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Preparation and development of equine hyperimmune globulin F(ab')$_2$ against severe acute respiratory syndrome coronavirus[1]

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Key words

severe acute respiratory syndrome coronavirus; neutralizing antibodies; hyperimmune globulin; cross protection; F(ab')$_2$ fragments

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Abstract

Aim: The resurgence of severe acute respiratory syndrome (SARS) is still a threat because the causative agent remaining in animal reservoirs is not fully understood, and sporadic cases continue to be reported. Developing high titers of anti-SARS hyperimmune globulin to provide an alternative pathway for emergent future prevention and treatment of SARS. **Methods:** SARS coronavirus (CoV)F69 (AY313906) and Z2-Y3 (AY394989) were isolated and identified from 2 different Cantonese onset SARS patients. Immunogen was prepared from SARS-CoV F69 strain. Six health horses were immunized 4 times and serum was collected periodically to measure the profile of specific IgG and neutralizing antibodies using indirect enzyme-linked immunosorbent assay and a microneutralization test. Sera were collected in large amounts at the peak, where IgG was precipitated using ammonium sulphate and subsequently digested with pepsin. The product was then purified using anion-exchange chromatography to obtain F(ab')$_2$ fragments. **Results:** The specific IgG and neutralizing antibody titers peaked at approximately week 7 after the first immunization, with a maximum value of 1:14210. The sera collected at the peak were then purified. Fragment of approximately 15 g F(ab')$_2$ was obtained from 1 litre antiserum and the purity was above 90% with the titer of 1:5120, which could neutralize the other strain (SARS-CoV Z2-Y3) as well. **Conclusion:** This research provides a viable strategy for the prevention and treatment of SARS coronavirus infection with equine hyperimmune globulin, with the purpose of combating any resurgence of SARS.

Introduction

Severe acute respiratory syndrome (SARS) emerged in Southeast Asia in late 2002 and subsequently spread internationally. The causative agent was quickly identified as a previously unknown member of the Coronaviridae family[1–3]. According to the World Health Organization, up to 2004 Apr 21, SARS coronavirus infected more than 8000 people in various countries worldwide and caused approximately 800 deaths[4]. Although SARS infection of human beings has been contained through infection-control measures, resurgence is still a threat because the causative agent remaining in animal reservoirs is not fully understood, and sporadic cases continue to be reported in Singapore[5,6], Taiwan[7] and mainland China[8,9].

There are no specific vaccines and effective drugs currently available for SARS-CoV[1,2,10,11]. Until an effective vaccine is developed, the best hope for the treatment of infection and the prevention and control of future outbreaks is the development of passive immunotherapy with SARS-CoV-specific antibodies[11]. Immunoglobulin is an effective method used in protection against animal coronavirus: transmissible

gastroenteritis virus (TGEV)[12,13], mouse hepatitis virus[14], and bovine coronavirus[15]. There is clinical evidence that serum from recovered patients is effective in infected individuals[16,17]. These observations suggest that hyperimmune serum could be developed for the passive treatment of SARS. The use of equine antisera for emergent prevention and treatment of infectious diseases has been proven to be an effective and safe strategy, such as in rabies virus[18,19]. Therefore, immunoprophylaxis and treatment of SARS coronavirus infection with equine hyperimmune globulin might be a viable strategy for controlling SARS.

Materials and methods

Virus strains Severe acute respiratory syndrome corona-virus Z2-Y3 (AY394989) and F69 (AY313906), isolated from the samples of 2 different Cantonese onset SARS patients in 2003, were sequenced and compared, showing certain differences (Table 1). Viral titres of SARS-CoV Z2-Y3 and F69 strains were determined to be $10^{6.5}$ 50% tissue-culture-infective doses ($TCID_{50}$)/mL and $10^{6.7}$ $TCID_{50}$/mL with the Reed-Muench method, respectively[20–22].

Antigen preparation F69 strain was used as antigen for immunization. African green monkey kidney (Vero-E6) cells, infected with SARS CoV F69 strain, were cultivated in serum-free minimum essential medium (MEM) (GIBCO**)** and observed periodically for cytopathic effect (CPE). When 75%–100% cytopathy was reached, infected Vero-E6 cells were frozen and thawed 3 times, which was subsequently centrifuged at 8000×*g* for 30 min, and then the cell debris was decanted. The supernatant was collected and stored at –70 ºC until used. The viral supernatant was then centrifuged at 30 000×*g* for 3 h. The precipitate was diluted with phosphate buffered saline (PBS), which was used as antigen for immunization.

Animal immunization Six 4–9 year-old healthy horses were provided by the Quartermaster University of PLA. Immunization of horses was performed according to the State Food and Drug Administration (SFDA) standard operating procedures. On d 0 and d 10, all horses were injected with 1.0 mL antigen intramuscularly (SARS-CoV F69) with complete Freund's adjuvant (FCA, Sigma). On d 21 and d 28, horses were injected with the same antigen 2.5 mL im, with incomplete Freund's adjuvant (FIA, Sigma). Eight batches of sera were collected from trachelo veins on d 0, d 10, d 21, d 28, d 35, d 42, d 49, and d 55 after the first immunization, which were stored at -20 °C for the measurement of antibody titers.

Enzyme-linked immunosorbent assay (ELISA) Severe acute respiratory syndrome coronavirus specific IgG was measured using an indirect enzyme-linked immunosorbent assay (ELISA) and whole purified SARS-CoV F69 as antigen. In brief, polystyrene micro well plates were coated with antigen (100 μL/well containing 1.0 μg/mL virus protein) in carbonate-bicarbonate buffer (pH 9.6). The wells were washed 3 times with PBS and then blocked with 15% bovine serum in

Table 1. Complete genomic sequence comparison between F69 and Z2-Y3. N1, atattaggtttttac; N2, caagaatgta; –, no nucleotide.

Locus	1–15	2015	3852	5455	6247	6760	7347	7777	8094
F69	N1	C	C	T	C	G	A	G	T
Z2–Y3	–	T	T	C	T	A	C	A	C

Locus	8591	9333	10265	11493	13470	14186	16959	17565	20374 -20383
F69	G	C	T	T	A	T	T	T	N2
Z2-Y3	A	A	C	C	G	A	C	G	–

Locus	21732	22233	24706	25275	25309	26488	27403	29358
F69	G	T	G	G	G	G	T	G
Z2-Y3	A	C	A	A	A	T	C	A

PBS containing 0.05% Tween-20 (PBST) at 37 °C for 1 h. After 3 washes with PBST, serially twofold diluted serum samples (from 1:100 to a final 1:51200) were added to the plates and incubated at 37°C for 1 h. Horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (Sigma, USA) diluted 3000-fold in PBS was added, followed by 3 washes. Following incubation at 37 °C for 1 h, the plates were washed as above and the substrate tetramethylbenzidine (TMB) solution (Sigma) was added to the wells. After incubation at 37 °C for 15 min, the reaction was stopped by adding 2.0 mmol/L sulfuric acid, and the absorbance value at 450 nm (A450) was measured with a microplate reader (Model 550, BioRad). IgG antibody titer was defined as the highest dilution of serum when the A450 ratio (A450 of negative serum) was greater than 2.0.

Microneutralization assay The neutralization assay was performed according to modified operating procedures of the Manual for the virological investigation of polio formulated by WHO/EPI/GEN/97.01. (http://www.who.ch/programmes/gpv/gEnglish/avail/gpvcatalog/catlog.htm). Each serum sample was serially diluted in twofold 1:10 dilution in MEM maintenance medium to a final dilution of 1:20 480 and incubated with an equal volume of 100 $TCID_{50}$/25 μL of purified F69 or Z2-Y3 strain for 3 h at 36 °C. The virus-antibody mix was then inoculated onto Vero-E6 cell (3×10^5 cells/mL) monolayers in 96-well plates at 37 °C for approximately 6 d. Wells for normal cell control and virus control were added to 100 μL maintenance medium and unneutralized active virus dilution, respectively. The plates were incubated until CPE developed in all the virus controls but the cell control remained normal. Neutralizing antibody titer was the highest dilution of serum, which protected 50% of the cultures against 100$TCID_{50}$ of the challenge virus, when the virus control (no serum) showed complete CPE.

Purification of immunoglobulin Horse antiserum was thawed in 37 °C water bath, added to 45% saturated ammonium sulphate solution, then mixed gently at 22 °C for 30 min, centrifuged at 10 000×*g*, and the precipitation generated was collected and stored at 4 °C overnight. The ammonium sulphate precipitation was diluted using an equal volume of 0.9% NaCl solution and dialyzed to remove the salt. Then pH was adjusted to 3.5 with 0.36 mol/L HCl. Then horse serum was added to 2% pepsin (Sigma) solution and digested at 37 °C for 8 h, 24 h, 36 h, 48 h, 60 h, and 72 h, respectively. The reaction was stopped by adjusting the pH to 8.0 using 1.0 mol/L NaOH. Then the digested material was ultrafiltrated. Anion-exchange separations of ultrafiltrated material were further performed using diethylaminoethyl (DEAE) Sepharose Fast Flow (Pharmacia) Column, pre-equilibrated with 200 mL of buffer A (50 mmo/L Tris-HCl, pH 8.0). The ultrafiltrated material was pumped down the column, while the A_{280} nm of the eluted material was monitored, followed by pumping fresh buffer A until the A_{280} nm returned to the baseline. All the unbounded material, corresponding to the $F(ab')_2$ fragments was collected and stored at 4 °C. Bound contaminants can then be eluted to regenerate the column using a gradient of buffer B (containing 50 mmo/L Tris-HCl, pH 8.0, 1 mmol/L NaCl, pH 8.0). The product obtained using anion-exchange chromatography was ultrafiltrated, concentrated and added to 0.3 mol/L aminoacetic acid to obtain stock solution. According to the demanded standards of biological product, the product characteristics (eg, pH, the protein concentration and bacterial endotoxin content) were detected using serial procedures.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Non-reducing SDS-PAGE gels, using the buffer system described by Laemmli (1970)[23], were used to monitor the digestion process and to check for traces of undigested IgG and other unwanted materials.

Results

Identification of SARS coronavirus The virus (SARS-CoV F69) was electron microscopically visualized, and the characteristic coronavirus particle form was observed (Figure 1).

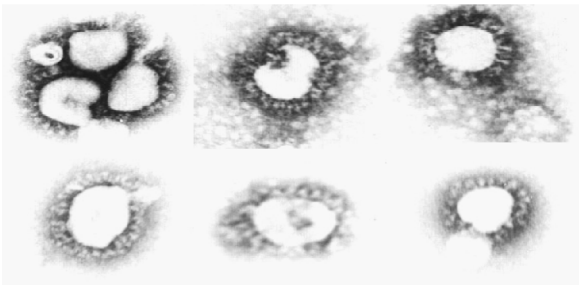

Figure 1. The virion form of severe acute respiratory syndrome coronavirus F69 isolate. 3×40 000.

Level of the specific IgG antibody Six health horses were immunized with purified SARS-CoV F69 strain. The titers of total anti-SARS-CoV IgG was measured using an indirect ELISA. The dynamic changes of specific anti-SARS-CoV IgG antibody titers are shown in Figure 2. On d 10, all sera distinctly showed positive reactions, with the range of specific IgG antibody titers from 1:160 to 1:980. Titers of specific IgG antibodies increased rapidly from week 4 and peaked at week 7 after the first immunization; the maximum value was

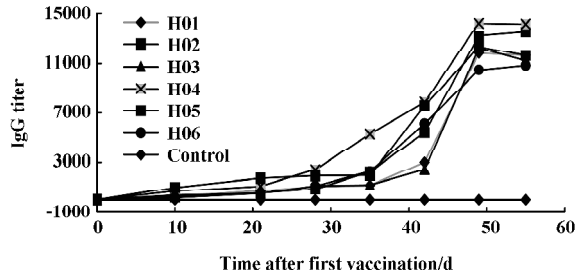

Figure 2. Changes of the specific anti-severe acute respiratory syndrome coronavirus IgG antibody titers. H, the immunized horse. The inoculation dates are on d 0, d 10, d 21, and d 28, respectively. The titers of serum specific IgG antibodies were shown on the highest dilution of serum at which the A450 ratio was greater than 2.0.

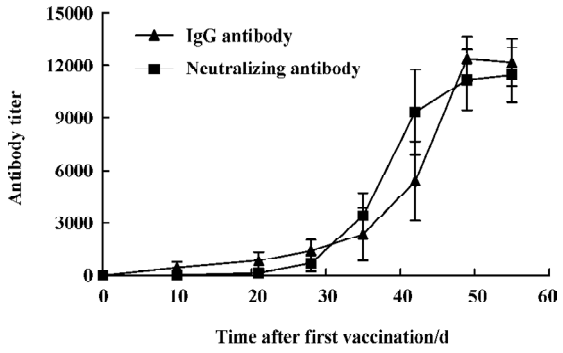

Figure 4. Comparison of the trend of change in neutralizing antibody titers with that of the specific IgG antibody titers. Each titer value was denoted as the mean of the titers of IgG and neutralizing antibody detected.

1:14210.

Titers of neutralizing antibodies The antiserum was measured using a micro-neutralization test. The kinetics of formation of neutralizing antibodies following immunization for horses were observed (Figure 3). The neutralizing antibodies were partially detectable on d 10 (from 1:10 to 1:60). After the third immunization, the neutralizing antibody titers of all the immunized horses increased rapidly on d 28. On d 48 after the first immunization, the neutralizing antibody titers of 4 of 6 equines reached the highest level. The other 2 continued increasing and reached the highest titer at 1:14240.

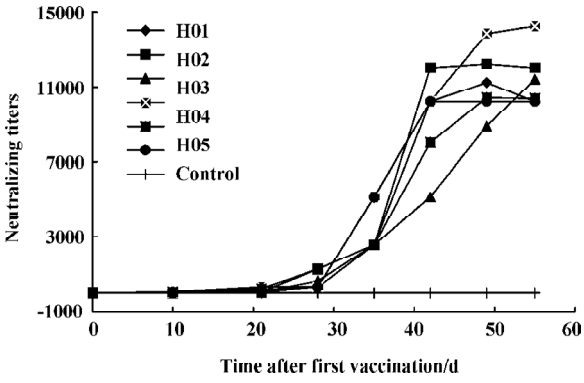

Figure 3. Kinetics of the specific anti-severe acute respiratory syndrome coronavirus neutralizing antibody titers. H, the immunized horse. The inoculation dates are on d 0, d 10, d 21, and d 28, respectively. Neutralizing antibody titers are shown on the highest dilution of serum, which protected 50% of the cultures against 100 50% tissue-culture-infective doses ($TCID_{50}$) of challenge virus.

Cross neutralization response Z2-Y3 strain was used in micro-neutralization test in vitro to measure the hyperimmune sera from the SARS-CoV F69 strain. The results indicate that the horse antiserum induced by the inactivated SARS-CoV F69 strain is capable of neutralizing the SARS-CoV Z2-Y3 strain completely.

$F(ab')_2$ preparation Digestion with pepsin at different time points was assessed using SDS-PAGE (Figure 5). The results indicate that IgG could be digested completely at pH 3.5 within 48 h and unwanted protein bands (eg albumin and transferrin) could be eliminated as well. The reaction was stopped by adjusting the pH to 8.0 using 1.0 mol/L NaOH. The anion-exchange chromatography with a salt gradient was performed to further remove high molecular weight aggregates and pepsin. Digested antisera in buffer A are separated into 3 peaks (Figure 6). Material from peak I was then concentrated. Finally, approximately15 g $F(ab')_2$ fragments were obtained from 1 litre antiserum with the purity

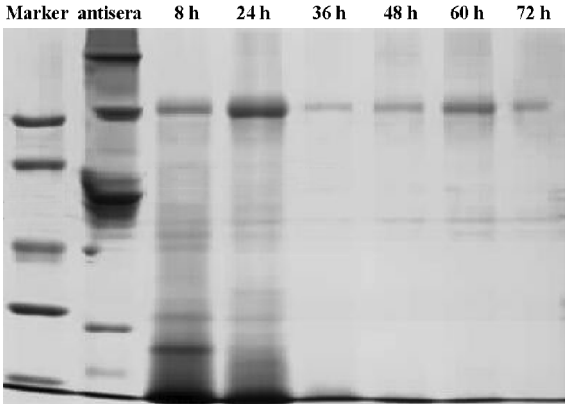

Figure 5. The digestion of equine antisera with pepsin, as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) under non-reducing conditions. Digestion samples at various time points, with molecular weight markers: 97 kDa, 66 kDa, 45 kDa, 30 kDa, 20.1 kDa. $F(ab')_2$: approximately 100 kDa.

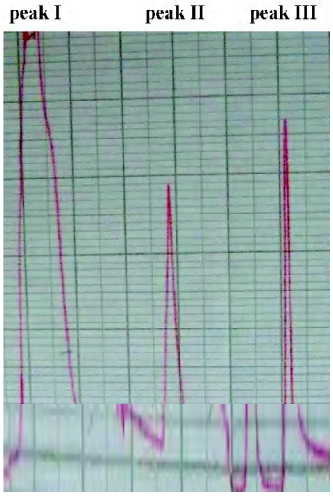

Figure 6. Removal of high molecular weight aggregate and pepsin using anion-exchange chromatography. diethylaminoethyl (DEAE) Sepharose Fast Flow ion-exchange separation of a diafiltered pepsin digested antisera. Peak I: $F(ab')_2$, Peak II: high molecular weight aggregate and Peak III: pepsin.

above 90%. The titer of neutralizing antibodies after purification was detected as 1:5120.

The product obtained above was dissolved in a suitable volume of 0.9% NaCl to adjust the protein concentration to 10 g/L. Then pH determined at 20 °C was 7.0. The bacterial endotoxin content was also detected at no more than 200 EU/mL. The terminal product according with the standard of SFDA [(2003) No.267], was stored at 4 °C.

Discussion

Severe acute respiratory syndrome has resulted in important challenges for the medical community. There are no available specific vaccines and effective drugs for use against SARS-CoV[11]. Control depends on prompt detection and isolation of cases, good infection control in hospitals, and the tracing and quarantine of contacts[24]. The widespread clinical successful application of immunoglobulins derived from heterogenous animals against rabies has a long history[25]. The passive administration of neutralizing antibodies could be an effective strategy for emergency prophylaxis and the treatment of SARS[26].

The results of our research indicate that healthy horses immunized with the SARS-CoV F69 strain can be induced to generate effective, specific and neutralizing antibodies. Analysis indicates that sequence difference existed among SARS-CoVs[27]. The sequence of the SARS-CoV F69 strain is different from that of the SARS-CoV Z2-Y3 strain (Table 1). Immunoglobulin prepared from SARS-CoV F69 strain isolated in April, 2003 could neutralize another SARS-CoV Z2-Y3 strain, which was isolated from SARS patient in February, 2003. This showed that SARS-CoV F69 and Z2-Y3 strain owned identical or similar neutralizing epitopes.

Heterogenous antisera used for treatment possibly result in anaphylactoid severe acute side-effects[28]. To avoid the side-effects caused by horse antiserum, IgG against SARS-CoV was digested with pepsin and purified with anion-exchange separations to exclude the immunogenicity of Fc fragments and to retain the special activity of binding the antigen of $F(ab')_2$ fraction. The titers of neutralizing $F(ab')_2$ against SARS-CoV was detected at higher level (1:5120). And approximately 15 g $F(ab')_2$ fragments were obtained from 1 litre antiserum, with the purity above 90%.

Until we have an efficacious vaccine and have implemented effective epidemiologic infection control measures, and given the presence of effective anti-SARS-CoV agents, SARS is likely to remain a major health threat to the world. In this article, we provide an alternative pathway of prevention and treatment of SARS, with the purpose of combating any resurgence of SARS. The profile of the antibody titer was observed, while an effective, specific and neutralizing hyperimmunoglobulin was prepared. The results indicate that the kinetics of the induced specific IgG and neutralizing antibodies are similar (Figure 4). This data paves the way for the development of an inactivated SARS vaccine.

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