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A novel rabies vaccine based-on toll-like receptor 3 (TLR3) agonist PIKA adjuvant exhibiting excellent safety and efficacy in animal studies

Yi Zhang [a], Shoufeng Zhang [b], Wei Li [c], Yuchi Hu [d], Jinyan Zhao [d], Fang Liu [a], Haixiang Lin [a], Yuan Liu [a], Liliang Wang [a], Shu Xu [a], Rongliang Hu [b,**], Hui Shao [a,*], Lietao Li [a,*]

[a] Yisheng Biopharma. Co., Ltd., Beijing, China
[b] Academy of Military Medical Sciences, Changchun, China
[c] National Center for Safety Evaluation of Drugs, Beijing, China
[d] Beijing Institute for Drug Control, Beijing, China

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ABSTRACT

Vaccination alone is not sufficiently effective to protect human from post-exposure rabies virus infection due to delayed generation of rabies virus neutralizing antibodies and weak cellular immunity. Therefore, it is vital to develop safer and more efficacious vaccine against rabies. PIKA, a stabilized chemical analog of double-stranded RNA that interacts with TLR3, was employed as adjuvant of rabies vaccine. The efficacy and safety of PIKA rabies vaccine were evaluated. The results showed that PIKA rabies vaccine enhanced both humoral and cellular immunity. After viral challenge, PIKA rabies vaccine protected 70–80% of animals, while the survival rate of non-adjuvant vaccine group (control) was 20–30%. According to the results of toxicity tests, PIKA and PIKA rabies vaccine are shown to be well tolerated in mice. Thus, this study indicates that PIKA rabies vaccine is an effective and safe vaccine which has the potential to develop next-generation rabies vaccine and encourage the start of clinical studies.

Introduction

Rabies is a zoonosis that is transmitted from animal to human through contamination of a bite or scratch with saliva-borne virus, or through intact mucous membrane (Manning et al., 2008). The mortality rate is almost 100% after the onset of the clinical symptoms and it remains one of the most lethal infectious diseases known (Rupprecht et al., 2002).

Current strategy to prevent rabies infection recommended by WHO includes post-exposure vaccination and inoculation of anti-rabies immunoglobulins (preferably human rabies immunoglobulin, HRIG) around the wound site for severe injury (World Health Organization, 2013). However, there are more than 55,000 deaths cause by rabies infection every year (Huang et al., 2015). A study evolving 725 human rabies cases in China, Guangxi province, showed that 197 death cases were caused by vaccination failure; amongst which 78.27% cases died before the 4th injection was administered (Wang, 2010). It is indicated that vaccination alone cannot effectively protect human from rabies virus infection. Rabies virus replicated rapidly and is capable of paralyzing the host central nervous system in days. If the victim suffered from severe multiple bites from an infected animal, the incubation period can be less than one week. Once signs of disease begin, the mortality rate is almost 100%. Therefore, vaccines must generate immediate and appropriate immune responses so that rabies virus could be eliminated before entering the nervous system. To eliminate the rabies virus, two requirements are needed, i.e., the production of rabies virus neutralizing antibodies (RVNA) for neutralizing the biological effects of rabies virus and the induction of cellular immunity for removing virus-infected cells (Huang et al., 2015; Jayakumar and Ramadass, 1990; Thraenhart et al., 1994). However, available vaccines failed to achieve this. Therefore, a more reliable and cost effective post-exposure vaccine which could induce earlier and higher titer of RVNA and strong cellular immunity is in need.

A novel adjuvant, PIKA, is introduced to the rabies vaccine in our study. The PIKA is a stabilized chemical analog of double-strand RNA capable of inducing non-specific immunity. Our previous studies showed that PIKA adjuvant acts through toll-like receptor 3 (TLR-3) and other cellular pathways, enhancing antigen presentation by antigen-presenting cells (APCs) and inducing production of pro-inflammatory cytokines (Lau et al., 2009, 2010). According to the standard of *Guideline on the Requirements for Quality Documentation Concerning Biological Investigational Medicinal Products* and *Chinese Requirements for Biological Products*

* Corresponding authors.
** Corresponding author.
E-mail addresses: ronglianghu@hotmail.com (R. Hu), hui.shao@yishengbio.com (H. Shao), lietao.li@gmail.com (L. Li).

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(CRBP), we developed a novel rabies vaccine called PIKA rabies vaccine (PIKA-RV). The PIKA rabies vaccine was tested in mice for efficacy of humoral immunity, cellular immunity and the protection after wild viral challenge. In addition, we developed an accelerated regimen which could be completed in 7 days post exposure. Safety of PIKA and PIKA-RV after single and repeated dose administration was also established.

Results

Evaluation of rabies-specific antibody response in vaccinated mice

To assess the immune response of PIKA-RV, rabies virus neutralization response in PIKA-RV-immunized mice was measured after 21 days of the first immunization. As shown in Fig. 1, high concentration PIKA-RV group (0.2 IU IPRV + 100 μg PIKA) produced almost 3-fold higher RVNA titer compared to IPRV alone (47.41 versus 16.01 IU/mL, respectively; $P < 0.001$). There was also a significant increase in RVNA titer in the group receiving median concentration PIKA-RV (0.02 IU IPRV + 10 μg PIKA) ($P < 0.01$). More interestingly, low concentration PIKA-RV (0.002 IU IPRV + 1 μg PIKA) also produced a slightly higher RVNA response.

Cell mediated immune response in vaccinated mice

Another critical feature of vaccines is the ability to induce cellular immunity, most effective in removing virus-infected cells (La Gruta and Turner, 2014; Visperas et al., 2014; Welten et al., 2013). To determine whether T cells were activated, the frequencies of IFN-γ-producing cells at the single-cell level by ELISPOT assay were determined following stimulation of mouse spleen lymphocytes with IPRV.

Splenocytes of high concentration PIKA-RV group (0.2 IU IPRV + 100 μg PIKA) generated approximately 73 SFU/3×10^5 of IFN-γ specific splenocytes, which was significantly higher than the group treated with IPRV ($P < 0.01$, Fig. 2). Similarly, the number of IFN-γ-secreting cells generated in mice primed with median concentration PIKA-RV group (0.02 IU IPRV + 10 μg PIKA) was also higher than that in mice primed with IPRV ($P < 0.001$).

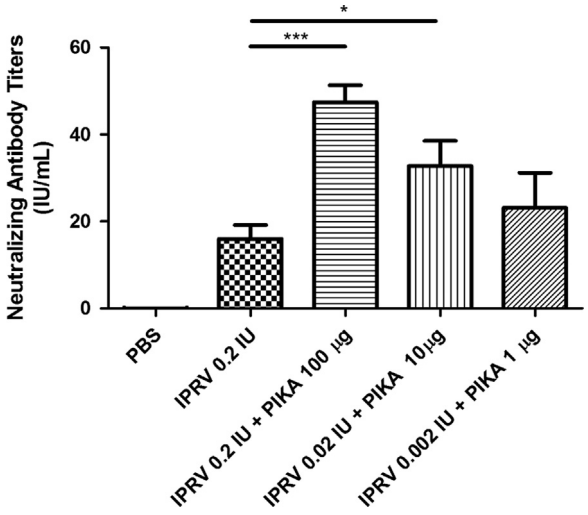

Fig. 1. PIKA enhanced antigen-specific antibody production. Mice ($n=6$) were immunized by i.m. injection of 100 μL PIKA-RV with different concentrations, IPRV, or PBS control. The preparations were injected on day 0 and 7. Blood was collected at day 21 after the first immunization. Rabies virus neutralizing antibody titer were evaluated using the FAVN test. Data are expressed as the mean ± SEM ($n=6$; $^{*}P < 0.05$; $^{***}P < 0.001$).

Protection of immunized animals against rabies virus challenge

To test whether PIKA-RV can elicit enough protective immunity against a robust rabies virus challenge in animal model, two challenge tests were performed.

In the first challenge test, Beagles and wild virus BD06 strain were employed. All Beagles were challenged with BD06 strain and immunized with different experimental materials post exposure. Their survival was monitored for 45 days. By day 9, animals began to develop clinic signs. Beagles died within 1–3 days after the onset of abnormal symptoms. Densely distributed florescent spots were observed after direct immunofluorescence staining of rabies virus in brain tissue, indicating death caused by rabies viral infection in CNS. Group of vaccine control only confer limited protection (30%), while PIKA-RV protected 70% of the animals as shown in Fig. 3.

In another challenge test, wild virus BD06 strain was prepared for infection at $50LD_{50}$. Golden hamsters in all testing group behaved normal 5 days after infection. Infected hamsters that did not survive started to show clinical symptoms, such as decreased activities, clumsiness, poor coordination, decreased food intake and anxiety, between day 6 and 14. Hamsters died within 4–6 days after the onset of abnormal symptoms. Densely distributed florescent spots were also observed, indicating death caused by rabies viral infection in CNS. The survival rate for all testing groups was demonstrated in Fig. 4. 80% of hamsters died in vaccine control

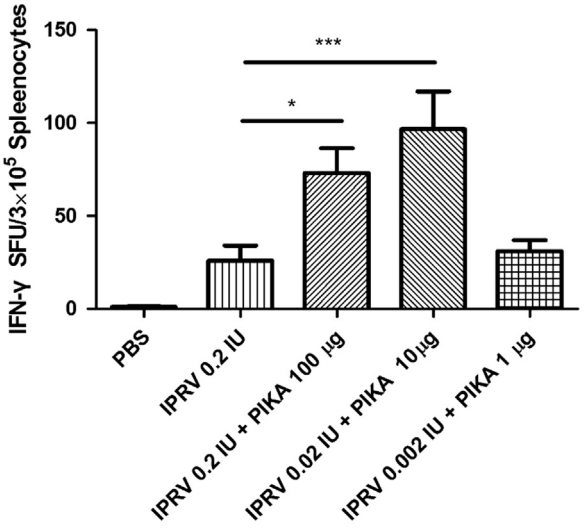

Fig. 2. Robust T-cell responses were generated upon re-stimulation with IPRV in vitro. Splenocytes from the immunized mice were isolated 21 days after the first immunization and were re-stimulated with IPRV in vitro for 20 h. The figure shows the number of IFN-γ secreting cells as determined by ELISPOT assay. Data are expressed as the mean ± SEM ($n=6$; $^{*}P < 0.05$, $^{***}P < 0.001$).

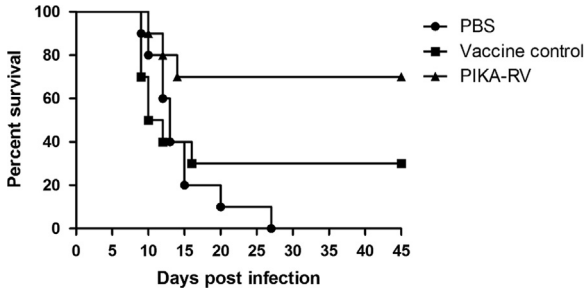

Fig. 3. Post-exposure test in Beagles. Animal (Golden Hamster) survival rates (%) after wild strain virus challenge followed by immunization with (1) PBS, (2) vaccine control and (3) PIKA-RV post exposure.

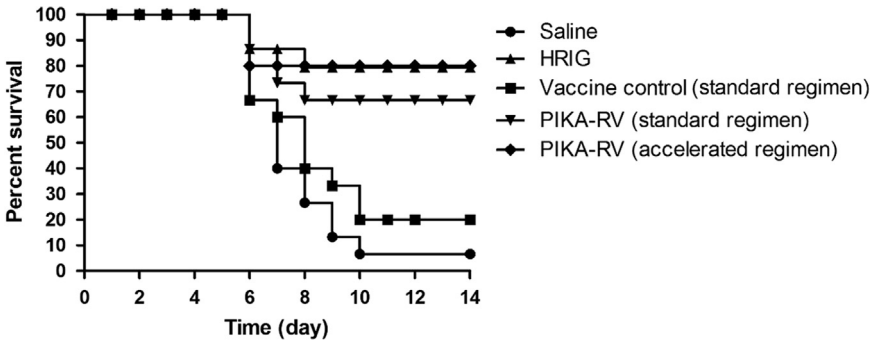

Fig. 4. Regimen study. Animal (Golden Hamster) survival rates (%) after wild rabies virus challenge followed by immunization with (1) saline solution by standard schedule, (2) vaccine control by standard schedule, (3) human rabies immunoglobulin (HRIG, 0.75IU administered at each site of right and left hind leg 2 h post exposure), (4) PIKA-RV by standard schedule), or (5) PIKA-RV by accelerated schedule post exposure.

group after viral challenge, whereas PIKA-RV could provide significantly higher protection using the standard regimen.

To further improve the protective activity and elevate patient compliance, we developed an accelerated regimen. Then we make a comparison between the accelerated regimen and the standard regimen. As shown in Fig. 4, the PIKA-RV administered using the accelerated regimen protected 80% of the mice, similar to that of passive immunization with HRIG. Compared with the standard regimen, the accelerated regimen enhanced the survival rate from 67.7% to 80%.

Acute toxicity test

Animals in PIKA and PIKA-RV groups did not show significant variations in food intake and body weight compared to the control group. No mortality was observed in any groups. All animals did not show observable toxic reactions and abnormalities during organ visual examination. The maximum dosage of PIKA adjuvant and PIKA-RV up to 10 mL/kg by body weight was well tolerated by test animals, which was equivalent to 300 times of human dosage.

Repeated dose toxicity

The ICH guideline on the Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals ("ICH S6") states that "the frequency of administration in laboratory animals may be increased compared to the proposed schedule for the human clinical studies in order to compensate for faster clearance rates or low solubility of the active ingredient". Therefore, in repeated dose toxicity study, our treatment schedule in mice was double dose administration on day 0 and day 2, and single dose administration on days 7, 28 and 42. This treatment schedule could be employed to study the repeated dose toxicity of the accelerated postexposure regimen and the four-dose regimen. For the accelerated postexposure regimen, the treatment schedule increased the frequency of vaccination on days 28 and 42. For the four-dose regimen, the treatment schedule increased the frequency of vaccination on day 42. The dosage of high dose of PIKA-RV group was equivalent to 242 times of human dosage.

There were no treatment-related differences in mean body weights and food consumption of males or females from any treatment group compared to control group.

Hematology

Blood samples from both genders were collected on day 44 and 56 for hematology analysis. Results of the 44th day for male and female mice in all testing groups including PIKA adjuvant and PIKA-RV groups are tabulated in Table 1a and b respectively. On day 44, male mice in PIKA-RV high dose group had lower level of LY and higher level of GR ($P<0.01$), whilst female mice in PIKA-RV high dose group had lower level of HGB ($P<0.01$) and HCT ($P<0.05$). However, there was no significant difference in PIKA group and PIKA-RV low dose group. On day 56, these difference had disappeared. No pathological association was established as the changes are within the normal reference range for mice.

Biochemistry

Blood samples were collected on the same day as the hematology test, they were used for blood biochemistry and electrolytes analysis. Male mice in PIKA adjuvant group showed increasing AST and CK value after final vaccination dose, while female mice showed a decrease level of ALP ($P<0.05$). For PIKA-RV low dose group, decreasing total bilirubin was observed among male mice and decreasing ALP was observed among female mice ($P<0.01$). Similarly for the high dose group, female mice had increasing ALP level compare to baseline ($P<0.01$).

The electrolytes for all animals in all testing groups did not show any significant fluctuation during the testing period. Though the above mentioned shifts in value of biochemistry parameters were statistically significant, they were still within the normal reference ranges for the species. The value for all biochemistry parameters and electrolytes are listed in Table 2a and b respectively.

Organ/body weight ratios

Mice in all study groups were sacrificed by the end of study period with organ weighted, dissected and examined for pathological changes. Spleen of male mice in PIKA-RV high dose group increased significantly compared to saline control ($P<0.05$). The weight of female mice spleen also increased for PIKA adjuvant group. The changes in the weights reflected the increasing immune response induced by vaccination and adjuvant activity. The weight changes for other organ were found to be statistically non-significant in all treated groups, thus had no pathological significance. Organ weights for both male and female mice in all study groups are presented in Table 3a and b.

Histopathology

The systemic pathological analysis found no treatment-related lethal changes. After the final vaccination, inguinal lymph nodes around injection site and spleen showed different degree of hyperplasia for mice in all study groups. The hyperplasia of lymph nodes and spleen was dosed-dependent. By the end of study period, hyperplasia of the organ returned to normal.

Table 1
Hematology parameter changes of the 44th day for male and female mice treated with different test materials.

	WBC ($\times 10^9$/L)	RBC ($\times 10^{12}$/L)	HGB (g/L)	HCT (%)	MCV (fL)	MCH (Pg)	MCHC (g/L)	PLT ($\times 10^9$/L)	RDW (%)	PCT (%)	MPV (fL)	PDW (%)	Ret (‰)	LY (%)	MO (%)	GR (%)
a. Male mice hematology parameters for 4 study groups																
Saline	2.5 ± 1.3	8.06 ± 1.59	125 ± 25	39.2 ± 7.8	48.7 ± 3.2	15.4 ± 0.9	317 ± 13	465 ± 144	13.4 ± 1.1	0.21 ± 0.07	4.6 ± 0.3	14.1 ± 0.3	17 ± 6	71.7 ± 7.6	11.1 ± 2.5	17.2 ± 7.2
PIKA	2.5 ± 1.0	8.52 ± 0.41	133 ± 8	40.2 ± 2.8	47.3 ± 3.5	15.6 ± 0.7	331 ± 22	549 ± 54	13.2 ± 0.4	0.26 ± 0.03	4.7 ± 0.2	13.7 ± 0.3	17 ± 6	65.9 ± 7.9	15.1 ± 3.7	19.0 ± 6.0
PIKA-RV (low dose)	2.9 ± 1.0	7.69 ± 2.59	132 ± 11	40.2 ± 3.1	46.4 ± 2.8	15.2 ± 0.6	328 ± 13	580 ± 125	12.4 ± 0.3	0.26 ± 0.07	4.6 ± 0.2	13.6 ± 0.4	17 ± 6	68.7 ± 4.3	14.3 ± 4.4	17.0 ± 7.7
PIKA-RV (high dose)	3.1 ± 1.7	8.73 ± 0.48	132 ± 8	41.3 ± 2.7	47.5 ± 3.6	15.2 ± 0.9	321 ± 21	596 ± 158	13.9 ± 1.5	0.28 ± 0.08	4.8 ± 0.2	13.5 ± 0.6	17 ± 5	59.4 ± 10.0**	11.9 ± 3.2	28.7 ± 10.3**
b. Female mice hematology parameters for 4 study groups																
Saline	3.3 ± 1.3	7.52 ± 3.77	158 ± 11	48.6 ± 1.9	51.2 ± 3.4	16.6 ± 0.5	326 ± 18	577 ± 67	13.0 ± 0.5	0.26 ± 0.03	4.7 ± 0.2	13.9 ± 0.4	6 ± 3	68.1 ± 9.0	12.9 ± 2.9	19.0 ± 7.8
PIKA	3.9 ± 2.0	7.84 ± 2.75	142 ± 14	45.2 ± 3.4	51.3 ± 4.4	16.0 ± 0.8	314 ± 20	536 ± 94	12.8 ± 0.4	0.26 ± 0.05	4.9 ± 0.2	13.8 ± 0.4	5 ± 4	62.2 ± 8.3	14.1 ± 4.2	23.7 ± 8.0
PIKA-RV (low dose)	3.7 ± 1.5	8.13 ± 2.71	146 ± 14	44.8 ± 6.4	48.9 ± 4.2	16.0 ± 0.7	328 ± 19	595 ± 84	13.3 ± 1.0	0.28 ± 0.04	4.7 ± 0.2	13.5 ± 0.5	5 ± 3	62.1 ± 11.8	12.2 ± 3.9	25.7 ± 9.3
PIKA-RV (high dose)	2.8 ± 0.6	8.73 ± 0.77	139 ± 10*	41.4 ± 3.2**	47.6 ± 2.3	15.9 ± 0.9	335 ± 6	531 ± 109	12.8 ± 0.5	0.26 ± 0.06	4.9 ± 0.4	13.9 ± 0.4	6 ± 3	61.4 ± 12.2	13.3 ± 2.8	25.3 ± 13.7

Values are expressed as mean ± standard deviation.
WBC – White Blood Cells, RBC – Red Blood Cells, HGB – Hemoglobin, HCT – Hematocrit, MCV – Mean Corpuscular Volume, MCH – Mean Corpuscular Hemoglobin, MCHC – Mean Corpuscular Hemoglobin Concentration, PLT – Platelets, RDW – Red Cell Distribution Width, PCT – Plateletcrit, MPV – Mean Platelet Volume, PDW – Platelet Distribution Width, Ret – Reticulocyte, LY – Lymphocyte, MO – Monocyte number, GR – Granulocyte.
** indicates significant increase or decrease in value with *p*.

Table 2
Clinical chemistry parameter changes of the 44th day for male and female mice treated with different test materials.

	ALT (U/L)	AST (U/L)	TP (g/L)	Alb (g/L)	T.BiLi (μmol/L)	Urea (mmol/L)	Cre (μmol/L)	Glu (mmol/L)	CK (U/L)	ALP (U/L)	CHO (mmol/L)	TG (mmol/L)	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)
a. Male mice biochemistry parameters for 4 study groups															
Saline	31 ± 9	84 ± 23	46.7 ± 9.4	15.5 ± 5.5	1.4 ± 1.0	9.37 ± 3.89	5.5 ± 14.0	6.46 ± 4.38	60 ± 40	26.5 ± 24.8	1.45 ± 1.50	0.37 ± 0.52	150.3 ± 4.4	6.89 ± 1.67	119.1 ± 2.8
PIKA	47 ± 23	142 ± 92*	49.6 ± 6.9	16.8 ± 1.2	1.4 ± 0.4	9.14 ± 1.99	17.0 ± 2.8	9.52 ± 3.58	204 ± 63*	47.5 ± 25.5	3.25 ± 1.64	0.68 ± 0.55	150.7 ± 6.5	6.35 ± 0.60	118.2 ± 5.8
PIKA-RV (low dose)	41 ± 20	75 ± 64	56.6 ± 11.1	10.9 ± 8.5	−0.1 ± 0.9**	5.52 ± 4.55	−2.9 ± 27.2	10.36 ± 10.54	104 ± 165	20.6 ± 22.5	2.42 ± 2.60	0.32 ± 0.56	149.9 ± 4.6	7.00 ± 1.66	118.2 ± 5.2
PIKA-RV (high dose)	35 ± 6	112 ± 34	46.2 ± 18.2	14.9 ± 5.7	0.8 ± 1.0	7.32 ± 3.03	9.9 ± 16.3	9.68 ± 5.67	94 ± 87	38.1 ± 16.7	3.32 ± 1.57	0.56 ± 0.26	152.3 ± 5.7	6.53 ± 0.56	121.5 ± 5.9
b. Female mice biochemistry parameters for 4 study groups															
Saline	24 ± 5	82 ± 13	52.9 ± 3.6	19.8 ± 1.2	0.6 ± 0.2	8.44 ± 1.73	25.3 ± 20.7	11.18 ± 1.33	54 ± 15	81.7 ± 18.5	3.00 ± 0.25	0.84 ± 0.43	162.3 ± 2.6	6.69 ± 0.87	129.2 ± 2.2
PIKA	24 ± 4	76 ± 8	51.4 ± 4.4	18.4 ± 1.4	0.2 ± 0.2	7.73 ± 1.90	12.3 ± 12.2	9.45 ± 3.60	39 ± 24	54.4 ± 31.1*	2.49 ± 0.94	0.64 ± 0.30	163.0 ± 2.9	6.34 ± 0.22	128.8 ± 2.8
PIKA-RV (low dose)	23 ± 4	78 ± 8	52.3 ± 3.3	17.6 ± 0.7	0.1 ± 0.2	8.76 ± 4.48	22.2 ± 5.8	12.91 ± 2.34	46 ± 11	54.3 ± 23.8*	2.46 ± 0.60	0.55 ± 0.28	163.8 ± 2.8	6.76 ± 0.40	130.1 ± 2.7
PIKA-RV (high dose)	23 ± 4	81 ± 9	53.5 ± 3.1	18.1 ± 1.4	0.1 ± 0.4	8.00 ± 1.86	18.3 ± 3.3	11.91 ± 1.48	51 ± 16	54.0 ± 18.9*	3.09 ± 0.36	0.83 ± 0.66	163.8 ± 1.6	6.82 ± 0.92	129.7 ± 0.3

Values are expressed as mean ± standard deviation.
ALT – Alanine transaminase (formly called glutamic-pyruvic transaminase), AST – Aspartate transaminase, TP – total protein, Alb – albumin, T.BiLi – total bilirubin, Urea – Urea, Cre – creatinine, Glu – glucose, CK – creatine kinase, ALP – alkaline phosphatase, CHO – cholesterol, TG – triglyceride.
* indicates significant increase or decrease in value with *p*.

Table 3
Organ/body weight ratios (mg/10 g body weight) of the 56th day for male and female mice treated with different test materials.

	a. Male mice organ/body weight ratios for 4 study groups								
	Heart	Liver	Spleen	Lung	Brain	Thyroid	Kidney	Testes	Epididymis
Saline	50.4 ± 6.0	466.1 ± 52.5	32.8 ± 4.5	69.1 ± 12.6	142.5 ± 9.0	12.5 ± 3.8	179.8 ± 24.6	73.1 ± 9.2	27.1 ± 2.9
PIKA	54.0 ± 7.4	436.2 ± 27.2	35.5 ± 4.6	63.0 ± 5.5	140.7 ± 18.6	10.7 ± 3.6	171.7 ± 18.5	75.7 ± 12.7	29.7 ± 5.0
PIKA-RV (low dose)	51.1 ± 5.4	457.9 ± 50.5	40.1 ± 5.3	59.8 ± 8.1	140.2 ± 9.6	12.4 ± 4.6	172.7 ± 11.9	70.4 ± 7.9	30.4 ± 3.8
PIKA-RV (high dose)	53.4 ± 5.9	475.7 ± 38.2	41.0 ± 10.3*	74.1 ± 12.5	145.9 ± 6.9	11.8 ± 2.9	193.4 ± 29.7	79.0 ± 12.1	28.1 ± 7.0
	b. Female mice organ/body weight ratios for 4 study groups								
	Heart	Liver	Spleen	Lung	Brain	Thyroid	Kidney	Uterus	Ovary
Saline	51.3 ± 4.8	495.6 ± 33.1	40.4 ± 5.0	73.2 ± 15.0	177.4 ± 17.6	16.3 ± 6.7	142.2 ± 9.3	55.8 ± 23.0	8.4 ± 1.4
PIKA	51.3 ± 4.2	500.7 ± 44.5	55.6 ± 15.8**	78.6 ± 18.7	182.7 ± 7.2	20.5 ± 4.5	142.2 ± 7.1	60.3 ± 26.2	8.9 ± 2.9
PIKA-RV (low dose)	54.6 ± 4.5	486.2 ± 20.5	49.3 ± 7.6	81.1 ± 13.2	186.6 ± 13.9	18.1 ± 4.4	147.4 ± 10.4	67.6 ± 38.5	7.5 ± 1.7
PIKA-RV (high dose)	53.4 ± 6.1	476.3 ± 32.4	50.4 ± 5.4	79.1 ± 10.1	177.5 ± 10.1	19.0 ± 3.7	143.5 ± 12.1	65.2 ± 21.6	8.4 ± 1.8

Values are expressed as mean ± standard deviation.
*, ** indicates significant increase or decrease in value with *p*.

Muscles around injection site of all treated groups showed different degree of myofibrosis and infiltration of inflammatory cells in a dose-dependent manner after the final vaccination. All animals returned to normal by the end of study period. No other abnormality was seen in other organs examined.

Discussion

Rabies remains one of the most important zoonotic disease in many countries. According to WHO recommendation, in order to protect people from developing rabies, those who were in category III of exposure should receive both an effective rabies vaccine and rabies immunoglobulin (RIG) (World Health Organization, 2013). However, rabies immunoglobulins are in short supply throughout the world and are virtually unaffordable in many developing countries (Wilde et al., 2002). The previous results showed immunization alone is not fully effective (Wang, 2010). Lin et al. (1993) compared the efficacy post-exposure of the aluminum adjuvanted vaccine and the PICKCa adjuvanted vaccine. They found aluminum adjuvanted vaccine did not enhance protection while PIKA adjuvanted vaccine did. Similar results that PIKA adjuvant vaccines are effective were also confirmed in this study (Figs. 3 and 4). In a study of Iran in 1954, anti-rabies serum and/or vaccine were administered to 17 patients that had incurred severe head wounds from one rabid wolf (World Health Organization, 2011). Among them, only one of 12 patients who received anti-rabies serum plus vaccine died of rabies, while three of five patients received only vaccine died of rabies. This phenomenon showed vaccination alone is not effective to protect human from rabies virus infection. The reasons are delayed generation of VNA and weak cellular immunity, which were critical to prevent rabies virus infection. So, it is important to develop a safer and efficacious vaccine against rabies.

As mentioned earlier, PIKA is a stabilized dsRNA that interacts with TLR3. There have been several reports as to the humoral immunity and cellular immunity induced by PIKA (Gai et al., 2011; Lau et al., 2009, 2010; Shen et al., 2007; Tang et al., 2014; Zhang et al., 2013). In this study, we first assessed the immunogenicity of PIKA adjuvanted rabies vaccine, including RVNA and cell mediated immune response in vaccinated mice. We found that even though the concentration of IPRV and PIKA was reduced to the standard concentration of 1/10 (0.2 IU/mL and 0.1 mg/mL), the RVNA titer was still significantly higher than the IPRV alone (2 IU/mL). The ELISPOT result also showed by formulating PIKA, antigen of 1/10 (0.2 IU) could significantly improve IFN-γ secreting level than the standard concentration of antigen (0.2 IU). Then we evaluated the protective effect of PIKA-RV against rabies in Beagles and mice. Compared with non-adjuvant vaccines, PIKA-RV enhanced the protective activity of rabies vaccine injected after wild rabies strain infection significantly. In the study of efficacy, PIKA-RV induced higher RVNA titer, stronger cellular immunity, and showed better protective effect, indicating the potential to develop next-generation rabies vaccine.

Previous studies showed that PIKA could promote maturation of dendritic cells (DCs) assessed by up-regulation of co-stimulatory molecules CD80, CD86 and CD40, and the induction of cytokines such as IL-12p70, IL-12p40 and IL-6 (Shen et al., 2007). DCs are professional antigen-presenting cells located in the skin, mucosa and lymphoid tissues. Their main function is to process antigens and present them to T cells to promote immunity to foreign antigens. The antigen signals from environment were recognized by PRR (pattern recognition receptors), which are expressed on DCs, inducing different immune responses toward the appropriate bias. TLRs are the best-studied PRRs (Beutler, 2009). Because DCs are activated by PRRs, then trigger the immune response cascade, TLRs are being interesting targets for adjuvants. Indeed, there are several adjuvants based on TLRs have been developed. AS04 (GSK) works by MPL-activation of TLR4 and used as a vaccine for HBV and HPV. ISS (Dynavax) works by activation of TLR9 and is used in vaccines against HBV and influenza. IC31 (Intercell) works by activation of TLR9 and is used as a vaccine against influenza and tuberculosis. PIKA is a mimicking viral dsRNA. It is recognized by TLR3 and is well known to induce stable maturation of DCs. This results in the induction of a Th1-type response, which potentially remove virus-infected cells. Our results showed PIKA vaccine protected mice against infection with wild rabies strains effectively.

Studies have also shown that rabies virus attacks the nervous tissue and appears to replicate almost exclusively in neuronal cells (Warrell, 1997). Once introduced through the skin or mucous membrane, the virus begins replicating in the striated muscles at the wounded site. To block the rapid replication and migration of rabies virus, vaccine should be administered to active the immune system as soon as possible. In addition, as stated in the ICH S6: “the route and frequency of administration should be as close as possible to that proposed for clinical use”. Taking these in mind, we proposed the accelerated regimen. Our results showed the accelerated regimen did enhance the survival rate, which indicates it is effective with the potential to develop a new regimen.

However, shorter dosing intervals and higher dose of vaccine may increase the risk of vaccine-induced toxicity due to increased inflammation and blood levels of vaccine. In view of developing

this new vaccine formulation with an accelerated regimen, as a safe, potent and affordable rabies vaccine, safety and pre-clinical toxicity parameters have been evaluated in animal species.

Based on the acute dose toxicity study, all experimental animals did not show any clinical sign of toxicity for the entire test period (14 days). Moreover, a repeated dose study was conducted in mice. It has been found that after the final vaccination, there were different degree of myofibrosis and infiltration of inflammatory cells in muscles around injection site of all treated groups. However, by the end of study, all animals returned to normal, which indicated that PIKA adjuvant and PIKA-RV did not show obvious toxicity in local injection site.

For systemic reaction, studies of hematology, blood biochemistry, nonimmune organ and systemic pathological were conducted. The results of nonimmune organ and systemic pathological did not show obvious change. In the study of hematology, we found there is the difference in HCT of high dose female group compared with saline group on 2 days after the last treatment. In our opinion, this difference lack biological significance, since other related values remain within normal range for the mice. HCT is the volume percentage (%) of red blood cells in blood. RBC and HGB can be quite helpful in evaluating a lower-than-normal HCT, because they can help the clinician to determine whether patients is suffering from anemia. However, the values of RBC and HGB of high dose female vaccine group were similar with saline group, and in normal range for the mice, which means the difference of single indicator HCT is not a sign of toxicity.

Other differences in hematology indexes compared with saline group were neutrophils and lymphocytes count. In our opinion, the differences between males of high dose vaccine group and the control group regards to neutrophils and lymphocytes count are induced by the inflammatory reaction at the injection site, thus provoking the mobilization of neutrophils to the affected area. Neutrophils are the first immune cells to arrive at a site of inflammation (Wang and Arase, 2014). Lymphocytes are the cells that determine the specificity of the immune response to infectious microorganisms and other foreign substances. Granulocytes may also help to initiate adaptive immune responses, as they play a key role in initiating adaptive immunity by releasing important cytokines that attract monocytes and dendritic cells and influence whether macrophages differentiate to a predominantly pro- or anti-inflammatory state (Mancebo et al., 2012). So the increased number of neutrophils and the decreased number of lymphocytes indicated the development of inflammation. By the end of study, these changes disappeared and the counts returned to normal range. Although the statistical analysis failed to show these differences in females, the observation of data in Table 1b illustrates clearly that this gender showed the same behavior.

ALT and AST levels are considered to be two of the most important parameters to detect liver injury (Goorden et al., 2013), and ALT is more specific for liver toxicity and is more commonly elevated rather than AST. In the study of blood biochemistry, male PIKA-treated group showed significantly higher level of AST than saline group 2 days after the last administration. To determine whether liver disease is present, AST levels are usually compared with results of other tests, such as ALP, total protein, and bilirubin. However, in this study, there was no obvious change in other related values of PIKA-treated group, especially ALT. Therefore, the change of AST is not an effective indication of toxicity. Furthermore, the level of AST in male PIKA-treated group returned to normal at the end of the study.

ALP is a group of enzymes primarily found in liver and bone (Song et al., 2011; Suzuki, 1986). They act by splitting off phosphorus (an acidic mineral) creating an alkaline pH. The primary importance of measuring alkaline phosphatase is to check the possibility of bone disease or liver disease. Because acid–alkaline is influenced by many other organs/glands including adrenals, uterus, prostate, and intestine, the implications of serum alkaline phosphatase levels must consider more than just bone and liver function. Although the serum levels of ALP in all female PIKA treated groups showed significant decrease compared to Saline group, none of other biomarkers showed alterations and none of the animals of treated groups showed organ lesions. Besides, the difference of ALP had also disappeared by the end of study. Thus, the temporary change of ALP level was not a sign of toxicity.

In the study of immune toxicity, immune organ and histopathology were observed. There was a significant increase of spleen weight in PIKA-treated and PIKA vaccine-treated groups when compared to saline group, in both gender, 2 days after last immunization. In addition, inguinal lymph nodes around the injection site and spleen showed different degree of hyperplasia. Considering increased neutrophil count and reduced lymphocytes, which was observed in this study, the increased weight in the spleen and the hyperplasia of lymph nodes were associated to stimulation of the immune system; this could be considered as indicators of the immunological action of the adjuvants. While hyperplasia of the spleen and lymph nodes returned to normal by the end of study period, which means PIKA adjuvant and PIKA-RV showed no toxicity in immune organs. In another related study, the immune toxicity of PIKA-RV in monkeys including antinuclear antibody test, CD4/CD8 level and histological examination of immune system (unpublished results) showed that PIKA adjuvant and PIKA-RV do not cause immune toxicity.

Conclusions

In summary, PIKA-RV enhance both humoral and cellular immunity. Administered with an accelerated regimen, the PIKA-RV protected 80% of the mice after viral challenge. According to the results of toxicity study, it is suggested that the PIKA and PIKA-RV are well tolerated in mice and can be considered safe. The presented preclinical results support the start of the first clinical studies with PIKA rabies vaccine. This would allow to further explore its safety in humans and also be a broad potential coverage against wild-type rabies strains.

Materials and methods

Animals

Specific pathogen-free (SPF) female BALB/c mice were purchased from the Beijing Laboratory Animal Center for evaluation of the immune response of PIKA-RV. Beagles were purchased from Beijing Rixin Technology Co., Ltd. for post-exposure efficacy test. Golden hamsters were purchased from Changchun Institute of Biological Products Co., Ltd. for post-exposure efficacy test. For acute and repeated dose toxicity studies, Kunming mice were purchased from Vital River Laboratories. Mice and hamsters were kept under SPF requirement. Beagles were kept under clean environment. All animals were treated according to the regulations of Chinese law and the local Ethical Committee.

Viruses

Wild virus strain BD06 in viral challenge test was isolated from infected dog and maintained by Veterinary Institute, Academy of Military Medical Sciences, China.

Vaccines

PIKA, inactivated purified rabies virus (IPRV) and PIKA-RV were produced by Liaoning Yisheng Biopharma Co. Ltd. The standard PIKA-RV containing 2.0 IU IPRV and 1.0 mg PIKA adjuvant was freeze-dried and reconstitute with 1.0 mL of water for injection (WFI) before use. The vaccine control used in animal (Golden Hamster) regimen studies was manufactured by Novartis, which contains inactivated rabies virus without adjuvant.

Evaluation of the immune response of PIKA-RV

Five groups of six mice (6–8 weeks old, weighted 18–22 g) were immunized by intramuscular (i.m.) injection with different concentration (0.2 IU IPRV+100 μg PIKA, 0.02 IU IPRV+10 μg PIKA, 0.002 IU IPRV+1 μg PIKA, or 0.2 IU IPRV) in a volume of 100 μl., or a PBS control. The preparations were injected into mice on days 0 and 7.

Serum samples were collected at day 21 after the first immunization and the virus neutralizing antibody (RVNA) titers were evaluated using FAVN test (fluorescent antibody virus neutralization test) as described previously (Cliquet et al., 1998), which is a standardized test recommended by World Health Organization (WHO).

Mice were sacrificed at day 21 after the first immunization, and splenocytes were prepared. The frequency of IFN-γ secreting cells was analyzed using commercial mouse IFN-γ ELISPOT kits (BD, America) following the manufacturer's instructions. Spots were counted using an automated ELISPOT reader (ChampSpot II, Sage Creation, China). The mean spot number ± SD of triplicate wells for each stimulation antigen or control was calculated.

Post-exposure efficacy test in Beagles

Three groups of Beagles, 10 Beagles in each group, were infected i.m. with 160,000 LD_{50} BD06 rabies virus and then immunized with different preparations post infection. Animals were administered with (1) PBS, (2) vaccine control or (3) PIKA-RV. For PBS and vaccine control groups, animals were injected on days 0, 3, 7, 14 and 21. For PIKA-RV group, animals were received double dose injection on day 0 and 3 post exposure and one dose injection on day 7, 10 and 14 post exposure. Infected and treated animals were observed for 45 days post-challenged for the development of rabies-specific symptoms or death confirmed by direct fluorescent antibody test (DFA) and percentage survival rate was calculated.

Post-exposure efficacy test in golden hamsters

Female golden hamsters (7–8 weeks old, weighted 100–150 g) were randomized into 5 groups with different immunization schedules. Animals were previously i.m. infected with 0.1 mL of 50 LD_{50} wild rabies viruses (BD06 strain) at the hind legs. Animals were treated with (1) saline solution by standard regimen, (2) vaccine control by standard regimen, (3) human rabies immunoglobulin (HRIG, 0.75IU administered at each site of right and left hind leg 2 h post exposure), (4) PIKA-RV by standard regimen, or (5) PIKA-RV by accelerated regimen respectively post infection. For standard regimen, animals were injected on day 0, 3, 7, 14, and 28 respectively. For accelerating regimen, animals were received double dose injection on day 0 and 2 post exposure and another injection on day 7 post exposure. All animals were observed for 45 days post-challenged for the development of rabies-specific symptoms or death confirmed by DFA test and percentage survival rate was calculated.

Acute toxicity test

Kunming mice weighted 17–20 g were divided into 4 groups with 20 mice in each group (10 male and 10 female) for single dose immunization. Mice were administered with 0.2 mL of (1) PBS negative control, (2) PIKA (1000 μg/mL PIKA), (3) PIKA-RV (1 IU/mL IPRV+500 μg/mL PIKA), or (4) PIKA-RV (2 IU IPRV+1000 μg/mL PIKA) respectively. They were observed closely for 4 h, immediate toxic reactions, death and abnormal clinical symptom were recorded. Mice were observed for another 14 days for body weight, food intake, behavior changes, death and abnormal clinical symptoms before sacrificed for macrophathology examination of organs.

Repeated dose toxicity test

Kunming mice weighted 16–22 g were divided into 4 groups with 40 mice in each group (20 male and 20 female) for repeated dose immunization. Mice were administered i.m. with (1) saline control, (2) PIKA adjuvant (1000 μg/mL PIKA), (3) low dose of PIKA-RV (1 IU/mL IPRV+500 μg/mL PIKA), or (4) high dose of PIKA-RV (2 IU IPRV+1000 μg/mL PIKA) respectively. The vaccination schedule evolved double dose (0.2 mL) administration at each side of the forelimbs on day 0 and day 2, and single dose (0.1 mL) administration in one of the forelimbs on days 7, 28 and 42.

The injection site and systemic clinical symptoms were observed 30 min before and after injection, once per hour up to 4 h and twice every day post-injection. Food intake and body weight were measured weekly throughout the study. Average food intake was calculated after each 7-day period to avoid daily fluctuations. Blood samples were obtained after the last vaccination dose on day 44 and during animal recovering period by day 56 for hematology and biochemistry tests.

Hematology parameters

The hematology parameters analyzed in the test include: white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), red cell distribution width (RDW), thrombocytocrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), reticulocyte count (Ret), lymphocyte (LY), monocyte (MO), and neutrophile granulocyte (GR). The analytical reagents were purchased from Shanghai Donghu Biomedicine Pte Ltd. (China).

Biochemistry parameters

The biochemistry parameters analyzed in the test include: Alanine Transaminase (ALT), Aspartate Transaminase (AST), total protein (TP), albumin, total bilirubin, urea, creatinine, glucose (Glu), creatinine kinase (CK), Alkaline phosphatase (ALP), cholesterol (CHO), triglyceride (TG), sodium (Na^+), serum phosphate (K^+), serum chloride (Cl^-). Various test kits purchased from BIOSINO Bio-technology and Science Inc. were used for analysis. The analytical reagents were purchased from Randox Laboratories (China).

Histology examinations

After the final vaccination and by the end of study period, 10 male and 10 female animals were randomly picked from each study, and they were sacrificed for histological examination. Sections of the following preserved tissues were processed by standard histological procedures: adrenals, aorta, axillary lymph node, bone marrow, brain, cecum, colon, duodenum, epididymides, esophagus, eyes, gross lesions, heart, ileum, inguinal lymph nodes,

jejunum, kidneys, liver, lungs, mammary glands, mesenteric lymph nodes, optic nerve, ovaries, pancreas, parathyroid glands, pituitary, prostate, rectum, salivary glands, seminal vesicles, spinal cord (cervical, thoracic and lumbar), spleen, stomach, testes, thymus, thyroid gland, trachea, urinary bladder, uterus vagina, seminal vesicle, sciatic nerve and skeletal muscle of the injection site.

The following organs were weighed: brain, thymus, lungs, heart, spleen, liver, adrenals, kidneys, testes (males), epididymides (males), uterus (females) and ovaries (females). The ratios of organ weight to terminal body weight were calculated.

Statistical analysis

The data on variations of antibodies and IFN-γ secreting cells were conducted using GraphPad Prism5 software (San Diego, CA, USA). Results have been expressed as mean ± SEM values. Differences between the treatment groups were evaluated by one-way ANOVA with significance determined by Tukey-adjusted *t*-tests. The data on variations of body weights, feed consumption, hematology, biochemistry and organ weight were analyzed between control groups and study groups by ANOVA using SPSS according to gender difference.

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