Introduction

Rabies though a major zoonotic yet a neglected disease, is typically caused by the rabies virus (RV). The World Health Organization (WHO) estimated that 40,000–100,000 human deaths are caused by rabies annually [16, 29]. Rabies occurs primarily in Asia and Africa where animal control, vaccination programs and effective human post exposure prophylaxis (PEP) are either not widely available or not effectively applied [3, 13]. Apart from annual doses of rabies vaccine, PEP remains the primary approach for preventing the development of rabies in humans after exposure to the virus [6, 21].

Modern PEP using effective biologics relies upon the neutralization of the virus by early administration of passive antibodies (immuno globulins of human or equine origin) and inactivated human rabies vaccines [6, 14, 29]. However, current schedules for human prophylaxis consist of 4 or 5 doses of inactive rabies vaccine over 14–28 days [31]. Attenuated vaccines are characterized by their low required doses and high efficacies for rabies PEP [5]. Live attenuated vaccines like ERAG333 and SPBAAN-GAS-GAS-GAS can protect 80% of mice after 24 hour PEP if administered through IM route [18]. However, live SPBAAN-GAS-GAS-GAS can protect 100% of mice after 2 days of PEP since live attenuated RV can stimulate extensive strong immune responses in mice when administered by IC route [12]. However, administration of RABV via IC route is not recommended by WHO [29]. Hence, we introduced a Chinese attenuated rabies virus SRV9, which is derived from an attenuated strain (SAD). For this experiment, forelimb
was selected as a site of injection with an aim to determine whether forelimb administration of attenuated RV vaccine is effective against the lethal CVS-11 infection in the CNS or not. Street rabies virus was selected for the same experiment in next work.

In present study, the safety of live attenuated SRV9 delivered by both (IC & IM) routes was evaluated in suckling mice of 18–22 days of age. Moreover, the efficacy of SRV9 treatment was confirmed by IM injection in the presence of CVS-11 infection in the brain. The primary data demonstrated that SRV9 treatment protected 40% of the mice from rabies when RV was detected in the brain suggesting that this treatment strategy could be a new and effective method of PEP in the future.

Material and methods

MICE

Female ICR (imprinting control region) mice [Mus musculus] were obtained from the Changchun H and N Animal Breeding Center for Medicine and acclimatized for a minimum of 72 hours. All animal handling and experimental procedures were performed in compliance with the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee guidelines.

VACCINES AND VIRUSES

A commercially available inactivated human diploid cell vaccine (HDCV) was selected randomly based on the availability of licensed products in France. Human rabies immune globulin (HRIG) was obtained from Imogam® Rabies-HT (Sanofi pasteur, 150 IU/ml).

The SRV9 strain [17, 10] (Gen Bank Accession No.AF499686), a candidate attenuated rabies vaccine strain was cloned from SAD B-19 while CVS-11 (Gene Bank Accession No.GQ918139) was a fixed pathogenic RV strain. CVS-11 (10^3 MICLD_{50}/30 µl) and SRV9 (10^7 TCID_{50}/ml) were prepared and subsequently stored at -80 °C.

VIRULENCE OF SRV9

Animal care and experimental procedures were performed in compliance with the Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Guidelines. Approximately, 18–22 days old ICR out bred suckling mice were divided into 5 groups by age (30 animals per group). Each group was subdivided into 3 subgroups, which were inoculated with 30 µl of SRV9 (10^7 TCID_{50}/ml) IC, 50 µl of SRV9 (10^7 TCID_{50}/ml) and PBS buffer (0.01 M, pH 7.4) IM respectively. Animals were observed daily for the signs of illness. Sick animals were euthanized by CO\textsubscript{2} inhalation followed by cervical dislocation. The brains of mice were removed for RV diagnosis by DFA test [26].

The above procedure of animal monitoring and euthanasia was applied in all experiments unless otherwise stated.

LOCALIZATION OF CVS-11 BY RT-PCR

The RT-PCR and quantitative real time PCR assays were applied to trace the spread of RABV from the site of inoculation to the CNS in the mice as previously described [5]. Three-week-old female ICR mice were divided into 5 groups with each group containing 10 mice. All mice were infected with 50 µl of 10^3 MICLD_{50}/30 µl CVS-11 via inoculation into the left hind limb gastrocnemius muscle, and then each group was euthanized by CO\textsubscript{2} inhalation at 2, 3, 4, 5, or 6 days of PE respectively. Brain samples were collected using sterile techniques to prevent cross-contamination. Total RNA was extracted using TRIzol reagent according to the manufacturer’s recommendations (Cat No. 15596-01, Invitrogen Corp). For reverse transcription (RT), 32 µl of total RNA was heated at 65 °C for 5 min, quickly chilled on ice for at least 2 min, and transferred to a reaction tube of Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, USA) with 1 µl of random primer [pd[N] 6, 0.2 µg/µl] (TaKaRa, Japan). After incubation at 37 °C for 60 min, the synthesized cDNA product was used for PCR. The PCR primers for the RV N gene [16] are listed in Table 1. PCR reaction setting was as follows: one cycle at 95 °C for 1 min for initial denaturation; 30 cycles of 95 °C for 15s, 55 °C for 20 s, and 72 °C for 30s; and a final termination at 72 °C for 10 min. The PCR products were identified on a 3% Ultrapure TM Agarose gel (Invitrogen, USA) and visualized by ethidium bromide staining under UV illumination with 20bp DNA markers (TaKaRa, Japan).

PROTECTION INDUCED BY SRV9, INACTIVATED HUMAN RABIES VACCINE OR HRIG DURING RV INFECTION.

Administration of CVS-11 by IM injection

Thirteen groups of mice (10 mice per group) were infected with 50 µl of 10^3 MICLD_{50}/30 µl CVS-11 via intramuscular injection into the left hind limb gastrocnemius muscle in group 1. Groups 2–13 were divided into 2 subgroups (10 mice per group), one subgroup was inoculated with 0.01 M, pH 7.4 PBS buffer (control) and the other subgroup was inoculated with 30 µl of SRV9 (10^7 TCID_{50}/ml) IC. Each subgroup was euthanized by CO\textsubscript{2} inhalation at 2, 3, 4, 5, and 6 days of PE respectively. Brain samples were collected using sterile techniques to prevent cross-contamination. Total RNA was extracted using TRIzol reagent according to the manufacturer’s recommendations (Cat No. 15596-01, Invitrogen Corp). For reverse transcription (RT), 32 µl of total RNA was heated at 65 °C for 5 min, quickly chilled on ice for at least 2 min, and transferred to a reaction tube of Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, USA) with 1 µl of random primer [pd[N] 6, 0.2 µg/µl] (TaKaRa, Japan). After incubation at 37 °C for 60 min, the synthesized cDNA product was used for PCR. The PCR primers for the RV N gene [16] are listed in Table 1. PCR reaction setting was as follows: one cycle at 95 °C for 1 min for initial denaturation; 30 cycles of 95 °C for 15s, 55 °C for 20 s, and 72 °C for 30s; and a final termination at 72 °C for 10 min. The PCR products were identified on a 3% Ultrapure TM Agarose gel (Invitrogen, USA) and visualized by ethidium bromide staining under UV illumination with 20bp DNA markers (TaKaRa, Japan).

**Table I: Primer sequences used for PCR**

<table>
<thead>
<tr>
<th>Primer Sequences</th>
<th>5’-CGCTGCATTTTYATCAAAGTCAAG-3’</th>
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<tbody>
<tr>
<td>fw [forward]</td>
<td>5’-TGMAYGGAGTTCAAGGGAGGAC-3’</td>
</tr>
<tr>
<td>rv [reverse]</td>
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injection to the left hind limb gastrocnemius muscle. One group was designated as the ‘virus only’ group; the other groups were inoculated with 50 µl (0.1 international unit, IU) of inactivated HDCV and 50 µl of 10⁷ TCID₅₀/ml attenuated live SRV9 or 50 µl of inactivated HDCV along with HRIG. Inoculations were performed by inoculation to the left forelimb deltoid muscle at 3, 4, 5 or 6 days after infection with CVS-11, while HRIG was inoculated by IM distant from the vaccination site. Animals were observed daily for 2 months and euthanized when clinical signs of rabies appeared. The survival rate of each group was calculated.

Inoculation of CVS-11 by IC injection

Ten groups of mice (10 mice per group) were intracerebrally infected with 30 µl of 10 MICLD₅₀/30 µl CVS-11. One group was designated as the ‘virus only’ group; the other groups were inoculated with 50 µl (0.1 international unit, IU) of inactivated HDCV, 50 µl of 10⁷ TCID₅₀/ml attenuated live SRV9, or 50 µl of inactivated HDCV in combination with HRIG. Inoculations were performed by injection to the left forelimb deltoid muscle at 0 h, 1 or 2 days after infection with CVS-11 and HRIG was inoculated as above mentioned. Animals were kept in daily observation for 2 months and then subsequently euthanized when clinical signs of rabies appeared. The rates of protection by each treatment were conferred and calculated.

Rapid Fluorescent Focus Inhibition Test (RFFIT)

The viral neutralizing antibodies of the survival mice were measured by RFFIT as described previously [23].

Statistical analysis

P-values were calculated using the χ² test and the continuity corrected χ² test.

Results

VIRULENCE OF SRV9

The results showed that no mortality was observed among SRV9 inoculating ICR mice that were 18–22 days old, neither by IC nor IM administration. All euthanized animals had RV antigens detected by Direct Fluorescent Antibody (DFA) test.

LOCALIZATION OF CVS-11 BY RT-PCR

Brain samples were collected from mice inoculated with CVS-11 at 2, 3, 4, 5 or 6 days post exposure. Amplification of the RV N gene demonstrated that no positive results were detected at 2nd days of PE. However, at 3rd day after PE, 80% (8/10) of the mice brains were found positive for RV N genes. After 3rd day, all the brain samples were reported positive (Figure. 1).

PROTECTION INDUCED BY SRV9, INACTIVATED HUMAN RABIES VACCINE OR HRIG DURING CVS-11 INFECTION.

Challenge with CVS-11 by IM injections

At 4, 5 and 6 days of post exposure, all control mice and HDCV along with HRIG treated mice developed rabies following an incubation period of 6–12 days from the initiation of PEP. These mice were euthanized when they exhibited signs and symptoms of rabies. Only 20% (2/10) of the mice survived when one dose of inactivated HDCV in combination with HRIG was administered at 3 days of PE. However, 40% (4/10), 40% (4/10), 30% (3/10) and 10% (1/10) of the mice survived when one dose of live attenuated SRV9 was administered at 3, 4, 5 or 6 days PE, respectively. Comparably, 20% (2/10), 10% (1/10), 0% (0/10) and 0% (0/10) of the mice survived when one dose of inactivated HDCV vaccine alone was administered at 3, 4, 5 or 6 days PE respectively. All euthanized animals had RV antigens detected by direct fluorescent antibody (DFA) test [26]. During this experiment, early death was observed in all treatment groups that exhibited significant signs of rabies following an incubation period of 6 days compared to control group (Figure 2a, 2b).
Challenge with CVS-11 by IC injections

The control mice and all mice that were administered with inactivated HDCV in combination with HRIG, developed rabies following an incubation period of 6–9 days regardless of the initiation of PEP. These mice were euthanized when they exhibited signs of rabies. However, 30% (3/10), 10% (1/10) and 0% (0/10) of the mice survived when PEP in the form of one dose of live attenuated SRV9 was administered at 0, 1 or 2 days PE. In comparison, 10% (1/10), 0% (0/10) and 0% (0/10) of animals survived when PEP in the form of one dose of inactivated HDCV vaccine alone was administered at 0, 1 or 2 days PE. All euthanized animals possessed RV antigens detected by direct fluorescent antibody (DFA) test. During all of these procedures, the “early death” phenomenon was observed again in all experimental treatment groups (Figure 3a, 3b).

Comparison of the survival rates among inoculums

The survival rates of mice that were administered live SRV9, inactivated HDCV or inactivated HDCV in combination with HRIG, were analyzed using the continuity corrected χ² test. The significance levels were calculated for the following comparisons: live SRV9 vs. inactivated HDCV (P<0.01), live SRV9 vs. inactivated HDCV in combination with HRIG (P<0.01), and inactivated HDCV vs. inactivated HDCV in combination with HRIG (P>0.05) (Table II).

Neutralizing antibodies in mice that survived infection

The average VNA titer in each surviving mice was measured at 15, 35 and 60 days. The titers in all surviving ICR mice were higher than 0.5 IU/ml, and the titers in mice inoculated with live SRV9 were 1 IU/ml higher than those inoculated with the HDCV vaccine (Figure 4A, 4B).

Discussion

Inactivated rabies vaccines have been extensively used to manage rabies in developed regions of the world. However, financial and delivery considerations dictate that a single-dose of rabies vaccine would be required to control rabies. This vaccine must be safe and able to confer long-lasting immunity after administration. In this study,
these considerations were fully abided by SRV9 which was investigated in these experiments.

When Live SRV9 was injected through both IM and IC routes to 18-22 days old ICR mice, it was observed that even not a single mice developed rabies which indicated that SRV9 was safe for adult mice.

After subsequent exposure to rabies virus, the spread of infection depends upon many factors including the RV genotype, pathogenicity of the RV, the route and the severity of the exposure, the host susceptibility to RV and the immune status of the host [1, 4, 25]. CVS-11, a genotype I and fixed pathogenic strain, multiplied well in the CNS. Thus, post-exposure prophylaxis of fixed strains must be carried out earlier than street strains. In this study, the PEP using SRV9 was performed at 3rd day of PE, in which CVS-11 was present in the CNS suggesting confirmation of the effects of SRV9.

Rabies virus can escape early recognition by the immune system in the periphery via limited replication, minimized glycoprotein expression [4], suppressed interferon response [18] and preventing anti-apoptotic stimulation. When the virus was injected into hind limb muscle, either it replicated at the site of inoculation or entered directly into the peripheral nerves [4, 18, 22], then spreads to the CNS using fast retrograde axonal transport at a rate of approximately 50–200 mm/day [4, 9]. For ensuring survival pathogenic RV must be cleared from the body, however, RV is very difficult to clear out from the body due to nervous barrier [15]. Our data showed that expression of RV N gene was detected in 80% mice brain on 3rd day after peripheral injection of CVS-11, 20% of the mice survived in one dose of HDCV in combination with HIRG. In contrast, the survival for one dose of live SRV9 was 40%. Until now, most examples of the clearance of lethal rabies virus from CNS had been associated with the permeability of the blood brain barrier BBB [4, 8, 9, 20]. Live attenuated rabies virus can increase the permeability of the BBB [3, 9], either by periphery or intracranially.

Upon Comparing with live SPBAANGAS-GAS-GAS, the higher survival of SRV9 might be due to following reason: 1) SRV9 had a Arg333 mutation, which was nonpathogenic for adult mice after IC inoculation, and those of Gly53→Glu53 . His192→Arg192 , Thr311→Ala311 mutations were also seen in the same gene [10, 19, 27, 28]; 2) the dose of CVS-11 used for PE by IM may be lower than Milosz, et al [18], although all the control mice succumbed to rabies. In this study, the high survival rate was further confirmed with 10 MICLD50 /30 µl by IC, resultantly, only 30% mice were survived. All these defined the fact that SRV9 could protect mice when pathogenic RV spread in the CNS. The characteristic of SRV9 would be further assessed under challenged by highly dose pathogenic RV.

As the normal route was always focused on IM but the effect of live SRV9 vaccine may be modified using different inoculation routes. Nevertheless, more functional sites can be explored like masseter muscle, especially for the challenged one. When inactivated rabies vaccine was used for human beings, deltoid muscle proved to be the best site of inoculation due to relatively close distance to CNS [24].

B cells and cytokines play important role in clearance of pathogenic RV from CNS [7, 32]. Mice that lack B cells developed rabies when they were infected through intranasal route with attenuated CVS-F3 [2, 8]. Similarly, results showed that IC administration of the attenuated RV SPBAAN-GAS-GAS-GAS and recombinant rabies virus expressing GM-CSF can eliminate pathogenic DOG4 RAVB infection in the brain [3, 9, 11], the SRV9 might also stimulate both of them.

In present PEP experiments, the demonstration of “Early death” phenomenon [30] is in accordance with many other studies. RV exposed mice that were treated with the HDCV vaccine, live SRV9 and HRIG, died earlier than the virus control mice that may be mediated by low levels of antibody against rabies virus.

To summarize our discussion, live SRV9 is efficacious and therapeutic tool in PEP, although there is room for further research. Our ultimate goal is to find an effective vaccine to protect against RAVB infection of the CNS. The work presented here demonstrates the first step toward this goal.

Acknowledgements

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