Introduction
Rabies is one of the most fatal central nervous system diseases and is a threat to humans and other mammals. The rabies virus (RABV) belongs to the Rhabdoviridae family and constitutes the prototype of the lyssa viruses[1]. Rabies kills more than 50,000 people and millions of animals worldwide every year[2]. The progress of infection is rapid, and the mortality rate is nearly 100%. The glycoprotein of the rabies virus (RABVG) has been studied extensively for many years. It is a crucial protein for determining the neurovirulent nature of the rabies virus and is an important antigen for inducing protective immunity[3]. Among the different antibodies elicited after immunization, neutralizing antibodies specific to the RABVG are thought to provide protection[4]. We screened out a human anti-RABVG single-chain variable fragment (scFv) from an immune phage antibody library[5]. Based on the discrepancies between the native conformations of scFv and IgG, if the scFv was shown to be a neutralizing antibody, we would not consider the IgG, for scFv having the same neutralizing activity. In addition, the small molecular weight, short half-life, and expression type of the inclusion body also restrained the therapeutic application of the scFv[6].

In the present study, the scFv was transformed into a Fab fragment with a larger molecular weight and longer half-life. Fab has the same native conformation as IgG. Accordingly, exploration of the immunological activity of Fab will be helpful in preparing the human IgG. In this study, we transformed the human anti-RABVG scFv into a Fab fragment and to analyze its immunological activity.

Materials and methods
The rabies virus strain CTN was provided by the Wuhan
were approved by the Ethics Committee on Laboratory Animals of Nanjing Medical University.

**Construction of human anti-RABV antibody Fab fragment**

The human V_H and V_L genes were amplified from the anti-RABVG scFv plasmid by PCR. The forward primer of V_H was V_HF: 5’-GCTGCCCACCCAGCCATGGCCAGCT-GGTGCAGCTGG-3’, which contained 21 complementary bases to the reverse primer of human IgG1 C_L (italicized). The reverse primer of V_H was V_HR: 5’-CGATGCGCTCTTGTTG-GAGGCCTAGAGACGGTGACCAGGGTTCC-3’, which contained 21 complementary bases to the forward primer of human IgG1 C_H1 (italicized) for overlap PCR. The human IgG1 constant domains were amplified using the forward primer V_HF: 5’-GGGCCCAGGCGGCCGAGCTGGACCAGCTCTGGCAGCTGG-3’, which contained the restriction endonuclease Sfi I site (underlined), and the reverse primer V_LR: 5’-GGGCCCAGGCGGCCGAGCTCTGGCAGCTGG-3’. The assembly of VH and C_H1 was performed for 2 min, followed by a final extension time of 10 min at 72 °C. The gene of C_L (italicized) for overlap PCR. The genes of C_H1 and C_L were joined to generate the Fd fragment by overlap PCR with a pair of primers — FdF: 5’-GCTGCCCACCCAGCCATGGCCAGCT-GGTGCAGCTGG-3’ and FdR: 5’-AGAAGCGTAGTC-ACCGAAGGC-3’.

The assembly of V_H and C_L was performed by PCR for 15 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 2 min, followed by a final extension time of 10 min at 72 °C. Similarly, human C_L and V_L fragments were joined to generate the light chain with the pair of primers LF: 5’-GGGCCCAGGCGGCCGAGCTTGACTGACATGTGCCACAGCT-3’ and LR: 5’-GGCCATGGCTGGTGGCCAGC-3’. The assembly of V_L and C_L was performed for 15 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 2 min, followed by a final extension time of 10 min at 72 °C. Similarly, human C_L and V_L fragments were joined to generate the light chain with the pair of primers LF: 5’-GGGCCCAGGCGGCCGAGCTTGACTGACATGTGCCACAGCT-3’ and LR: 5’-GGCCATGGCTGGTGGCCAGC-3’.

**SDS-PAGE and Western blot analysis**

The purified Fab091 fragment was resolved by 12% SDS-PAGE under reducing conditions with β-mercaptoethanol (β-ME). For Western blot analysis, Fab091 was detected by HRP-conjugated goat anti-human IgG (1:2000), and the blot was developed using the DAB/H_2O_2 system.

**Antigen binding assays for Fab091**

**Indirect ELISA**

A 96-well microplate (Costar, Washington, DC, USA) was coated with 2 μg/mL of rabies virus strain CTN. The Fab091 fragment (0.2 mg/mL) and _E coli_-negative supernatant was added to antigen-coated wells in serial two-fold dilutions (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640), and bound antibodies were detected by HRP-conjugated goat anti-human IgG (1:5000). Each sample had two duplicate wells. PBS was used as a blank control, and its OD_{450} value was assigned as 0.
Competitive ELISA (C-ELISA) of Fab091 and scFv

Fab091 (0.5 mg/mL) was added into a 96-well microplate coated with RABV. Then the original anti-RABVG scFv was added at dilution ratios of 1:1, 1:5, 1:25, and 1:125 and incubated in these wells for 1 h at room temperature. After washing five times with PBST (20 mmol/L PBS, 0.05% Tween 20), HRP-conjugated mouse anti-human IgG (Fab specific) was added to the wells and incubated for 1 h at room temperature. An anti-Met scFv antibody was used as a negative control in lieu of anti-RABVG scFv, and the blank control lacked scFv. The final results were expressed as percentages of inhibition (PI). The PI values for the Fab091 were calculated with the following formula:

$$\text{PI} = 1 - \frac{(OD_s - OD_b)}{OD_b} \times 100\%$$

(s: sample, b: blank control)

A competitive ELISA kit was used to detect the neutralizing titer of Fab091. For the ELISA, the standard serum was obtained from volunteers who had been inoculated with rabies vaccine. The titers of neutralizing antibodies in the serum were detected by the FAVN method, and the serum was diluted to 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 IU/mL. The neutralizing antibodies in the ELISA were murine monoclonal antibodies against RABV conjugated with horseradish peroxidase. One hundred microliters of HRP-conjugated antibodies against RABV were mixed with 100 μL of different titers of standard serum (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 IU/mL) and added to a RABV-coated ELISA microplate. Meanwhile, 100 μL of HRP-conjugated antibodies and 100 μL of Fab091 were mixed in the coated microplate. Every sample had two duplicate wells. Following incubation for 45 min at 37 °C, the plates were washed, and a tetrathylbenzidine (TMB) substrate solution was added. After incubation for 15 min at 37 °C, the reaction was stopped by adding 2.0 mol/L sulfuric acid, and the OD450 was measured by a Multiskan Spectrum Microplate Photometer. The neutralizing antibody titer of Fab091 was calculated according to the OD450 values and the titers of the standard samples.

Immunoprecipitation (IP) and mass spectrometry (MS)

A mixture of 20 μL of Fab091 and 40 μL of CTN was resuspended in 1 mL of PBS and incubated for 1 h at 4 °C. Protein G magnetic beads (30 μL) were added to the mixture for immunoprecipitation (IP). The mixture was whirled gently and incubated with agitation at 4 °C for 12 h. The beads were collected by centrifugation at 4000×g for 5 min and then washed three times with PBST. The supernatant was removed. Finally, the beads were resuspended with loading buffer and boiled, and the proteins were resolved by 12% SDS-PAGE. The protein solution was added. After incubation for 15 min at 37 °C, the reaction was stopped by adding 2.0 mol/L sulfuric acid, and the OD450 was measured by a Multiskan Spectrum Microplate Photometer. The neutralizing antibody titer of Fab091 was calculated according to the OD450 values and the titers of the standard samples.

Affinity analysis by surface plasmon resonance (SPR)

SPR was performed on a BIACore T100 (GE, Piscataway, NJ, USA) analytical system. RABV was diluted to 5 μg/mL with acetate buffer (10 mmol/L sodium acetate, pH 5.5, GE, Piscataway, NJ, USA) and immobilized on the surface of a CM5 sensor chip (GE, Piscataway, NJ, USA) to capture purified Fab091. Fab091 was diluted by HBS-EP buffer (GE, USA) at concentrations ranging from 31.25 to 1000 nmol/L and performed at a constant flow rate of 30 μL/min for 3 min at 25 °C. The association time was 180 s, and the dissociation time was 600 s. The constants for association (k_a) and dissociation rate (k_d) and the equilibrium constant for dissociation (K_D=kd/ka) were obtained using global fittings from the Langmuir binding model (1:1 binding model). The sensor grams were evaluated using the BIACore T100 evaluation software (GE, Piscataway, NJ, USA).

Detection of Fab091 neutralizing activity

To perform the FAVN test, BHK-21 cells and the RABV strain CVS-11 were used. The positive control serum against RABV was obtained from adult dogs vaccinated with rabies vaccine. The neutralizing antibody titers were determined by the Weybridge Laboratory (UK), a rabies reference laboratory of OIE, the World Organization for Animal Health. The serum was then diluted to 0.5 IU/mL and used as a standard serum. Serial three-fold dilutions of the positive and negative control sera and the Fab091 were made in 100-μL volumes in microplate wells. Each sample was added to four adjacent wells and serially diluted five times using a multichannel pipette. Fifty-microliter challenge virus suspensions containing 100 TCID50 were added to each well. The microplates were incubated for one hour at 37°C in a humidified incubator with 5% CO2. Following incubation, 2×10^3 BHK-21 cells in 50 μL of culture medium were added to each well, and the plates were washed three times with PBST, the membrane was incubated with the HRP-conjugated goat anti-mouse IgG (Sigma, St Louis, MO, USA). Then the original anti-RABVG scFv, and the blank control lacked scFv. The final results were expressed as percentages of inhibition (PI). The PI values for the Fab091 were calculated with the following formula:

$$\text{PI} = 1 - \frac{(OD_s - OD_b)}{OD_b} \times 100\%$$

(s: sample, b: blank control)
were incubated for another 48 h. Thereafter, the medium was discarded, and the plates were rinsed in PBS (pH 7.2) and in acetone (80% in distilled water). The plates were then fixed in 80% acetone for 30 min at room temperature and air-dried. The staining was carried out by adding 50 μL of fluorescein-isothiocyanate-conjugated anti-rabies serum. After incubation for 30 min at 37°C, the plates were washed twice with PBS. The FAVN result was assessed with a fluorescence microscope (image A; Zeiss, Germany). The total area of each well was examined. The well was considered positive if one or more fluorescent cells were observed; otherwise, it was considered negative. The 50% endpoint of the antibody (D50) content of the test sample and virus titers (TCID50) were calculated according to the Spearman-Karber method[17]. The neutralizing titer of Fab091 was calculated based on the number of negative wells and their dilutions, compared with those of the standard serum included in the test[18].

Animals and inoculation
A lethal animal model mimicking rabies exposure was used as described elsewhere[19–21]. The mice were treated as described in Table 1. Four-week-old pathogen-free Kunming mice (10–12 g, 8 mice/group, 9 groups) were infected with 100 LD50/0.05 mL CVS-24 on d 0 and d 7. The mice were inoculated in the hind limb with rabies vaccine (Sanofi-PASTEUR SA, France). In addition, human rabies immunoglobulin (HRIG, Taibang Health, Taian, China) at 20 IU/kg and Fab091 (treated with 32, 20, 8, 2, and 0.5 IU/kg) were administered on d 0. The mice were evaluated for 28 d for clinical signs of neuritis and death. In the control group, the mice were injected with 100 LD50/0.05 mL of the CVS-24 strain. The experiments using the CVS-24 strain were performed in a Biosafety Level 3 Laboratory (BSL-3)[22].

Table 1. Rabies virus postexposure prophylactic trial.

| Treatment   | Rabies Virus dose (IU/kg) | HRIG+ vacc | Fab091+ vacc | PBS |
|-------------|---------------------------|------------|--------------|-----|
| A           | 0                         | 0.5        | 2            | 8   | 20  | 32  | 20  | 20  | 0   |
| B           |                           |            |              |     |     |     |     |     |     |
| C           |                           |            |              |     |     |     |     |     |     |
| D           |                           |            |              |     |     |     |     |     |     |

+ = disposed; – = undisposed; HRIG, human rabies immunoglobulin; PBS, phosphate-buffered saline.

Statistical analysis
Kaplan-meier method was used for survival analysis of Kunming mice after rabies virus challenge. Statistical analyses were performed with SPSS 11.5 statistical software package (SPSS Inc, Chicago, IL, USA). A P value <0.05 was accepted as statistically significant.

Results
Amplification of the human anti-RABV antibody Fab gene fragment and construction of the expression vector
The human anti-RABV antibody Fab gene fragment was amplified in three rounds of PCR, as described in Materials and methods. The cloning strategy is shown in Figure 1.

Purification and detection of soluble Fab fragment
Purified Fab091 was resolved by 12% SDS-PAGE under reducing conditions. Both the Fd (34 kDa) and L chains (26 kDa) were detected in the monomeric form (Figure 2A). The expression of Fab091 was confirmed using Western blotting (Figure 2B).

Specific binding of Fab091 fragment to RABV
Binding of the Fab091 fragment to RABV was detected by indirect ELISA as shown in Figure 3. The OD450 values showed a gradient change that was accompanied by a decreasing concentration of Fab091.

In the competitive ELISA, as shown in Figure 4A, the PI of Fab091 ranged from 73.65% to 11.83% concomitant with the decreasing concentration of scFv. Less than 10% inhibition was observed for the anti-Met scFv negative control. A standard curve for the competitive ELISA is shown in Figure 4B. The range of the standard curve was 0 to 8 IU/mL. If the OD450 value was between 0 to 8 IU/mL, the neutralizing antibody titers could be read from the curve. In Figure 4B, the titers of standard serum decreased with increasing OD450 val-
ues. The OD_{450} of the test sample was 1.022, so the result was negative.

IP was also used for the detection of the specific binding of Fab to the rabies virus strain CTN (Figure 5A). Four peptide sequences (Table 2) matched with RABVG by MS analysis (Figure 5B) were found when the identified peptides were compared with the known sequences of RABVG in the SWISS PROT database. Mass tolerance was allowed within 0.05%.

Table 2. Amino-acid residue sequences of matched peptides.

| Relative intensity | Amino-acid residue          |
|-------------------|-----------------------------|
| 1                 | 983.431 TCGFVDER            |
| 2                 | 1070.575 STQHGLGGTGR        |
| 3                 | 2118.972 YEESLHNYPDYHWLR    |
| 4                 | 2708.251 YVLMASGLJALMLTFLMTCCR |

Figure 2. SDS-PAGE and Western blot of the purified Fab091 fragment after purification. The purified Fab091 was resolved in 12% SDS-PAGE under reducing conditions and stained with Coomassie blue. The heterodimer was dissociated into light chain (26 kDa) and Fd (34 kDa). (A) Lane 1: protein marker (#0671, Fermantas, Burlington, Ontario, Canada); Lane 2: the purified Fab091 fragment. (B) Electroblotted Fab091 fragments were detected by goat anti-human IgG-HRP conjugate (1:2000 dilution).

Figure 3. Indirect ELISA of the different dilutions of Fab091 fragment. The ELISA plate was coated with rabies virus strain CTN at 2 μg/mL. (1–8): The virus reacted with Fab091 which was in serial two-fold dilution (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320) and incubated for 2 h at room temperature. E. coli Top10F' supernatant was used as control. The plate was washed five times with PBST, followed by incubation with goat anti-human IgG HRP-conjugated (1:5000).

Figure 4. Competitive ELISA detection. (A) The rabies virus strain CTN was used as coated antigen for Competitive ELISA of scFv and Fab091. (1–4): The scFv was diluted to 1:1, 1:5, 1:25, 1:125, and incubated with Fab091. PI of Fab091 ranged from 73.65% to 11.83% (73.65%, 60%, 24.95%, and 11.83%) with decreasing concentration of scFv. Less than 10% inhibition was observed for negative anti-Met scFv. (B) Standard curve for Competitive ELISA: The 96-well microplate was coated with RABVG. HRP-conjugated antibodies against RABVG were mixed with different titers of standard serum (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 IU/mL). Meanwhile, HRP-conjugated antibodies and Fab091 were mixed in the coated microplate and incubated. The mean OD_{450} value of Fab091 well was 1.022, according to the standard curve ranging from 0 to 8 IU/mL, so the corresponding titre was 0.

Protein matching with a MASCOT score of >69 was considered statistically significant (P<0.05).

Affinity assay of Fab091
The binding affinities between rabies protein and purified Fab091 were analyzed by Biacore T100 (GE, Piscataway, NJ, USA). Fab091 had a high affinity with a K_D of 6.268E-10 M. (χ^2=1.09, U=5). The interaction between Fab091 and rabies virus is shown in Figure 6.

Neutralizing activity of Fab091
In the FAVN, no fluorescence was seen in the wells until the standard serum was diluted nine times. There were still two negative wells when the serum concentration was at 1:27 dilution. For the test sample Fab091, no fluorescence was seen until the wells were diluted to 1:81 (12 μg/mL). There was only one negative well at 1:243 dilution. According to the dilution rates and the total number of negative wells, compared with the standard serum (0.5 IU/mL), the neutralizing antibody titer of Fab091 was calculated to be 10.26 IU/mL.

Activity identification in vivo
The number of deaths observed in Kunming mice after infec-
Figure 5. IP and MS analysis of RABVG. (A) Proteins immunoprecipitated by Fab091 were separated by SDS-PAGE and probed with RAB-50 by Western blot. (1): Protein marker; (2–3): One protein was recognized by RAB-50. The molecular weight of the protein was about 67 kDa. (4): BHK-21 lysate was used as the negative control to replace Fab091 in the IP. (B) The corresponding 67 kDa band on the polyacrylamide gel was analyzed by mass spectrometry, which was identified as RABVG. MS spectrums of fragment ions were from the 67 kDa protein. Four major \(m/z = 983.431, 1070.575, 2118.972, 2708.251\) ions were detected.

Figure 6. SPR analysis of Fab091 and rabies virus interaction. Fab091 (1 mg/mL) was subjected to SPR analysis. CM5 sensor chip was activated by the injection of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS). Rabies virus was coated on the CM5 chip, and the surface was washed with 10 mmol/L HEPES (pH 7.4) for 200 s and then Fab091 was injected. Dilution rates of Fab091 were 31.25, 62.5, 125, 250, 500, 1000 nmol/L (Chi\(^2\) = 1.09, U = 5, \(R_{\text{max}}\) = 13.05 RU). (A) Association stage; (B) Dissociation stage.

Figure 7. Kaplan-Meier survival curve for Kunming mice after rabies virus challenge. Mice (n=8 per group) were challenged with CVS-24 strain on d 0. Three hours later (d 0), the mice in treatment groups were inoculated with rabies vaccine and treated either with 32, 20, 8, 2, 0.5 IU/kg Fab091 or 20 IU/kg HRIG. The treatment groups also included 20 IU/kg Fab91 group. The mice in control groups received only PBS. The mice were evaluated twice daily and were sacrificed when neurological signs appeared. Kaplan-Meier survival curves were shown for d 0 to d 28. (A) Vaccine; (B) Vaccine+0.5 IU/kg Fab091; (C) Vaccine+2 IU/kg Fab091; (D) Vaccine+8 IU/kg Fab091; (E) Vaccine+20 IU/kg Fab091; (F) Vaccine+32 IU/kg Fab091 (P<0.05, Logrank test); G: 20 IU/kg Fab091; H: Vaccine+20 IU/kg HRIG (P<0.05, Logrank test); I: PBS.
received vaccine only. The group treated with vaccine and HRIG had a survival rate of 62.5% (5/8). The survival rates were 50% (4/8), 50% (4/8), 37.5% (3/8), 37.5% (3/8), and 25% (2/8) when the mice were treated with 32, 20, 8, 2, and 0.5 IU/kg Fab091, respectively, and vaccine. The groups that received vaccine and 32 IU/kg Fab091 (Figure 7) or vaccine and 20 IU/kg HRIG (Figure 7) were provided with a level of protection against the rabies virus compared to the PBS group (P=0.0485 and P=0.038, respectively; Logrank test).

Discussion

Equine anti-RABV immunoglobulin (ERIG) and human anti-RABV immunoglobulin (HRIG) have been used for passive rabies immunotherapy. However, ERIG can lead to allergic reactions and blood diseases, and HRIG is expensive and often in short supply.[23–25]. Recombinant DNA technology and bacterial expression systems used to obtain active antibodies were very attractive compared to hybridoma technology, and antibody molecules can be further engineered to increase their binding affinity. Smaller antibody fragments (such as Fab and scFv) can be effectively used for diagnostic and therapeutic application due to their low immunogenicity and better tissue penetration.[27–30]. ScFv fragments are composed of V_H and V_L with a connecting peptide link. Fab fragments are more stable due to an additional domain-domain interface resulting from the C_H1-C_L association. This structure resulted in higher affinity of purified antibodies.[27–30].

Expression of heterologous proteins in bacterial systems can be influenced by many factors such as codon usage, DNA-protein interactions, regulatory factors for transcription and translation, and culture conditions. In the present study, the amber stop codon (cross) enabled the production of soluble Fab fragments in a non-suppressor strain of E. coli. We optimized the expression conditions and found that Fab could be expressed in a soluble form at room temperature (22 to 25 °C).

If the inducing temperature was 30 or 37°C, Fab was most often expressed in the form of inclusion bodies. In the process of purification, it was found that the amount of purified Fd fragments was less than that of the light chain. This result was possibly due to the pH value of the binding or washing buffer used for purification being unsuitable for purification of the Fd fragment. Furthermore, we found that the Fab091 fragment was easily eluted by phosphate buffer with a concentration of 100 mmol/L imidazole and that the eluted protein had a higher purity. In Figure 3, OD_{450} values showed a gradient change that was accompanied by a decreasing concentration of Fab091, which illustrated the high specificity of Fab091 against the RABV. Furthermore, IP and MS analysis results also demonstrated that Fab091 binds specifically to RABVG. The competitive ELISA indicated that Fab091 and the original scFv shared the same epitope. The neutralizing antibodies in the ELISA kit had different epitopes against RABVG from Fab091.

The most critical property of Fab091 was its neutralizing potency to inhibit the entry of CVS-11 into BHK-21 cells. A sufficient level of antibody to inhibit entry is considered to be 0.5 IU/mL, according to the recommendations of the World Health Organization and the Centers for Disease Control and Prevention.[31]. Compared with the reported neutralizing concentrations of FabRV01 and FabRV02, which neutralized the CVS-11 at 146 μg/mL,[32], Fab091 was used to neutralize the virus at a concentration of only 12 μg/mL.

Vaccination alone in the in vivo study was not sufficient to protect mice from rabies, whereas the treatment of mice with both vaccine and HRIG (20 IU/kg)/Fab091 (32 IU/kg) provided better protection against rabies than vaccine alone (P<0.05, Logrank test). However, the survival rates of the
groups treated with vaccine and Fab091 (32 IU/kg) were lower than that of the vaccine and HRIG (20 IU/kg)-treated group. This result could be interpreted as follows. First, the in vivo clearance of RABV is a complicated process. Compared with small-molecular-weight antibodies, the IgG antibody (HRIG), with antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity, has a greater scavenging capacity compared with antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity. However, Fab091 can only bind to a single epitope, limiting its ability to remove the virus.

In future studies, the Fab091 and human IgG Fc fragments could be fused and expressed in eukaryotic cells. The reconstitution of Fab091 to IgG is a strategy that could be used for future passive immunotherapy against RABV infection. Although Fab091 is different from scFv and IgG in biological function, tissue penetration, and half-life, the three together will not retain the same binding specificity but also play complementary functional roles. They, together with other monoclonal antibodies, in accordance with the WHO-recommended “cocktail” therapy, can be used for post-exposure prophylaxis of rabies virus.

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Author contribution
Chen LI carried out the molecular biology experiments and drafted the manuscript. Jin ZHU designed the study. Zhen-qing GUAN and Xiao-hong GUAN were involved in the design of the study, and Feng ZHANG, Hong LIN, Jin ZHU, Zhong-can WANG, Xian-jian LIU, Zhen-qing FENG, and Xiao-hong GUAN helped to draft the manuscript.

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