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Efficiency of live attenuated and inactivated rabies viruses in prophylactic and post exposure vaccination against the street virus strain

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Summary. – Rabies remains an enigmatic and widely discussed global infectious disease and causes an increasing number of deaths. The currently used highly effective prophylactic and post exposure (p.e.) vaccination depends solely upon inexpensive, effective and safe vaccines to counteract the spread of the disease. In this study, the potential of an attenuated Chinese rabies vaccine (SRV9) strain in prophylactic and p.e. vaccination against the street strain of rabies virus (RV) was evaluated in mice. Prophylactic vaccination consisting of one intramuscular (i.m.) dose of SRV9 protected 100% of mice from intracerebral (i.c.) challenge with a lethal dose of the street virus. The latter was detected in the brain of mice at day 6 post challenge by RT-PCR. Post exposure vaccination was performed at days 1, 2, 3, 4, 5 and 6 post infection (p.i.) with either SRV9 or inactivated rabies vaccine. The survival rates after i.m. inoculation of SRV9 at the indicated days were 70%, 50%, 30%, 20%, 10%, and 0%, respectively; the corresponding survival rates for the inactivated rabies vaccine were 30%, 20%, 10%, 0%, 0%, and 0%, respectively. However, 100%, 90%, 70%, 50%, 20%, 10%, and 10% of mice survived after i.c. inoculation of SRV9 at the indicated days. The increased permeability of the blood-brain barrier and the infiltration of CD19+ B cells into the central nervous system after i.c. inoculation of SRV9 are regarded as prerequisites for the clearance of the street virus. The obtained data suggest that SRV9 is a promising candidate for prophylactic and p.e. vaccination against rabies infection and that it exhibits a potential for the control of rabies in China.

Keywords: attenuated rabies vaccine; inactivated rabies vaccine; post exposure vaccination

Introduction

Rabies is a well known zoonotic disease throughout the world. Each year, about 55,000 human deaths occur due to this disease (WHO, 2005). In most developing countries, dogs are considered as the major rabies reservoir (Faber *et al*., 2009). In developed countries, where canine rabies has been eliminated, wild animals like raccoons and skunks are still an important target species and these have become the main target species particularly in the USA (Dietzscholdand and Schnell, 2002; Dietzschold *et al*., 2004). Until now, especially for the species at risk of rabies virus (RV) infection, prophylactic and p.e. vaccinations remain the main and sole approach to prevent the disease (Franka *et al*., 2009; Wu *et al*., 2011).

Inactivated rabies vaccines, which have been always praised by their safety, have a long history in rabies control. However, producing large amounts of high titer vaccines would be a large economic burden in developing countries (Knobel *et al*., 2005; Hampson *et al*., 2011). Therefore, high efficacy and cheap rabies vaccines are still an urgent need in

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Abbreviations: BBB = blood-brain barrier; CNS = central nervous system; i.c. = intracerebral(ly); i.m. = intramuscular(ly); p.i. = post inoculation/infection; p.e. = post exposure; PEP = post exposure prophylaxis; PrEP = pre exposure prophylaxis; RV = rabies virus

those areas. In Europe and North America, several live attenuated rabies vaccines such as SAD B19, SAG-1 and SAG-2 (Orciari *et al*., 2001; Faber *et al*., 2009; Muller *et al*., 2009; Prager *et al*., 2011) have been successfully applied as PrEP (pre exposure prophylaxis) by oral immunization to wildlife. These vaccines are derived from the Street Alabama Dufferin (SAD) field strain, isolated from a dog in North America in 1935 (Geue *et al*., 2008). To date, rabies has been reported in livestock such as swine, cattle, horses and sheep in developing countries (Liu *et al*., 2008), but unfortunately limited data about p.e. vaccination of animals has been reported (Hanlon *et al*., 2002). Moreover, existing data demonstrated that SAD-modified live RV vaccines could be used in p.e. vaccination of animals. A genetically engineered vaccine SPBAAN-GAS-GAS-GAS, derived from attenuated vaccine SADB-19, could protect 60% and 100% of mice when applied i.m. and i.c. 1 day p.e., respectively (Faber *et al*., 2009). SRV9 is a Chinese attenuated RV strain, which was subcloned from SADB19 and had been previously reported by many researchers (Geue *et al*., 2008; Ming *et al*., 2009; Jiao *et al*., 2011; Wang *et al*., 2011). Since there is, however, still just few data available related to its characteristics in prophylactic and p.e. vaccination against street RV, the presented study was mainly focused on this issue.

Materials and Methods

Mice. Female 18-22 day-old ICR mice were obtained from the Changchun H. and N. Animal Breeding Center for Medicine. They were acclimatized before experiments for a minimum period of 72 h after arrival and all animal handling and experimental procedures were carried out in accordance with the Centers for Disease Control and Prevention (China) and Institutional Animal Care and Use Committee guidelines.

Vaccine and viruses. Rabigen®mono (Virbac), a commercial inactivated rabies vaccine containing at least 1 IU/ml was used. SRV9 strain (GenBank Acc. No. AF499686), a Chinese vaccine strain candidate subcloned from SAD B-19 (Gueue *et al.* 2008; Jiao *et al.* 2011) had a titer of 107 TCID50/ml. The street RV HuNPN01 (GenBank Acc. No. DQ496219.1) of 102.5 LD50/50 µl was a gift from Dr. Changchun Tu, Institute of Veterinary Sciences, Academy of Military Medical Sciences. The viruses were stored at -80°C.

Testing of virus virulence. Mice were divided into 5 groups of 30 animals and each group was divided in 3 subgroups of 10 animals. The mice in subgroups were inoculated with (a) 30 µl of SRV9 (107 TCID50/ml) i.c., (b) 50 µl of SRV9 (107 TCID50/ml) i.m. and (c) 50 µl of 0.01 mol/l PBS pH 7.4, respectively. Animals were checked daily during 60 days for the signs of illness. All inoculated mice were euthanized by CO_2 intoxication, followed by cervical dislocation. The brains were removed for RV diagnosis by fluorescent antibody test and RT-PCR (Wang *et al*., 2011; Wu *et al*., 2011). Such procedures of animal monitoring/euthanasia were used in all experiments unless otherwise stated.

Prophylactic vaccination. Prophylactic vaccination of mice was performed as previously described (Morimoto *et al*., 2001; Wu *et al*., 2011). Three-week-old mice were divided into 3 groups of 10 mice, which were injected i.m. (a) with 50 µl of the attenuated SRV9 virus (107 TCID50/ml) at day 0 and with 50 µl of PBS at days 7 and 21, (b) with three 50 µl doses of inactivated virus at similar intervals and (c) with 50 µl of PBS on days 0, 7 and 21 (control), respectively. Three weeks after immunization, each animal was challenged i.m. with a lethal dose (102 LD50/50 µl) of the street virus. The survival of animals was calculated at 2 months post challenge and routine diagnosis was made by fluorescent antibody test.

Post exposure vaccination. Three-week-old mice were divided into 13 groups of 10 mice. All mice were inoculated i.m. with 102 LD50 of the street virus in 50 µl. Six groups of mice were injected with a single i.m. dose of SRV9 at 1, 2, 3, 4, 5 and 6 days p.e., respectively. Other six groups of mice were injected i.m. or i.c. with a single dose of inactivated virus, while a control group of mice were given PBS. The animals were observed daily for 2 months and euthanized when clinical signs of the disease appeared. A routine diagnosis was made by fluorescent antibody test.

RT-PCR for the street virus. The assay was carried out as previously described (Franka *et al*., 2009). Mice were divided into 3 groups of 4 animals and all were infected i.m. with 103 LD50 of the street virus in 50 µl and euthanized at days 5, 6 and 7 p.e., respectively. Brain samples were collected and total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. For reverse transcription, total RNA (32 µl) was heated at 65°C for 5 min, chilled on ice and transferred to a tube with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, USA) with 1 µl (0.2 µg) of random primer pd(N)6 (TaKaRa, Japan). After incubation at 37°C for 1 hr, the cDNA product was subjected to PCR with primers for the street virus N gene (Table1). The PCR consisted of one cycle of 95°C/1 min (initial denaturation), 30 cycles of 95°C/15 sec, 55°C/20 sec (annelation) and 72°C/30 sec (amplification) and termination at 72°C for 10 min. The PCR products were separated by agarose gel electrophoresis using 3% UltrapureTM Agarose (Invitrogen) and visualized by ethidium bromide staining and UV illumination. Twenty bp DNA ladder (TaKaRa, Japan) was employed as marker. Assay of blood brain barrier permeability was carried out by a modification of a previously described technique, in which a low molecular weight fluorescent marker (fluorescein of MW 376) was used as tracer (Phares *et al*., 2006; Fabis *et al*., 2008; Faber *et al*., 2009). Briefly, mice were injected with 100 µl of 100 mg/ml Na-fluorescein under anesthesia via intravenous route. Ten min later, peripheral blood was collected and mice were perfused with PBS for 10 min. Then 50 µl of serum was mixed with an equal volume of 15% trichloroacetic acid (TCA), followed by centrifugation for 10 min at 10,000 rpm and the supernatant was harvested. The uptake of Na-fluorescein by tissue was determined by homogenizing brain tissue in 7.5% TCA and centrifuging for

10 min at 10,000 rpm to remove insoluble precipitates. After the addition of 30 µl of 5 N NaOH to 120 µl of the supernatant the fluorescence was determined using a BioTek Spectrophotometer (Bio-Tek Instruments Inc) with excitation at 485 nm and emission at 530 nm. Na-fluorescein uptake by tissue was expressed as (mg fluorescence of spinal cord/mg protein)/ (mg fluorescence of serum/ml blood) to normalize uptake values for blood levels of the dye at the time of tissue collection.

Real-time RT-PCR for CD19 mRNA. To determine the expression of immunocyte CD19 after i.c. inoculation of SRV9, a SYBR Green-based real-time PCR was carried out in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Brain tissue was removed from infected mice after perfusion at indicated time points, and frozen on dry ice before storage at -80°C. Total RNA was extracted with Trizol and used for real-time RT-PCR as described previously (Wang *et al.*, 2005, 2011). Each reaction was carried out in duplicate with approximately 100 ng of DNase-treated RNA and 5 nmoles of primers using SYBR Green real-time RT-PCR Master Mix Kit (TaKaRa, Japan) according to the manufacturer's instructions (Table 1). For CD19 gene expression, mRNA copy number of a particular gene was normalized to GAPDH as housekeeping gene. Levels of gene expression in samples are presented as a fold increase over mock-infected controls.

Fluorescent antibody test for titration of virus neutralizing antibodies was carried out as previously described (Smith *et al.*, 1973; Franka *et al.*, 2009).

Statistical analysis. The $\chi 2$ test, two-tailed Fisher exact test and Mann-Whitney test were employed (Faber *et al.*, 2005; Wang *et al.*, 2011; Wu *et al.*, 2011).

Table 1. Primers used in RT-PCR and real-time RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
RV nucleoprotein	CTGGCAGACGACGGAACC	CATGATTCGAGTATAGACAGC
GAPDH	GGAGAAGCTGCCAATGGATA	TTACGCTTGCACTTCTGGTG
CD19	GAGCTCAGAGCCATGAAACA	CAAGGTTGGAGTCGTTCTCA

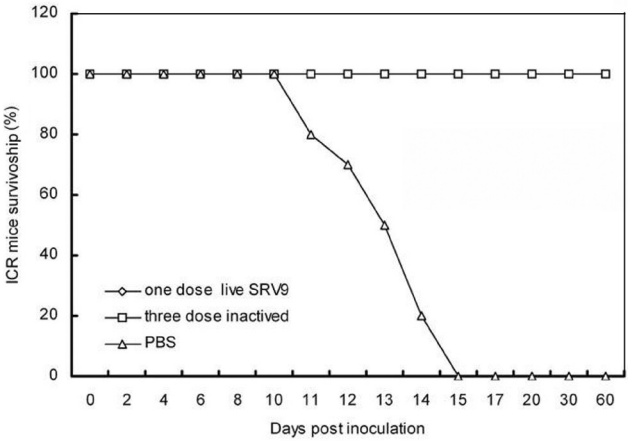

Fig. 1
Survivorship of mice after infection with HuNPN01 using PrEP
Mice were administered 1 dose of live attenuated SRV9 or 3 doses of inactivated RV as pre exposure prophylaxis.

Results

Avirulence of the attenuated SRV9 vaccine

The SRV9-inoculated mice were observed for 18–22 days p.i. but neither i.c. nor i.m. inoculation resulted in death or any clinical signs of sickness. Moreover, brain tissues tested negative for the virus infection by both fluorescent antibody test and RT-PCR.

Prophylactic vaccination

Mice were immunized i.m. with one dose of attenuated SRV9 vaccine or three doses of inactivated vaccine at 0, 7 and 21 days or with PBS (control) and challenged i.c. with a lethal dose of the street virus. All vaccinated mice showed no signs of illness, while control mice developed symptoms of rabies and were euthanized at days 11–15 p.e (Fig. 1). All survived mice tested negative for the virus by both fluorescent antibody test and RT-PCR. Real-time RT-PCR showed that the N gene of the street virus was expressed in the brain of all mice at days 6 and 7 but not at day 5 p.e. (Fig. 2).

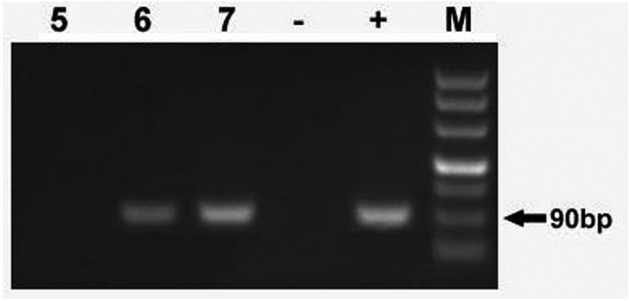

Fig. 2
Expression of N gene in the brain of mice prophylactically vaccinated with HuNPN01
RT-PCR and agarose electrophoresis of PCR products. The N gene (89 bp) expression in the brain of mice at days 5, 6 and 7 p.e. (lanes 5, 6 and 7). Positive control (lane +), negative control (lane -), 100 bp ladder (lane M).

Post exposure vaccination

Mice were inoculated i.m. with a lethal dose of the street virus. A part of them were then injected with a single i.m. dose of attenuated SRV9 vaccine at 1, 2, 3, 4, 5 and 6 days p.e., re-

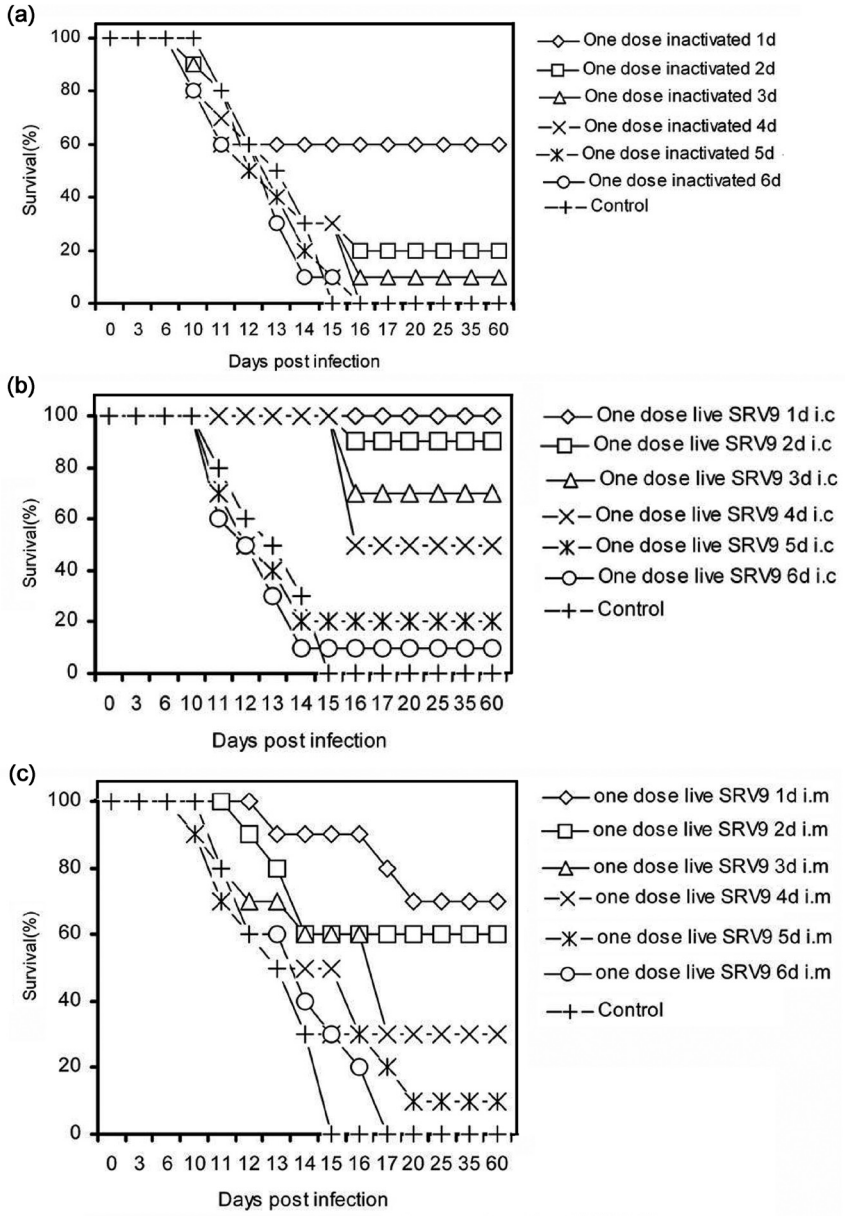

Fig. 3
Survival of mice after post exposure vaccination
Mice were infected with the virus HuNPN01 and administered PEP with 1 dose of live attenuated SRV9 and 1 dose of inactivated rabies vaccine at 1–6 days p.e. (a) Survival of mice (%) after i.m. administration of inactivated virus. (b) Survival (%) after i.c. administration of SRV9. (c) Survival (%) following i.m. administration of SRV9.

spectively, while other part were injected i.m. or i.c. with a single dose of inactivated vaccine. The mice given PBS served as the control. The animals were observed daily for 2 months and were euthanized when clinical signs of rabies appeared. Routine diagnosis was then made by fluorescent antibody test.

The application of attenuated vaccine at indicated days resulted in 70%, 50%, 30%, 20%, 10%, and 0% survival. In case the inactivated vaccine was used, its i.m. application led to 30%, 20%, 10%, 0%, 0%, and 0% survival (Fig. 3c), while its i.c. application resulted in 100%, 90%, 70%, 50%, 20%, and 10% survival, respectively (Fig. 3b). All survived mice tested negative for the virus by fluorescent antibody test and RT-PCR. These results clearly proved that live attenuated vaccine was more effective than inactivated vaccine (Table 2). Hence, it is evident from these results that attenuated SRV9 vaccine is more effective when applied i.c. (Table 2).

Table 2. Survival of mice after post-exposure vaccination

Vaccine	Day of vaccination p.e.						Total survivors	P-value
	1	2	3	4	5	6		
Live SRV9 vaccine applied i.m.	7/10	5/10	3/10	2/10	1/10	0/10	18/60	0.012*
Live SRV9 vaccine applied i.c.	10/10	9/10	7/10	5/10	2/10	1/10	34/60	0.010**
Inactivated vaccine applied i.m.	3/10	2/10	1/10	0/10	0/10	0/10	6/60	

*Live vs inactivated vaccine, both applied i.m. **Live (i.c.) vs live (i.m.) vaccine.

Effect of vaccination on blood-brain barrier permeability

Previous studies have demonstrated that live attenuated RV applied i.c. enhances the blood-brain barrier (BBB) permeability (Faber *et al*., 2009; Wang *et al*., 2011). The BBB permeability is one of the important mechanisms by which immune effectors enter into the CNS to clear RV. In analyzing the enhancement of the BBB permeability by the attenuated SRV9 vaccine, which generally occurs 6–8 days after immunization, the leakage of Na-fluorescein from the circulation into the CNS was proved in the cortex and cerebellum (Fig. 4a) (Faber *et al*., 2009).

The capacity to induce the mechanisms that deliver rabies-specific immune effectors into CNS tissues is an important feature that differentiates effective vaccine variants from pathogenic RV (Faber *et al*., 2009; Wang *et al*., 2011). B lymphocytes are such effectors that can be evaluated via CD19 as their marker. The real-time RT-PCR assay of CD19 mRNA in the brain of mice immunized with attenuated SRV9 vaccine showed its increase at 6–8 days post immunization (Fig. 4b).

Immune response of mice to vaccination

To test the immune response of mice to RV vaccines, serum virus-neutralizing antibodies were determined. The mice given 1 dose of live attenuated SRV9 vaccine or 3 doses of inactivated vaccine showed titers up to 0.5 IU at days 28 or 44 post vaccination. Noteworthy, the i.c route of administration was superior to the i.m. one, both in regard to the titer and its duration (Fig. 5a,b).

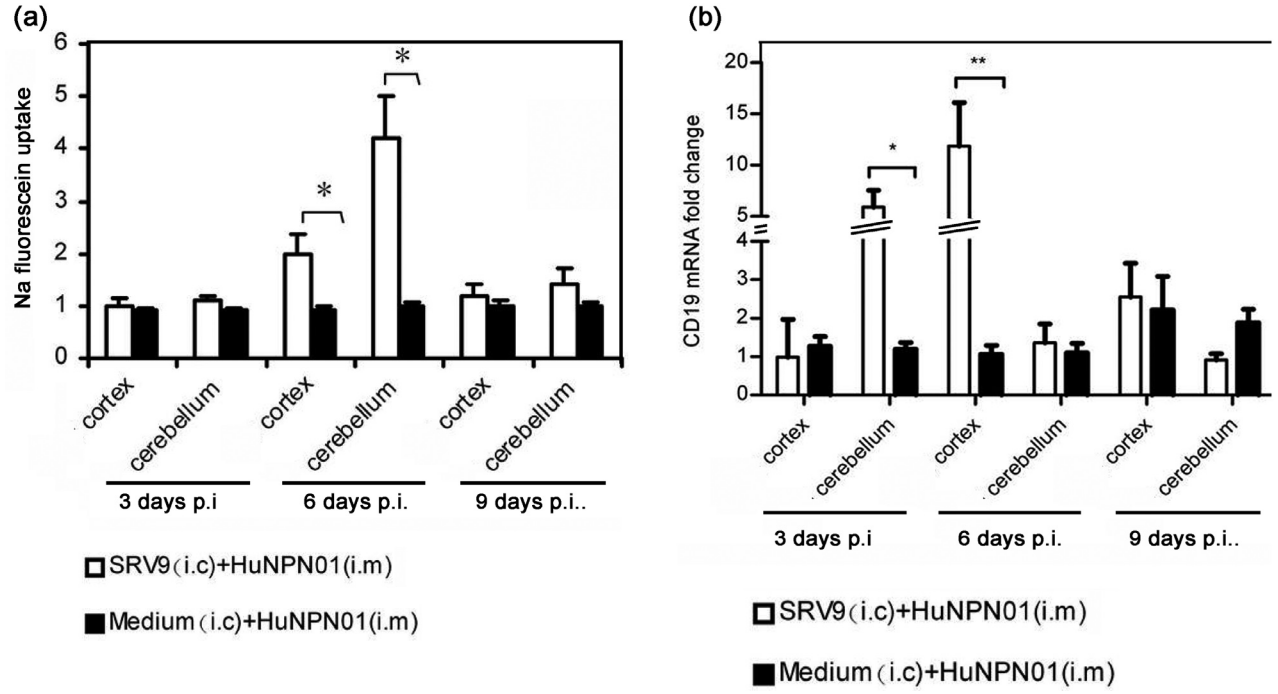

Fig. 4
Induction of BBB permeability with attenuated SRV9 vaccine
For the experiment see Results. (a) The BBB permeability was assessed in the cortex and the cerebellum 6 and 8 days after application of the vaccine. It is expressed as the amount of Na-fluorescein in the infected tissue normalized to the amount in uninfected tissue. (b) CD19 mRNA in the cortex and in cerebellum was assayed by real-time RT-PCR. CD19 mRNA levels are expressed as the fold increase in infected over uninfected tissue. The significance of differences was assessed by the Mann-Whitney test.

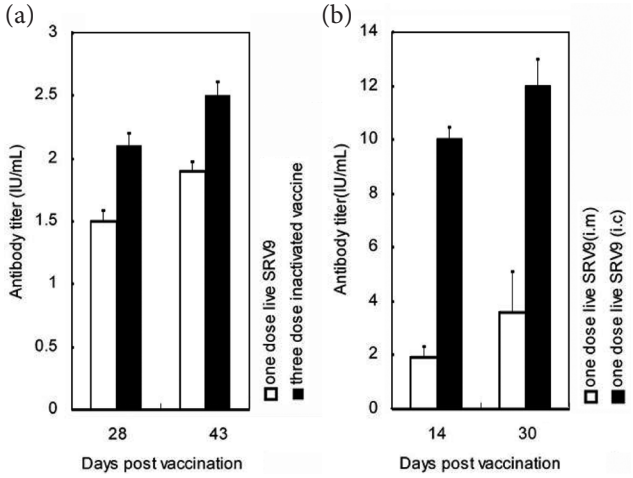

Fig. 5
The immune responses of mice to vaccines
The serum virus-neutralizing antibody titers in mice at different time periods after vaccination. (a) Mice were administered one dose or live attenuated SRV9 or three doses of inactivated rabies vaccine as PrEP. The antibody titer was determined at 28 and 43 days after vaccination. (b) Mice received one dose of live attenuated SRV9 vaccine i.m. or i.c. and the antibody titer was determined at 14 and 30 days after vaccination.

Discussion

With the development of science and technology, the shortcoming of traditional inactivated rabies vaccine was obviously its high price and low efficacy. In this regard the attenuated rabies vaccine proved highly efficient at low doses, inducing relatively better immune responses, and useful in oral immunization of wildlife or stray dogs (Lodmeel *et al.*, 2004; Franka *et al.*, 2009; Wu *et al.*, 2011). The attenuated SRV9 vaccine just fulfilled these requirements very well.

Namely, the mice inoculated i.m. or i.c. with SRV9 showed no rabies symptoms and antibody titers reached up to 0.5 IU. The results also showed that SRV9 was safe for adult mice but, safer for younger mice than the previously reported recombinant virus derived from SADB19 (Morimoto *et al.*, 2001; Dietzschold *et al.*, 2004). Moreover, our results are also in accord with previous reports (Wang *et al.*, 2011) and indicate that SRV9 could be used in prophylactic as well as p.e. vaccination.

Some characteristic mutations were found in the glycoprotein gene of SRV9, namely Arg333Gly, Gly53Glu, His192Arg and Thr311Ala (Ming *et al.*, 2009; Wei *et al.*, 2010).

In this study, mice were used to evaluate the effects of attenuated SRV9 vaccine in prophylactic and p.e. vaccination. Typical clinical features of rabies were observed in control mice, while mice receiving a single dose of attenuated SRV9 vaccine or 3 doses of inactivated vaccine did not show any clinical signs or death. Thus, these results showed that a single dose of SRV9 could protect mice from lethal RV infection. In case of p.e. trials, 70%, 50%, 30%, 20%, 10%, and 0% protection rates were observed at 1, 2, 3, 4, 5 and 6 day p.e., respectively, with a single dose of SRV9. This protection was considerably higher than that with inactivated vaccine. Similar findings for the protection with inactivated vaccine had previously been reported. Namely survival rates for the SPBAAN-GAS-GAS-GAS vaccine applied i.c. were 100%,100%, 80%, 50% and 20% at 4 hr and 2, 3, 4, and 5 days p.e., respectively (Faber *et al.*, 2009). It implies that i.c. inoculation of attenuated RV could evoke a rapid protective immune response. Although the i.c. route is not practical for large scale trials, it may be suitable for endangered or seriously exposed species. Our work had shown that the protection rates with a single i.c. dose of SRV9 applied at 1, 2, 3, 4, 5, and 6 days p.e. were 100% ,90%, 70%, 50%, 20%, and 10%, respectively; i.e. these protection rates were significantly higher than those with traditional i.m. inoculation.

The high survival induced by SRV9 may be ascribed to a large amount of expressed G protein, which further stimulates cytotoxic CD8 T cells in addition to B and CD4 T cells and CD8(+) T cells. The latter cells provide direct perforin and gamma interferon-mediated antiviral activity. However, current commercial inactivated RV vaccines for animals can stimulate CD4+ helper T-cell as well as humoral B-cell responses (Franka *et al.*, 2009).

The main factors behind the high efficacy of SRV9 inoculated i.c. post exposure to lethal street RV could be a rapid production of antibodies, improved BBB permeability and CD19+B cell infiltration of CNS. These factors may lead to the clearance of any pathogenic viruses from CNS.

Some previous studies reported that the BBB permeability peaked 6–8 days after i.c. administration of live attenuated rabies vaccine (Faber *et al.*, 2009; Wang *et al.*, 2011). In present experiments, we also observed such a kinetics and higher levels of B cell mRNA in CNS of SRV9-vacinated mice. Mice lacking either B and T cells or B cells alone developed a progressive disease and succumbed to rabies following intranasal infection with an attenuated CVS-F3 RV (Hooper *et al.*, 1998, 2009). The mice lacking CD8+ T cells, IFN receptors or complement components C3 and C4 did not show any significant difference in survival compared with intact counterparts, suggesting a lesser role of cellular immune responses in RV clearance. Similarly, others findings showed that immunocompetent mice, but not those lacking B cells or having defective type I IFN, TLR or IL-1 receptor signaling pathways survived an i.c. infection with a mixture of pathogenic DOG4 RV and attenuated SPBAAN-GAS-GAS-GAS RV (Franka *et al.*, 2009; Faber *et al.*, 2009).

These facts explain why SRV9 could not protect mice 6 days after infection with the street virus regardless of the inoculation route. Evidently, during this time lapse the virus had chance to spread in CNS. These results are similar to those reported previously (Franka *et al.*, 2009). The virus

has evolved its own specific mechanisms to escape early recognition by the immune system via limited replication, minimized glycoprotein expression, suppression of IFN response, antiapoptotic stimulation and solely neuronal transportation (Finke and Conzelmann, 2005; Brzozka *et al.*, 2006). Some researchers have reported early deaths in p.e. vaccination (Willoughby, 2009), but we could not confirm them. Immunoglobulin has always been used in routine p.e. vaccination of humans as recommended by WHO. Its high price, however, does not allow its application to animals in developing countries (Kreindel *et al.*, 1998; Hampson *et al.*, 2011). Moreover, WHO did not approve the administration of immunoglobulin to animals (WHO, 2005). Besides, it has been shown that immunoglobulin either alone or combined with inactivated vaccine could not effectively protect dogs from street RV (Hanlon *et al.*, 2002). Thus, immunoglobulin was entirely excluded from this study. Furthermore, the SADB19 has been reported safe for many carnivores irrespective of the route of administration (Vos *et al.*, 1999). The SRV9, as a derivative of the SADB19, may also be safe for many carnivores and some species of mammals (Wei *et al.*, 2010). So, with the help of this study, the use of the SRV9 in p.e. vaccination of dogs, livestock and wildlife is worth of testing.

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