expressed as absolute copies of N gene per gram of lung tissue. $*p < 0.01$, compared with nonimmune equine antibody control (50 mg/kg).

ilar to that on day 3 (Fig. 3A). qRT-PCR confirmed these results (Fig. 3B). The pathological observations and IHC assay showed that a saturated dose (50 mg/kg, as shown in Fig. 3A and B) of anti-SARS-CoV F(ab′)$_2$ could considerably relieve the pathological lesions of the animal lungs (data not shown).

DISCUSSION

We have previously shown that equine anti-SARS-CoV F(ab′)$_2$ prevents infection in cultured Vero E6 cells and in a BALB/c mouse model (17). Furthermore, the antibody can provide therapeutic protection to SARS-CoV-infected Vero E6 cells (17). However, before this antibody can be used in clinical studies, it requires evaluation in as many animal models as possible. In this study, we established a new animal model susceptible to SARS-CoV. We succeeded in infecting the Chinese hamster, which is readily obtained in China, with SARS-CoV and found that this virus could replicate effectively in animal lungs.

Importantly, although clinical manifestations were not observed, the pathological changes in the lungs of infected animals were definitive. At the early stage (day 1 p.i.), a little lung tissue was infected, but without overt inflammation. At the inflammatory stage (days 3–5 p.i.), there was a large amount of virus in the lung, accompanied by apparent inflammatory reaction. At the lung consolidation stage (days 7–9 p.i.), focal to multifocal lung consolidations, accompanied by focal necrosis, were observed. Finally, at the convalescence stage (from day 9 p.i. onward), lung consolidation still existed, but the inflammatory reaction and the viral load in lungs decreased dramatically, and in some animals (two of four) the virus was undetectable. Most importantly, the severity of pathological changes in the animal lungs was closely associated with the level of viral burden in the same lung. This observation will be helpful for evaluation of the efficacy of anti-SARS-CoV infection agents in animal models that are susceptible to infection but do not develop the actual disease.

Interestingly, the pathology observed in Chinese hamsters had also been observed in golden Syrian hamsters (21). However, the highest mean viral titer in golden Syrian hamster lungs was 7.2 $\log_{10}$ TCID$_{50}$/g on day 2 and the high titer of virus was sustained only to day 5 p.i. In contrast, Chinese hamsters displayed a viral titer of $1 \times 10^8 - 1 \times 10^9$ TCID$_{50}$ /g on days 3–5 p.i. and more than 1×10^6 TCID$_{50}$/g on day 7 p.i. (Fig. 2). These differences may result from several factors, including the virulence of each strain and the batch of SARS-CoV, the

lower dose of virus used in golden Syrian hamsters (about 10-fold lower than in this study of Chinese hamsters), and might also be animal strain dependent.

Other animals have also been reported to be susceptible to SARS-CoV, including young and adult mice (4,22), ferrets and domestic cats (23), and rhesus, cynomolgus, and African green monkeys (24,25). However, in contrast to both golden Syrian and Chinese hamsters, ferrets, for example, display lower viral propagation efficacy and pathological severity. Also, primates can only provide very limited data as they show no obvious illness, only mild pathological lung changes, low-level virus replication, and rapid recovery from infection (within 4 days p.i.).

Although detailed tissue studies of SARS-CoV infection in the lungs of patients have not been performed, based on the symptoms and signs, indirect evidence including radiographic features, and partial direct evidence including throat wash and autopsies (6,26,27), it appears that the pathological course of infection is similar to that observed in hamsters. Thus, although no evidence of disease can be observed in the Chinese hamster, based on the detailed tissue studies described here and previously, both the Chinese and golden Syrian hamsters appear to represent good animal models for SARS-CoV infection.

A study of the humoral immune response of SARS patients indicated that patients with a longer course of illness showed a lower neutralizing antibody response than did patients with a shorter illness duration (28). This indicates that neutralizing antibodies in patients play a pivotal role in SARS-CoV clearance *in vivo*. Accordingly, we have generated equine anti-SARS-CoV $F(ab')_2$ and shown a noticeable preventive effect against SARS-CoV infection in a BALB/c mouse model. Here we describe its excellent preventive role in the Chinese hamster model. At 10 mg/kg body weight, this antibody could completely protect animal lungs from infection with SARS-CoV (Fig. 2C and D). The protected animals did not show pathological changes in their lungs (data not shown). These results confirmed those observed in the BALB/c model (17). Significantly, besides the preventive role, the equine anti-SARS-CoV antibody also displayed a therapeutic role. The saturated amount of antibody, 50 mg/kg body weight, could decrease the viral load in the lungs by about 4 $\log_{10}$ (Fig. 2). This level of protection, although not complete, is remarkable, considering that the circulating antibodies can neutralize only the extracellular SARS-CoV, and also suggests that in therapeutic settings it may be necessary to combine passive antibody transfer with other methods such as antiviral drugs and vaccines. Importantly, similar therapeutic effects were also observed by Roberts *et al.*, by using MAb201 in the golden Syrian hamster model, although the viral copies in the lungs were not measured (29).

We have confirmed that equine anti-SARS-CoV $F(ab')_2$ has an excellent preventive effect and even a considerable therapeutic role in SARS-CoV infection; however, the possibility that heterogeneous antibody might evoke a strong host immune response may inhibit its application in a clinical setting. Thus, the development of human or humanized antibody against SARS-CoV is theoretically the ideal strategy to prevent infection, as it would be recognized as a "self" component by human hosts and would not elicit an immune response in the host. For this reason and other considerations such as the difficulty of finding immune human donors and the risk related to the use of human blood products, human and humanized monoclonal antibodies against SARS-CoV components had been developed and have exhibited effective preventive roles against SARS-CoV infection *in vitro* and *in vivo* (30–34). Nevertheless, the major obstacle for the application of these mAbs in the clinic is the yield of mAb products. However, the heterologous antibodies, for example, equine IgGs, have an advantage in this respect. Furthermore, one potential advantage of the polyclonal IgGs is the broader antigenic coverage and the lower likelihood of the emergence of escape mutants, although this theoretical advantage of equine anti-SARS-CoV $F(ab')_2$ has not been studied here. In addition, the heterology of specific IgGs can be decreased through the preparation of $F(ab')_2$ fragments by cutting off the Fc fragment, and thus $F(ab')_2$ should have higher specific neutralizing activity than complete IgG molecules, which means a smaller quantity of $F(ab')_2$ would be needed to neutralize the virus and this would reduce the possibility of an immune response against the $F(ab')_2$, although skin testing for hypersensitivity and possible desensitization (8) may still be necessary in practice. It is possible that equine anti-SARS-CoV $F(ab')_2$ fragments could satisfy the need for large-scale production of antibodies during an emergency to salvage infected patients, combined with the use of antiviral drugs, and other techniques as appropriate.

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