Brief Report

Inhibition of Severe Acute Respiratory Syndrome-Associated Coronavirus Infection by Equine Neutralizing Antibody in Golden Syrian Hamsters

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ABSTRACT

Equine anti-severe acute respiratory syndrome-associated coronavirus F(ab′)2 has been verified to protect mice from infection with severe acute respiratory syndrome-associated coronavirus (SARS-CoV). However, before potential clinical application, the antibody needs to be tested in as many animal models as possible to ensure its safety and efficiency. In this study, after verification by various methods that the golden Syrian hamster constitutes a model susceptible to SARS-CoV infection, we confirmed that the antibody could protect animals completely from SARS-CoV infection in the preventive setting. More importantly, the antibody could reduce viral titers or copies by approximately 103- to 104-fold in animal lung after virus exposure, compared with negative control. These data provide further evidence to warrant clinical studies of this antibody in the treatment and prevention of SARS.

INTRODUCTION

Severe acute respiratory syndrome (SARS) is now controlled, and widespread infections between humans and/or animals have not been observed since the initial outbreak in 2002 and 2003 (3,4). However, its mysterious animal origins (11) and strong infectivity necessitate further studies on how to control the replication of the SARS-associated coronavirus (CoV) in infected individuals. Furthermore, it is possible for an SARS outbreak to reoccur, for example, as a result of the virus evolving into variants that can be transmitted from human to human or as a result of laboratory accidents and biological warfare. These issues mandate further study of SARS prevention and treatment.

Various interventions to prevent and treat SARS...
should be explored, including vaccines, passive immune therapy, RNA interference, and antiviral drugs. A prospective study of the humoral immune response of SARS patients indicated that patients with a longer illness showed a lower neutralizing antibody response than did patients with a shorter illness duration (5), indicating that neutralizing antibodies in patients play a pivotal role in SARS-CoV clearance in vivo. However, the neutralizing antibody titers decreased markedly after month 16 of infection (6), suggesting that persons who were exposed to SARS-CoV may become reinfected later. All this evidence indicates that humoral immunity is critical in preventing and treating SARS-CoV infection, which also suggests the direction of vaccine research. On the basis of this evidence, we developed an equine anti-SARS-CoV F(ab′)2 that was able to provide excellent protection from this virus in a BALB/c mouse model (15). However, before any possible future clinical applications, this kind of antibody must be tested rigorously in as many animal models as possible to ensure its efficacy and safety.

To date, several animal models susceptible to SARS-CoV infection, including mouse, ferret, golden Syrian hamster, rabbit, and monkey, have been established (1,2,8–10,13,14,16,17). Among these animals, hamster seems to be a good platform for evaluation of various anti-SARS strategies because it can maintain infection longer, with higher titers of SARS-CoV and more evident pathological changes in experimental animal lungs, than do other animals (9,10). In this study, we evaluated the equine anti-SARS-CoV F(ab′)2 in a golden Syrian hamster model to provide more evidence for potential clinical testing of this antibody.

**MATERIALS AND METHODS**

**Virus, antibody, and animals**

SARS-CoV (strain BJ01, GenBank accession number AY278488, isolated February 10 to March 15, 2003) was maintained at the Institute of Microbiology Epidemiology (Academy of Military Medical Sciences [AMMS], Beijing, China) and propagated in Vero cells. The virus was released from infected cells by three freeze–thaw cycles and the titer was determined to be \(1.13 \times 10^7\) TCID\(_{50}\) (50% tissue culture infective doses)/mL. All operations with SARS-CoV were performed in the Biosafety Level 3 (BSL-3) laboratory.

We prepared serum IgGs and their pepsin-digested F(ab′)2 fragments from horses inoculated with purified SARS-CoV (BJ01 strain), as described previously (15). The neutralizing titer of the F(ab′)2 was 1:1600–1:3200, determined in cultured Vero E6 cells by cytopathic effect (CPE), 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and plaque-forming assays, respectively (15).

To evaluate the susceptibility of golden Syrian hamsters to SARS-CoV infection, after light anesthetization with isoflurane, an SARS-CoV particle suspension (\(1 \times 10^4\) TCID\(_{50}\) in 100 μL) was administered intranasally to the animals on day 0. Four hamsters from each group were killed on days 1–7 postinfection. The lungs of experimental animals were removed and homogenized in a 10% (w/v) suspension of Leibovitz 15 medium (Invitrogen, Carlsbad, CA). Viral titers and copies in the homogenates were then determined in CPE and TaqMan real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays, as described below. The pathology and localization of SARS-CoV in the lungs of infected animals were determined by pathological observation and immunohistochemistry (IHC), as described below.

To evaluate the therapeutic role of anti-SARS-CoV F(ab′)2 against SARS-CoV infection in golden Syrian hamsters, the animals received, intraperitoneally, a 100-μL suspension containing \(1 \times 10^4\) TCID\(_{50}\) of SARS-CoV on day 0, followed by intraperitoneal injection of 0.5 mL of the F(ab′)2 solution containing 70 or 35 mg/kg body weight of this kind of antibody, or normal horse antibody (70 mg/kg) lacking neutralizing activity as a negative control on day 1 postinfection. The viral titer, copy number, and localization, as well as pathologic changes in the infected animal lungs, were determined at the indicated times.

To investigate the preventive role of the equine anti-SARS-CoV F(ab′)2 against the SARS-CoV infection, after anesthetization, the hamsters were injected intraperitoneally with the anti-SARS-CoV F(ab′)2 (10 mg/kg body weight) or nonimmune normal horse antibody (10 mg/kg body weight), as a negative control, on day −1, the day before viral infection. Twenty-four hours later (day 0), the golden Syrian hamsters were challenged intranasally with \(1 \times 10^4\) TCID\(_{50}\) of SARS-CoV, and were killed on the indicated time points. The viral titer, copy number, and localization, as well as pathologic changes in the infected animal lungs, were then determined on the basis of CPE, qRT-PCR, IHC, and observation of pathology.

**Cytopathic effect and qRT-PCR assay**

Real-time quantitative TaqMan PCR and CPE assays were used to determine SARS-CoV titer and copy number in the lungs of SARS-CoV-infected or mock-infected hamsters. As described previously (15), the number of SARS-CoV N gene copies was determined by qPCR and the CPE assay was conducted on cultured Vero E6 cells to determine the viral titer.
**Histopathology and immunohistochemistry**

Hamsters were anesthetized with isoflurane and killed by cervical dislocation on the indicated day after virus administration or anti-SARS-CoV F(ab')2 injection. Lung tissue was fixed with 10% neutral buffered formalin, embedded in a paraffin block, and processed for routine histology and IHC (avidin–biotin–peroxidase complex [ABC] technique) detection as described (12). For IHC, the purified equine anti-SARS-CoV IgG (diluted 1:10,000) was used as the detecting antibody and 3,3’-diaminobenzidine (DAB) was used as the chromogen substrate.

**Statistical analysis**

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

**RESULTS**

As the golden Syrian hamster has not been studied extensively, we validated this animal model not only with the CPE assay used by Roberts and colleagues (9,10), but also with a quantitative TaqMan reverse transcription polymerase chain reaction (qRT-PCR) method that could provide accurate copy numbers of propagated virus, as described previously (7,15). Hamsters (weighing 80–100 g each) were inoculated intranasally with $1 \times 10^4$ TCID$_{50}$ of SARS-CoV on day 0. We then determined viral titers and copies in the same lung tissue on days 1–7 postinfection, using the CPE assay and qRT-PCR assay, respectively. Viral titers increased from day 1 postinfection, and peaked (about $1 \times 10^8.5$ TCID$_{50}$/g lung tissue) on day 3 postinfection (Fig. 1A). Viral titers sustained a high level until day 5 postinfection (about $1 \times 10^7$ TCID$_{50}$/g), but decreased rapidly thereafter to...
undetectable levels by day 7 postinfection (Fig. 1A), results that were similar to previously published results (9,10). The qRT-PCR assays verified that the course of the viral titer changes was concordant with the course of viral copy changes (Fig. 2A). On days 1–3 postinfection, the lung viral titers peaked at $1 \times 10^{10}$ copies/g lung tissue and remained high until day 5 postinfection. However, on day 7 postinfection, when viral titers were undetectable by CPE assay, several thousand viral copies could still be detected by qRT-PCR (Figs. 1A and 2A), which reflects the higher sensitivity of real-time RT-PCR versus CPE. Thus, more information has been generated with respect to the golden Syrian hamster as a model of SARS-CoV infection.

Because of the close association between the degree of pathological change and viral titer (9,10), we believe that the efficiency of various interventions against SARS-CoV infection, such as antiviral drugs or passive antibody transfer, can be evaluated by detecting the viral titer or copy number in infected animal lungs. For assessment of therapeutic efficacy of the equine anti-SARS-CoV F(ab')$_2$, each hamster was inoculated intranasally with $1 \times 10^4$ TCID$_{50}$ of SARS-CoV on day 0, and received an intraperitoneal injection of the indicated dose of F(ab')$_2$ on day 1. The therapeutic role of the F(ab')$_2$ fragment was then investigated at the indicated times. We have determined that the half-life ($t_{1/2}$) of the F(ab')$_2$ fragment is about 60 h in rat and 40 h in macaque (18). As hamsters are more closely related to rats than to macaques, we assumed 60 h as the $t_{1/2}$ of our F(ab')$_2$ fragments. On the basis of the $t_{1/2}$, we measured the therapeutic role of the antibody on days 3–5 postinfection to provide exact data for its potential future clinical applications. The CPE assays indicated that equine anti-SARS-CoV F(ab')$_2$ at a concentration of 70 mg/kg body weight could exert a significant therapeutic effect on the inhibition of SARS-CoV propagation in animals. Animals protected with this dose of antibody could decrease about virus in their lungs by 4 log$_{10}$ TCID$_{50}$ (Fig. 1C), compared with controls ($p < 0.01$) (Fig. 1B). As expected, the therapeutic efficiency of this antibody on day 4 postinfection was similar to that on day 3 postinfection (Fig. 1C). However, on day 5 postinfection, past the half-life of this antibody, the protective rate did not decrease.

FIG. 2. Inhibition of SARS-CoV infection in the golden Syrian hamster, as determined by qPCR. Animals were inoculated intranasally with $1 \times 10^4$ TCID$_{50}$ of SARS-CoV in 100 μL of PBS on day 0, and SARS-CoV copies were determined by qPCR on days 1–7 postinfection (A). For therapeutic effect assays, $1 \times 10^4$ TCID$_{50}$ of SARS-CoV was inoculated intranasally into each hamster on day 0, the indicated nonimmune equine F(ab')$_2$ control or equine anti-SARS-CoV F(ab')$_2$ was injected intraperitoneally on day 1 postinfection, and viral copies were examined on the following 6 d (days 2–7, B), or on days 3–5 postinfection (C and D). For the preventive role investigation, 10 mg/kg body weight of equine anti-SARS-CoV F(ab')$_2$ or nonimmune F(ab')$_2$ was administered intraperitoneally on day −1 and $1 \times 10^4$ TCID$_{50}$ of SARS-CoV was inoculated intranasally into each hamster on day 0. Viral copies were then investigated on days 1–7 postinfection (F), with 10 mg/kg body weight of nonimmune equine F(ab')$_2$ as a negative control (E). Viral copies are expressed as N gene copies of SARS-CoV. Each experimental group contained three hamsters and the panels show mean viral copies from three independent experiments. Error bars indicate standard errors. *$\Delta p < 0.01$ (one-way ANOVA) compared with 70 mg/kg of nonimmune equine antibody control. Solid wedge, the day the antibody was administered; sun symbol, the day the virus was inoculated.
about 1010 copies of SARS-CoV N genes could be de-
in lung homogenates at this dose (Fig. 2F). In contrast,
because N gene copies of SARS-CoV were undetectable
of the SARS-CoV-specific antibody could completely
PCR assay also demonstrated that 10 mg/kg body weight
SARS-CoV infection (Fig. 1E). Accordingly, the qRT-
0 and pathological changes were analyzed by hema-
toxin–eosin (H&E) staining and immunostaining on
days 1, 3, and 5 postinfection. On day 1 postinfection,
the pathological changes were mild and the lung tissue
architecture remained roughly normal, except for some
small vessel dilation and congestion, and edema in alve-
olar spaces (Fig. 3A), compared with normal mouse lung
(see Fig. 3I). The virus invaded bronchial epithelial cells
and, to a lesser extent, alveolar epithelial cells adjacent
to the bronchi (Fig. 3B). On day 3 postinfection, there
was a large degree of inflammatory cell infiltration,
mainly involving mononuclear cells and lymphocytes
(Fig. 3C). There also were focal hemorrhages around
small vessels (Fig. 3C), and besides bronchial epithelial
cells, alveolar epithelial cells were infected extensively
(Fig. 3D). On day 5 postinfection, there was considerable
inflammatory cell infiltration that surrounded bronchi
(Fig. 3E and F). The interstitial pneumonia had become
more severe than that observed on day 3 postinfection
and there appeared to be apparent focal consolidation
(Fig. 3E and F). A large viral load also could be observed
in animal lungs on this day (Fig. 3F).

When the equine neutralizing antibody was adminis-
tered after virus inoculation, antibody at 35 mg/kg ap-
parently relieved the pathological lesions (Fig. 3G) on
day 4 postinfection, compared with virus control (Fig. 3C
and E). On the same day, animal lungs showed mild to
moderate interstitial pneumonia and lung consolidation
(Fig. 3G and H). The viral load in animal lungs on this
day was much less than that in the viral control (Fig. 3D
and F). For preventive effect assay, antibody at 10 mg/kg
completely neutralized the inoculated virus. No patho-
logical changes or virus could be detected in animal lungs
(Fig. 3I and J).

**DISCUSSION**

Although the ideal immunoglobulin for adoptive trans-
fer would be of autologous or homologous origin for the
lowest anti-antibody response, the bottleneck of low mAb
yield must be overcome before it can be used in practice.
On the other hand, heterologous immunoglobulins can
surmount the bottleneck of antibody yield. In general,
10,000–20,000 mL of whole blood can be obtained from
one horse on a weekly basis, and about 100,000 mL of
whole blood can be harvested if the horse is killed. One
milliliter of blood contains about 7.23–16.85 mg of IgG
and the recovery rate of F(ab′)2 from IgG is about 50%.
Thus a relatively high amount of antibody can be ob-
tained from each horse. Furthermore, polyclonal IgGs
would possess broader antigenic coverage and a lower
likelihood of emergence of escape mutants, although this
potential advantage of the equine anti-SARS-CoV
F(ab′)2 has not been studied here.
Heterologous immunoglobulins from several species including horse have been used for adoptive humoral therapy for several diseases such as toxicosis from spider and caterpillar, pure red cell aplasia, multiple sclerosis and systemic sclerosis, myelodysplasia, rabies, hepatitis virus, and human immunodeficiency virus (HIV), which have been discussed previously in detail (15). The immunogenicity of heterologous equine immunoglobulins must be given seriously consideration when used in humans. We can take some measures to reduce the immunogenicity of equine antibody, for example, by cutting off the Fc fragment. This way, the immune response of the host to the equine F(ab')2 can be decreased to a tolerable level. Our experimental data (19) from 18 monkeys that were injected intravenously every day for 4 wk showed that equine F(ab')2 had stimulated the host immune response against this kind of antibody itself and caused mild hypersensitivity. No experimental animals died and the motivated host secondary lymphoid organs, the spleen and lymph nodes, recovered 3 wk after the last injection. Because the host anti-F(ab')2 antibody appears 2 wk after F(ab')2 administration, there is enough time for the equine antibody to exert a protective effect at the early stage of SARS-CoV infection, thus producing a marked effect during a large-scale SARS outbreak.

**FIG. 3.** Pathological effects on the golden Syrian hamster lung. Golden Syrian hamsters were inoculated intranasally with 1 × 10^4 TCID50 of SARS-CoV on day 0, with or without antibody protection. At the indicated times, the experimental animal lungs were removed and fixed with formalin and then stained with hematoxylin and eosin (H&E) for pathological observation, or subjected to IHC with anti-SARS-CoV F(ab')2 as the primary antibody (diluted 1:10,000) and DAB as the chromogenic substrate. (A–F) Establishment of the animal model. After viral inoculation, animal lungs were examined on days 1, 3, and 5, postinfection by H&E staining and by IHC assay. To assay the therapeutic effect, animals were treated with antibody at 35 mg/kg after viral exposure and the lungs were analyzed on day 4 postinfection (G and H). For preventive assays, animals were administered equine anti-SARS-CoV F(ab')2 at 10 mg/kg before viral exposure and the lungs were analyzed (I and J); as no pathological changes were observed, this tissue was also used as a normal control. Data shown are representative of five animals per group.
In our animal model, equine F(ab’)2 could only partially but significantly inhibit SARS-CoV propagation in the therapeutic setting. As SARS-CoV is an intracellular virus and equine anti-SARS-CoV F(ab’)2 will neutralize mainly the extracellular virus, it is reasonable that a saturating concentration of this antibody, 35 mg/kg as determined in this study, could not completely neutralize all viral particles. However, this effective neutralization of extracellular virus will inhibit other normal tissue from being infected further and thus halt the transmission of the virus in vivo. This will enable the host to produce an immune response against the virus and clear it thor-
oughly. However, we currently cannot verify this scenario because there are no appropriate animal models that can be infected for so long a time to evaluate an active immune response to SARS-CoV infection. Finally, the rapid clearance of SARS-CoV by the F(ab’)_2 in vivo will provide the chance for other interventions such as antiviral drugs, RNA interference (RNAi), and vaccines to eliminate the virus from the host.

Roberts et al. reported that some animals injected with neutralizing antibody did not display this antibody in the serum (9). However, we have not observed this phenomenon. It may be due to the following reasons. The antibody used in this study has been truncated by removing the Fc fragment from IgG, and therefore the specific neutralizing activity of the F(ab’)_2 per dose of antibody solution used is higher than that used by Roberts et al., which consisted of intact immunoglobulin (9). This means the 35-mg/kg dose of F(ab’)_2 used in this study will have higher neutralizing activity than the 40-mg/kg dose of IgG used by Roberts et al. (9), and thus F(ab’)_2 at 35 mg/kg should exceed the dose needed to neutralize extracellular SARS-CoV in the golden Syrian hamster. In addition, the diffusible feature of F(ab’) may represent another advantage over intact IgG, thus presenting better protective efficiency than the latter.

In conclusion, in this study we have verified the golden Syrian hamster model by several methods. We have confirmed that equine anti-SARS-CoV F(ab’)_2 can protect animals completely from SARS-CoV infection when adequate doses of antibody are administered before viral inoculation. More importantly, the antibody also exhibits an excellent therapeutic effect after viral exposure. Together with its proven protective role against SARS-CoV infection in mice (15), more evidence now exists to warrant clinical studies of this antibody in the treatment and prevention of SARS.

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**REFERENCES**


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