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Preparation of equine immunoglobulin F(ab')$_2$ against smallpox and evaluation of its immunoprotective effect

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Abstract

Objective: Smallpox is a severe infectious disease caused by the smallpox virus, and the death rate is as high as 30% within 15—20 days post infection.

Methods: We prepared and tested pepsin-digested $F(ab')_2$ fragments of serum IgGs from horses.

Results: Transmission electron microscopy showed that the purified virus conformed to the morphology of VVTT. The titer was above 1.0×10^7 PFU/mL. The purity quotient of purified antigen is more than 90% by HPLC. After purification and cutting, and the yield of the purified product $F(ab')_2$ was about 1.3%, the purity of $F(ab')_2$ was more than 90%, and the neutralizing antibody titer was over 1:3200. $F(ab')_2$ fragments have a good preventive and therapeutic effect in mice at antibody dosages of 5.2 mg/mL and 2.6 mg/mL. Compared with the model group, the viral loads of the drug-treated mice were all suppressed to varying degrees, with the higher dose groups (5.2 and 2.6 mg/mL) showing a 2–3 folds reduction in viral loads.

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Conclusion: The process for producing equine immunoglobulin F(ab')$_2$ against VVTT was established. The prepared horse anti–smallpox immunoglobulin product has a good neutralizing antibody effect on VVTT. The highly purified preparation should be a potential candidate for smallpox treatment.

Keywords: Smallpox, Equine immunoglobulin F(ab')$_2$ fragments, Needle–free, Immunoprotective

Introduction

Smallpox is a severe infectious disease caused by the smallpox virus, which can be transmitted from an infected person to another, and the death rate is as high as 30% within 15—20 days post infection (1, 2). Since the global eradication of smallpox was announced in 1980, the vaccine has been discontinued (3). At present, most people are unvaccinated, and vaccine–induced immunity in people vaccinated before 1980 is waning (4, 5). And because of the events of September 11, 2011 in America, the concern has risen that terrorist organizations might use smallpox as a biologic weapon (6). Smallpox virus is retained in high security biosafety laboratories in the United

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States and Russia, and recently the biosafety accidents in high–level laboratories also remind us that we must be fully prepared for Smallpox (7, 8). Although there are chemosynthetic drugs for the treatment of smallpox patients, they are not specific drugs, and there are some key problems such as drug resistance and liver injury caused by long–term usage (9). The United States recently approved a non–replicating smallpox vaccine, but only for emergency use. And vaccine immunizations might not protect people who have already been infected with smallpox. In addition to vaccination for prevention, it is urgent to study a specific biological treatment drug to protect comprehensively.

Passive immunotherapy with sera of animal origin has been used for more than 120 years to treat viral and bacterial infections, as well as drug intoxications (10). The equine antisera, especially the $F(ab')_2$ fragments, which are easy to purify in large quantities and relatively inexpensive for urgent prevention and treatment of infectious diseases, have been proven to be effective and safe, such as in the case of the rabies virus (11–13).

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In this study, modern biopharmaceutical technology was applied to optimize the production, purification, and healthy horse immunity, preparation and purification of $F(ab')_2$ of horse immunoglobulin against VVTT (Vaccinia Virus TianTan, VVTT); and finally, the purified immunoglobulin was prepared. The purity of the production can be more than 90% by High Performance Liquid Chromatography (HPLC); the neutralizing antibody titer of the production against VVTT can reach more than 1:3200 when it is determined on the sensitive cell; the production has a good protective effect both in the prevention mode and in the treatment mode in the sensitive animal model. According to the test results of Joinn Laboratories (China) Co., Ltd., the safety of the product meets the requirements of the Chinese Pharmacopoeia. These results in our study will provide the basic experimental data for future clinical research.

Methods

1. Virus, cells and animals

VVTT was provided by the Institute of Virology of the China Center for Disease Control and Prevention. It was originally isolated from the blister scabs of smallpox patients in

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1926 and obtained by successive generations of virus reduction. Specific pathogen–free (SPF) chicken embryos were purchased from Beijing Meiliweitong Experimental Technology Co., Ltd. Primary chicken embryo fibroblasts (CEF) are derived from freshly harvested, 9—11–day–old SPF chicken embryos. Cells were received in a suspension of DMEM containing 5% newborn calf serum. Vero cells, provided by the cell bank of the Beijing Institute of Microbial Epidemiology. Ten healthy horses, ranging in age from 3 to 5 years, have been quarantined to meet the requirements of the Chinese Pharmacopoeia. Six–week–old BALB/c mice, weighing 18—20 g, were provided by SPF Experiment Animal Technology Co., Ltd. All the animals were approved for the experiments by the Institutional Animal Welfare Committee (No. LL20220603).

2. Production, purification and identification of VVTT

Chicken embryo fibroblast cells were cultured at 37°C, and VVTT solution was inoculated with monolayer cells at 0.1 MOI for 96—120 h, and the virus was harvested within 24 h after the whole cell was broken. The 300 kD Millipore ultrafiltration

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membrane was concentrated 100 times. The virus was centrifugated at 30000 g in the sucrose zone for 6 hours, and the protein peak was collected by Sepharose 4FF gel chromatography. A transmission electron microscope was used to observe the virus morphology of VVTT; thioglycolate medium, nutrient agar slant medium, and modified Martin medium were used for the sterility test. For the mycoplasma test, half fluid and half broth medium were used. Mice, chicken embryos, and cells were inoculated for the exogenous factor test. HPLC was used for the purity test.

3. Preparation of antigens for immunity and production of raw plasma

In scheme 1, the purified inactivated virus antigen (1:1) was mixed with ISA206 adjuvant of equal volume, emulsified, and immunized at least 10–poins injection immunization through submaxillary and inguinal lymph nodes of horses. The first four basic immune doses were 1, 2, 3, and 4 mg, respectively. The interval was 21 days each time, and the titer of neutralizing antibodies was determined 14 days after the last immunization. In scheme 2, purified virus antigen was injected into submaxillary and inguinal lymph nodes of horses at least 10–poins injection immunization with phosphate buffer

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solution (PBS), and the first four basic immune doses were 5×10^7, 6×10^7, 7×10^7 and 8×10^7 PFU respectively. The interval between each vaccination was 21 days, and the titer of neutralizing antibodies was determined by ELISA (9) 14 days after the last immunization (schema in Supplementary Figure 1).

4. Preparation and verification of F(ab')2 products

In the GMP plant of Shanghai serum Biotechnology Co., Ltd., according to the production process of immunoglobulin $F(ab')_2$, the immunoglobulin $F(ab')_2$ product of horse against smallpox virus was prepared. The protein concentration of immune plasma and $F(ab')_2$ was determined by Lowry assay (14), the protein yield of the final product was calculated according to the initial volume of plasma and the final volume of $F(ab')_2$, and the purity of the final product $F(ab')_2$ was determined according to the established product quality regulations (15).

5. The neutralization effect of horse anti smallpox virus immunoglobulin F(ab')2 raw plasma and final product on VVTT by MTT method

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Vero cells were inoculated into 96 well plate, each well was 100mL, containing 2 $\times 10^4$ cells / well, cultured at 37℃ for 24 hours, and the cells grew into a monolayer. The immunoglobulin $F(ab')_2$ of equine anti smallpox virus was diluted twice with DMEM medium, the initial titer was 1:800, six gradients in total, and then the VVTT 1×10^5 PFU, which was diluted with serum-free DMEM medium, was incubated at 37℃ for 1h, and then 200μL/well was successively added with the cell wells which drew the supernatant in advance, each dilution was set with four multiple wells, and normal cell control (without virus, without $F(ab')_2$), virus control (with virus, without $F(ab')_2$), normal horse immunoglobulin control (with virus, with normal horse immunoglobulin) were set. When cells in the virus control group were dead, add MTT (0.25 mg/mL) to 200 mL/cell for 4h, suck off the liquid, and add termination solution (10% SDS + 0.01 mHCL) 200 mL/cell dissolution for 8h, and detected the OD value at 570 nm (16).

6. Prevention effect of equine anti smallpox immunoglobulin in animals

Twenty BALB/c mice were divided into 5 groups, with 4 mice per group. Four concentrations of anti-smallpox virus immunoglobulin were prepared by PBS dilution,

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which were 5.2, 2.6, 1.3, and 0.65 mg/mL, respectively; the same amount of normal saline was used as a control. Each mouse was given 0.5 mL subcutaneously (s.c.) in the abdomen via a needle-free injection device (POK-V DART, Boker BioTe, China) according to the manufacturer's instructions, then Six days later, VVTT 1×10^6 PFU was injected intravenously. The weight change, scab formation, and death of mice were observed. Peripheral blood was collected from the lateral tail vein on days 1, 3, and 6 after the virus challenge. Plaque assays were conducted as previously described to detect viral loads in peripheral blood.

7. Therapeutic effect of equine anti smallpox immunoglobulin in vivo

Twenty BALB/c mice were divided into 5 groups, with 4 mice per group. Each mouse was injected intravenously with 1×10^6 PFU of VVTT. Six days later, each mouse was given 0.5 mL via needle-free injection subcutaneously (s.c.) in the abdomen (5.2, 2.6, 1.3, or 0.65 mg/mL); the same amount of normal saline was used as a control. Peripheral blood was collected from the lateral tail vein on days 1, 3, and 6 after the immunoglobulin injection. Plaque assays (17) were conducted.

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8. Safety evaluation

According to the requirements of the Chinese Pharmacopoeia, Joinn Laboratories (China) Co., Ltd. was entrusted to carry out sterility testing, pyrogen testing, abnormal toxicity testing, general pharmacological testing, acute toxicity testing, immune toxicity testing, hemolysis testing, and vascular stimulation testing on the final product of horse anti smallpox virus immunoglobulin $F(ab')_2$.

9. statistical analysis

Statistical analyses were performed using Prism 9.0. Data are presented as the mean values ±SD or SEM. Two-way ANOVA analysis of repeated measurements was used to compare among three or more groups. $p < 0.05$ was considered significant.

Results

1. Production, purification and detection of VVTT

Transmission electron microscopy showed that the purified virus conformed to the morphology of the vaccinia virus (Fig. 1A); the purified virus was sterile and had no

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mycoplasma, or exogenous factors; and the purity of the purified antigen was more than 90% by HPLC (Figure 1B). The results showed that the antigens for immunization met the standard of the Chinese Pharmacopoeia.

2. Yield and purity of the final product $F(ab')_2$

The product $F(ab')_2$ of horse anti-smallpox virus immunoglobulin was tested, and the results (Figure.2) showed that the yield of purified product $F(ab')_2$ was about 1.3% and the purity of $F(ab')_2$ was more than 90%.

3. Determination of neutralization effect of $F(ab')_2$ on VVTT by MTT

The prepared horse anti-smallpox immunoglobulin product has a good neutralizing antibody effect on VVTT, and the neutralizing antibody titer is 1:3200 (Table 1).

4. The preventive effect of $F(ab')_2$ in animals

Four groups of mice in the prevention experiment group were given the immunoglobulin $F(ab')_2$ via needle-free injection, and the mice in the antibody dosage groups of 5.2 mg/mL and 2.6 mg/mL showed the lowest body weight on the 6th day

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after VVTT challenge, and then gradually increased to normal; the mice in the antibody dosage groups of 1.3 mg/mL and 0.65 mg/mL showed an upward trend after the weight was reduced to the lowest, but it was not obvious (Fig. 3A). The results showed that a certain amount of horse anti smallpox virus immunoglobulin has a good preventive effect on mice. In the PBS control group, the mice began to have acne on their limbs on the 6th day after the VVTT challenge, and grew all over the body on the 8th day, with poor life status. At the end of the experiment, there was no body weight recovery and no scab disappearance. As shown in Figure 3B, the virus titer in the peripheral blood of mice in the model group was significantly higher after the challenge. In contrast, the viral loads were suppressed in prevention mice, with the higher dose groups (5.2 and 2.6 mg/mL) showing a 2–3 folds reduction in viral loads. Meanwhile, the viral proliferation capacity of the higher dose group was significantly inhibited.

5. Therapeutic effect of equine anti smallpox immunoglobulin F(ab')$_2$ in animals

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On the 6th day after intravenous injection of VVTT, the bodies of the five groups of mice began to develop pox, and the four groups of mice injected with immunoglobulin $F(ab')_2$ showed weight changes. The weight of mice in the experimental group after injection of immunoglobulin $F(ab')_2$ with 5.2 mg/mL, 2.6 mg/mL, and 1.3 mg/mL and slowly recovered to that before the experiment, while 0.65mg/mL mice in the experimental group recovered slowly until the 8th day after injection of immunoglobulin $F(ab')_2$. There was still a little scab. However, the mice in the PBS group began to have acne on their limbs on the 6th day after the VVTT challenge and grew all over the body on the 8th day, with poor life status. At the end of the experiment, neither weight recovery nor scab formation had disappeared (Fig. 4A). As shown in Figure 4B, the viral loads of the treated mice were all suppressed to varying degrees compared with the model group. Meanwhile, the decrease was more significant in the high–dose group.

Discussion

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Smallpox is one of the most infectious and pathogenic diseases in the world. Because the smallpox virus has great infectivity and lethality, and countries have stopped vaccinating, humans have lost the protection of smallpox virus immunity; therefore, the smallpox virus has become a powerful biological weapon of extreme forces (6). At present, there are two places in the world, FDA of the US Centers for Disease Prevention and Control in Atlanta and the Russian National Center for Virology and Biotechnology Research (7, 8). In recent years, safety accidents often occurred in these centers, which aggravated the concerns of countries about biosafety. Although there is a chemosynthetic drug cidofovir for smallpox patients, but the efficacy is limited, and side effects are strong, so far there is no specific drug. In view of the current biosafety situation, the United States approved a non-replicating smallpox vaccine in 2019, in case of accidents. However, most people in China do not have immunity to smallpox. With the increase of overseas interests and the expansion of foreign exchange, the smallpox virus or a similar monkeypox virus has become the most realistic threat to biological security.

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Therefore, the study of specific drugs for smallpox has become an urgent and important social demand. In view of the experience of human history in the struggle against infectious diseases, one of the most effective and economical means is the passive immunotherapy of antiserum, which is still used in the treatment and prevention of some important infectious diseases and plays an irreplaceable role. The advantages of traditional horse serum are: 1. the preparation process is mature, simple, and convenient for large-scale production; 2. the antibody is cleaved by a gastric enzyme and retains the $F(ab')_2$ functional group, which can remove most of the IgG whole molecules and Fc fragments that cause side effects, with high stability and strong affinity; and 3. the heterogeneity of horse serum to humans is weaker than that of other animals. Its disadvantages are low purity of immunoglobulin $F(ab')_2$ and 1% side effect (18).

The Beijing Institute of Microbiology and Epidemiology and Shanghai Serum Biotechnology Co., Ltd. have successfully developed anti-smallpox immunoglobulin $F(ab')_2$ products with their own advantages. The purity of the final products can reach

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more than 90%, and the side effects can be reduced from about 1% to less than 0.01% (19).

The core of therapeutic antibody research and development is the selection and preparation of immune antigens. VVTT is a virus isolated from the blister scabs of smallpox patients in 1926 and obtained by successive generations of virus reduction. The use of VVTT has cleared smallpox in China, which shows that it has an immune protection effect on the smallpox virus. The technology for large-scale culture, purification, and identification of VVTT is very mature. We chose chicken embryo fibroblasts to culture the smallpox virus. After density gradient centrifugation and chromatography purification, we prepare qualified antigens, whose purity is more than 80%. The morphology conforms to the characteristics of the smallpox virus and meets the requirements of the Chinese Pharmacopoeia for antigens for immunization.

For the immune program of healthy horses, we choose two programs: live virus immunization or inactivated vaccine plus adjuvant immunization. Although both of them can achieve the final immune effect, considering the biological safety concerns and

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high–cost factors of the live virus, we finally choose the inactivated vaccine plus adjuvant program.

The bottleneck in the development of products for the prevention and control of smallpox virus and other virulent pathogens is relying on high–level biosafety laboratories and smallpox virus. With the help of VVTT, we established two methods: the cell infection model and the animal infection model, which broke through the bottleneck. Although the two models cannot completely replace the model established by the smallpox virus, considering the same immune protection mechanism, they can be used as an important and useful reference (20).

This study innovatively employed needle–free injection device for immunoglobulin delivery and produced the same immunoprotective effect as needle–based injection. The needle–free injection device is simple, fast, and portable, and is suitable for use in a wide range of vaccinations and battlefield self–help medical systems. In addition, it also addresses the pain and needle–phobia caused by needle injection (21).

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In a word, we have successfully prepared equine anti-smallpox immunoglobulin $F(ab')_2$ products by using the latest technology of modern biopharmaceuticals, which is expected to become a "life-saving drug" for the smallpox virus. Clinical approval has been obtained, and relevant clinical trials are in intense progress.

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Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure 1. Detection results of purified antigens of vaccinia virus Tiantan strain. (A)TEM results. Transmission electron microscopy showed that the purified virus conformed to the morphology of vaccinia virus. (B) HPLC test results. The purity of purified antigen was 98.4% by HPLC.

Figure 2. Test results of horse anti smallpox virus immunoglobulin $F(ab')_2$. (A)SDS–PAGE of equine purified $F(ab')_2$. Lane 1–3,purified $F(ab')_2$. (B) HPLC of purified $F(ab')_2$ with the $F(ab')_2$ peak indicated.

Figure 3. (A) The change of average body weight of mice in prevention experiment of horse anti smallpox virus immunoglobulin $F(ab')_2$. Four concentrations of horse anti smallpox virus immunoglobulin were prepared by PBS dilution, which were 5.2, 2.6, 1.3 and 0.65 mg/mL respectively; the same amount of normal saline was used as control. After each mouse was given 0.5 mL subcutaneously by needle–free devices, VVTT 1×10^6 PFU was challenged intravenously. The weight changes of mice were shown in

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the figure. (B) Determination of viral loads in mice' peripheral blood at various time points post challenge.

Figure 4. (A) Changes of average body weight of mice treated with horse anti smallpox virus immunoglobulin $F(ab')_2$. Twenty BALB/c mice were divided into 5 groups. Each mouse was injected intravenously with 1×10^6 PFU VVTT and observed for one week. Four concentrations of horse anti smallpox virus immunoglobulin were prepared by PBS dilution (5.2, 2.6, 1.3, 0.65 mg/mL), each mouse was injected 0.5 mL subcutaneously by needle–free devices, the same amount of normal saline was used as control. The weight changes of mice were shown in the figure. (B) Determination of viral loads in mice' peripheral blood at various time points after the treatment.