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Artesunate enhances the immune response of rabies vaccine as an adjuvant

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ABSTRACT

Rabies is an ancient zoonosis that continues to be an important health problem worldwide. Vaccination with rabies vaccine is the most important strategy to prevent rabies. Adjuvants contribute to the immune response of viral vaccine. The aim of this study was to investigate whether artemisinin derivatives artesunate and dihydroartemisinin could enhance the immunogenicity of inactivated rabies virus in mice. Administration of artesunate or dihydroartemisinin by intramuscular injection at a dose of 5 mg/kg did not cause body weight loss and unusual symptoms in mice. Mice were immunized with inactivated CVS-11 or inactivated rHEP-dG together with either artesunate or dihydroartemisinin through intramuscular injection. Blood samples were collected to investigate the virus-neutralizing antibody (VNA) titers, and challenge assays were then conducted. The results showed that the rabies VNA titers in mice co-treated with artesunate rather than dihydroartemisinin were significantly higher than in the control animals treated with the phosphate buffered saline (PBS). In addition, mice co-treated with artesunate survived from lethal rabies virus challenge compared with those treated with PBS. In contrast, co-treatment with dihydroartemisinin did not improve the survival rate of the challenged mice. The findings indicate that artesunate could be used as a new candidate adjuvant for rabies vaccination.

1. Introduction

Rabies is an ancient zoonosis and causes more than 59,000 human deaths each year. The domestic dog is the main source of human rabies [1]. Although rabies is associated with a high mortality rate in both humans and dogs once clinical signs are apparent, rabies vaccines can be effectively used to control the disease. However, most rabies cases occur in the developing countries/regions, especially in Asia and Africa, and the high costs of these vaccines limit their use in those places [2]. Currently, the World Health Organization (WHO) recommends inactivated rabies vaccine based on their safety. To develop more efficient and cheaper inactivated rabies vaccines, it is imperative to improve immunogenicity of vaccines and better adjuvants.

Artemisinin was originally used as an antimalarial drug [3]. Several artemisinin derivatives (termed artemisinins), such as dihydroartemisinin (DHA) and artesunate (ART), are currently used for clinical treatment of malaria [4–6]. Previous studies have demonstrated that artemisinin and its derivatives possess potent anticancer effects [7–9]. Recently, more studies have focused on the immune regulatory function of artemisinins. It has been shown that artemisinin derivatives inhibit Th1 and Th17 cells *in vitro* and enhance the expansion and functions of Treg cells [10,11]. In addition, artemisinin and its derivatives can enhance T lymphocyte-mediated immune responses *in vivo* [12]. Furthermore, artemisinin can activate NK cells [13], and shift the lymphocyte responses from Th1 to Th2 [14]. Therefore, artemisinins may contribute to humoral immunity response *in vivo*. However, so far, it remains unknown whether any of artemisinin derivatives can enhance viral vaccine immune response as vaccine adjuvants.

This study was set to determine whether ART and DHA can act as adjuvants to enhance immunogenicity of inactivated rabies vaccine in mice. Our previous study has indicated that rabies virus (RABV) encoding two G (rHEP-dG) induces stronger immune response than that encoding one G [15]. In the present study, CVS-11 strain and rHEP-dG strain were inactivated and were then used to immunize mice in combination with ART or DHA. We found that ART rather than DHA enhanced the virus-neutralizing

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antibody (VNA) level induced by inactivated rabies vaccine. In addition, ART preferably protected vaccinated mice from lethal RABV challenge. Therefore, ART has a great potential to be a viral vaccine adjuvant.

2. Materials and methods

2.1. viruses, reagents and animals

Rabies strains rHEP-dG [15] and CVS-11 (a gift from Dr. Xianzhu Xia, Academy of Military Medical Sciences, Beijing, China) were propagated in neuroblastoma NA cells (obtained from the Wuhan Institute of Biological Products, China). ART (TCI AmericaPortland, OR, USA) was directly dissolved in Dulbecco's modified Eagle's medium (DMEM), while DHA (TCI AmericaPortland) was firstly dissolved in dimethyl sulfoxide (DMSO) and then diluted in DMEM for use. 3–4 weeks of age or 6–7 weeks of age female Kunming (KM) mice, which is an outbreeding strain from Swiss mice, were purchased from the Center for Laboratory Animal Science of the Southern Medical University (Guangzhou, China). Mice were housed in the Laboratory Animal Center of the South China Agricultural University. All animal experiments were approved by the Ethics Committee for Animal Experiments of the South China Agricultural University, and conducted in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

2.2. Effect of ART and DHA in mice

To investigate whether ART or DHA was toxic *in vivo*, KM mice (3–4 weeks of age) were treated with ART or DHA once by intramuscular (i.m.) injection at a dose of 5 mg/kg. Phosphate buffered saline (PBS) treatment was used as a control. Each group contained 6 mice. The body weights and symptoms (piloerection, appetite, tremble, weakness et al.) of mice were monitored every day for 15 days after treatment.

2.3. Immunization assay

RABV CVS-11 strain and rHEP-dG strain were inactivated by UV and the effectiveness of inactivation was assessed in NA cells using a direct fluorescent antibody assay with FITC-labeled anti-RABV N antibodies (Fujirebio Diagnostics, Malvern, PA, USA) after inactivated RABVs treatment. Then, the inactivated CVS-11, inactivated rHEP-dG, or PBS was mixed thoroughly with DHA or ART. KM mice (6–7 weeks of age) were immunized by i.m. injection of the mixture of 1.0×10^5 FFU inactivated RABV and 5 mg/kg ART, DHA or PBS. Specifically, groups are rHEP-dG + ART, rHEP-dG + DHA, rHEP-dG + PBS, CVS-11 + ART, CVS-11 + DHA and CVS-11 + PBS. Mice received PBS injection without RABV were mock infection. Each group contained 6 mice. Peripheral blood was collected at 7 and 14 days post immunization (dpi) and the serum was used to determine VNA levels by the fluorescent antibody virus neutralization (FAVN) test as described previously [16].

2.4. Challenge assay

KM mice (10 mice each group) were immunized with the mixture of inactivated rHEP-dG + ART, rHEP-dG + DHA, or rHEP-dG + PBS, respectively, through i.m. route. A group of 8 mice received PBS injection without RABV (mock). At 14 dpi, mice were i.m. challenged with 3.16×10^5 FFU of CVS-11. Survival rates were recorded daily for 3 weeks, whereby death during the first 4 days of observation was considered non-specific.

2.5. Statistical analysis

Data were presented as mean values ± standard error (SE), and analyzed using GraphPad Prism 6 (GraphPad software, San Diego, CA, USA). The statistical significance was determined using one-way ANOVA, Student's *t* test or Logrank Mantel-Cox test. $P < 0.05$ was considered to be significantly different.

3. Results

3.1. Treatment with ART or DHA does not cause loss of body weight in mice

To investigate whether ART and DHA have side effects *in vivo*, KM mice were treated i.m. with ART or DHA at a dose of 5 mg/kg every day for 15 days. The body weights of the mice were monitored every day. As shown in Fig. 1, ART or DHA treatment did not result in loss of body weight in mice, compared to PBS treatment. Furthermore, no side effects such as piloerection, decreased appetite, tremble, weakness et al. were noticed in all mice treated with DHA, ART or PBS. The results indicate that a dose of 5 mg/kg treatment of ART or DHA was safe to mice.

3.2. ART enhances VNA induced by inactivated RABV

VNA induced by RABV plays an important role in rabies prevention. Next, we investigated whether ART or DHA enhances the induction of VNA after RABV vaccination. As shown in Fig. 2A, mice immunized with inactivated CVS-11+ART showed a significantly higher level of VNA in the peripheral blood at 14 pdi, compared to those treated with CVS-11+PBS. Similarly, ART treatment also significantly enhanced the induction of VNA in mice immunized with inactivated rHEP-dG, compared with PBS treatment (Fig. 2B). To our surprise, treatment with DHA failed to potentiate the induction of VNA in mice immunized with inactivated CVS-11 or rHEP-dG (Fig. 2).

3.3. ART helps rabies vaccine to provide a better protection against RABV challenge

To investigate whether ART or DHA treatment can really help rabies vaccine to improve the protection against a virulent RABV challenge, KM mice were treated i.m. with ART or DHA along with

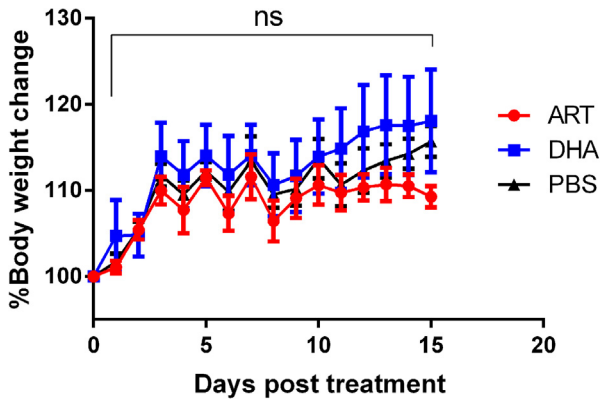

Fig. 1. Effects of ART and DHA on mouse body weight. ART or DHA were administered to KM mice (3–4 weeks of age; n = 6 per group) once by i.m. injection at a dose of 5 mg/kg or PBS (control). Body weight was monitored daily for 15 days. Changes of body weight are shown as the percentages compared to the body weight at day 0 (100%). Data are presented as mean values ± standard error (SE), and calculated by one-way ANOVA with the Holm-Sidak method. ns: none significant differences.

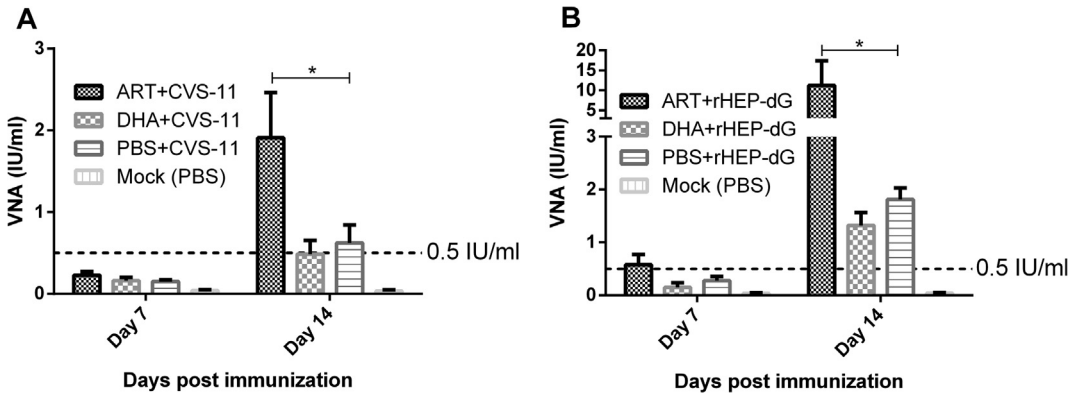

Fig. 2. Immunogenicity induced by inactivated RABVs together with ART or DHA *in vivo*. KM mice (6–7 weeks of age, n = 6 per group) were immunized once i.m. with the mixture of 1.0×10^5 FFU of inactivated CVS-11 + ART (5 mg/kg), CVS-11 + DHA (5 mg/kg), CVS-11 + PBS, rHEP-dG + ART (5 mg/kg), rHEP-dG + DHA (5 mg/kg), rHEP-dG + PBS, or only PBS without RABV (mock). (A) Inactivated CVS-11 and (B) inactivated rHEP-dG. Peripheral blood was collected and the concentrations of serum VNA were determined by FAVN assay, as described in *Materials and Methods*. Data are presented as mean values ± SE, and analyzed by Student's *t* test. $^*P < 0.05$.

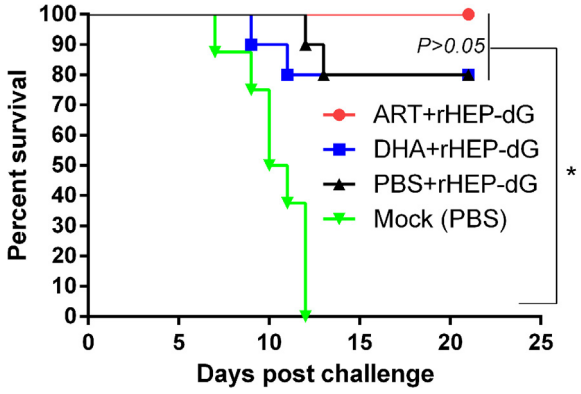

Fig. 3. Survival rates of mice challenged with CVS-11. KM mice (6–7 weeks of age, 10 mice each group) were immunized i.m. with the mixture of 1.0×10^5 FFU of inactivated rHEP-dG + ART (5 mg/kg), rHEP-dG + DHA (5 mg/kg), or rHEP-dG + PBS. Control group of 8 mice received PBS injection without RABV. At 14 dpi, mice were i.m. challenged with 3.16×10^5 FFU of CVS-11 and were observed for 21 days. Survival rates were recorded daily. Data are analyzed by Logrank Mantel-Cox test. $^*P < 0.05$.

inactivated rHEP-dG. As shown in Fig. 3, mice treated with rHEP-dG+ART had a higher survival rate ($P < 0.05$) than those treated with rHEP-dG+PBS. In line with its effect on the induction of VNA (Fig. 2), rHEP-dG+DHA treatment did not alter the survival rate significantly, compared with rHEP-dG+PBS treatment. No mice treated with PBS survived the challenge. The results indicate that ART is able to enhance the protective effect of rabies vaccine, improving the survival rate in response to a virulent RABV challenge.

4. Discussion

Rabies vaccines play an important role in rabies elimination by providing protective immunity. To enhance the immune response of rabies vaccines, we were interested in identifying a safe and effective vaccine adjuvant. Artemisinins have been used as antimalarial drugs in many countries. In this study, we found that treatment with the artemisinin derivatives ART and DHA at 5 mg/kg showed no obvious side effects in mice. This is consistent with the previous observations that ART and DHA show good safety *in vivo* [17–20]. RABV CVS-11 strain and HEP-Flury strain have been used to make inactivated rabies vaccines [21,22]. Previous studies have shown that recombinant RABVs carrying two G genes induce stronger immune responses *in vivo* [15,23–25]. In this study, CVS-11 strain and rHEP-dG strain, which carries two G genes, were used to investigate whether ART and DHA can really enhance the immune response to rabies vaccination in mice. We observed that treatment with ART indeed enhanced the immune response induced by either inactivated CVS-11 or rHEP-dG. Our results suggest that the ART-enhanced immune response induced by RABV was strain independent.

Both ART and DHA belong to artemisinin derivatives. In this study, we found that ART enhanced the VNA level induced by rabies vaccine, but DHA showed no effect on rabies vaccination. Th2 cells play an important role in humoral immunity. A previous study demonstrated that DHA treatment inhibits the production of IL-4, which induces Th2 proliferation and shifts the immune response towards Th1 [26]. However, ART enhances the secretion of IFN-γ in Th1 cells and IL-4 in Th2 cells [27]. Moreover, ART treatment could increase the number of Treg cells [28]. Therefore, we speculate that ART, but not DHA, could enhance the VNA level induced by rabies vaccine possibly through inducing different cytokines and subtype of Th cells. VNA induced by rabies vaccine is critical in counteracting the lethal RABV challenge [29]. In agreement with this, here we noticed that only ART was able to enhance the protective effect of rabies vaccine, improving the survival rate of mice challenged by a virulent RABV.

In summary, here we show that the artemisinin derivative ART enhanced immune response of inactivated rabies vaccine when used as an adjuvant. In addition to artemisinin, DHA and ART, there are more artemisinin derivatives in the market, such as arteeter, artemether, and artemisone. Further research is needed to determine whether any of other artemisinin derivatives is superior to ART as a vaccine adjuvant. This study provides a reference for the research of viral vaccine adjuvants.

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Author contributions

Xiaofeng Guo and Jun Luo designed the research. Jun Luo, Hongling He, Yue Zhang and Qing Liu performed the experiments. Jun

Luo, Xiaofeng Guo, and Shile Huang analyzed the data and wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, et al. Estimating the global burden of endemic canine rabies. PLoS Negl Trop Dis 2015;9(4):e3709.
[2] Fu ZF. Rabies and rabies research: past, present and future. Vaccine 1997;15 (Suppl):S20–4.
[3] Tu Y. The development of new antimalarial drugs: qinghaosu and dihydroqinghaosu. Chin Med J (Engl) 1999;112(11):976–7.
[4] Lalloo DG, Shingadia D, Bell DJ, Beeching NJ, Whitty C, Chiodini PL. UK malaria treatment guidelines 2016. J Infect 2016;72(6):635–49.
[5] White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM. Malaria. Lancet 2014;383(9918):723–35.
[6] WHO. Guidelines for the Treatment of Malaria. 3rd ed. World Health Organization; 2015.
[7] Jia JG, Zhang LG, Guo CX, Wang YG, Chen BL, Wang YM, et al. Artemisinin inhibits proliferation of gallbladder cancer cell lines through triggering cell cycle arrest and apoptosis. Zhonghua Wai Ke Za Zhi 2016;54(3):222–7.
[8] Odaka Y, Xu B, Luo Y, Shen T, Shang C, Wu Y, et al. Dihydroartemisinin inhibits the mammalian target of rapamycin-mediated signaling pathways in tumor cells. Carcinogenesis 2014;35(1):192–200.
[9] Wang L, Liu L, Wang J, Chen Y. Inhibitory effect of artesunate on growth and apoptosis of gastric cancer cells. Arch Med Res 2017;48(7):623–30.
[10] Hou LF, He SJ, Li X, Wan CP, Yang Y, Zhang XH, et al. SM934 treated lupus-prone NZB x NZW F1 mice by enhancing macrophage interleukin-10 production and suppressing pathogenic T cell development. PLoS ONE 2012;7(2):e32424.
[11] Li X, Li TT, Zhang XH, Hou LF, Yang XQ, Zhu FH, et al. Artemisinin analogue SM934 ameliorates murine experimental autoimmune encephalomyelitis through enhancing the expansion and functions of regulatory T cell. PLoS ONE 2013;2;8(8):e74108.
[12] Yang SX, Xie SS, Gao HL, Long ZZ. Artemisinin and its derivatives enhance T lymphocyte-mediated immune responses in normal mice and accelerate immunoreconstitution of mice with syngeneic bone marrow transplantation. Clin Immunol Immunopathol 1993;69(2):143–8.
[13] Houh YK, Kim KE, Park S, Hur DY, Kim S, Kim D, et al. The effects of artemisinin on the cytolytic activity of natural killer (NK) cells. Int J Mol Sci 2017;18(7).
[14] Khakzad MR, Ganji A, Ariabod V, Farahani I. Artemisinin therapeutic efficacy in the experimental model of multiple sclerosis. Immunopharmacol Immunotoxicol 2017;39(6):348–53.
[15] Liu X, Yang Y, Sun Z, Chen J, Ai J, Dun C, et al. A recombinant rabies virus encoding two copies of the glycoprotein gene confers protection in dogs against a virulent challenge. PLoS ONE 2014;9(2):e87105.
[16] Cliquet F, Aubert M, Sagne L. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods 1998;212(1):79–87.
[17] Choi WH. Novel Pharmacological activity of artesunate and artemisinin: their potential as anti-tubercular agents. J Clin Med 2017;6(3).
[18] Li HJ, Wang W, Li YZ, Qu GL, Xing YT, Tao YH, et al. Effects of artemether, artesunate and dihydroartemisinin administered orally at multiple doses or combination in treatment of mice infected with Schistosoma japonicum. Parasitol Res 2011;109(2):515–9.
[19] Li HJ, Wang W, Qu GL, Li YZ, Tao YH, Xing YT, et al. Effect of the in vivo activity of dihydroartemisinin against Schistosoma mansoni infection in mice. Parasitol Res 2012;110(5):1727–32.
[20] Tasai S, Saiwichai T, Kaewthamasorn M, Tiawsirisup S, Buddhirakkul P, Chaichalotornkul S, et al. Artesunate-tafenoquine combination therapy promotes clearance and abrogates transmission of the avian malaria parasite plasmodium gallinaceum. Vet Parasitol 2017;233:97–106.
[21] Morimoto K, Kawai A, Sato Y, Ohkubo A. A unique transcription mode of rabies virus high egg passage-Flury strain detected in infected baby hamster kidney-21 cells. Microbiol Immunol 2011;55(8):558–64.
[22] Patel AC, Upmanyu V, Ramasamy S, Gupta PK, Singh R, Singh RP. Molecular and immunogenic characterization of BHK-21 cell line adapted CVS-11 strain of rabies virus and future prospect in vaccination strategy. Virusdisease 2015;26 (4):288–96.
[23] Faber M, Pulmanausahakul R, Hodawadekar SS, Spitsin S, McGettigan JP, Schnell MJ, et al. Overexpression of the rabies virus glycoprotein results in enhancement of apoptosis and antiviral immune response. J Virol 2002;76 (7):3374–81.
[24] Hosokawa-Muto J, Ito N, Yamada K, Shimizu K, Sugiyama M, Minamoto N. Characterization of recombinant rabies virus carrying double glycoprotein genes. Microbiol Immunol 2006;50(3):187–96.
[25] Tao L, Ge J, Wang X, Wen Z, Zhai H, Hua T, et al. Generation of a recombinant rabies Flury LEP virus carrying an additional G gene creates an improved seed virus for inactivated vaccine production. Virol J 2011;8:454.
[26] Noori S, Hassan ZM. Dihydroartemisinin shift the immune response towards Th1, inhibit the tumor growth in vitro and in vivo. Cell Immunol 2011;271 (1):67–72.
[27] Lee SH, Cho YC, Kim KH, Lee IS, Choi HJ, Kang BY. Artesunate inhibits proliferation of naive CD4(+) T cells but enhances function of effector T cells. Arch Pharm Res 2015;38(6):1195–203.
[28] Meng QF, Zhang XX, Zhang Z, Chen W, Li XL, Wang YJ, et al. Therapeutic potential of artesunate in experimental autoimmune myasthenia gravis by upregulated T regulatory cells and regulation of Th1/Th2 cytokines. Pharmazie 2018;73(9):526–32.
[29] Koraka P, Bosch BJ, Cox M, Chubet R, Amerongen G, Lovgren-Bengtsson K, et al. A recombinant rabies vaccine expressing the trimeric form of the glycoprotein confers enhanced immunogenicity and protection in outbred mice. Vaccine 2014;32(36):4644–50.