FULL PAPER *Laboratory Animal Science*

Adjuvant effects of recombinant giant panda (*Ailuropoda melanoleuca*) IL-18 on the canine distemper disease vaccine in mice

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(Received 5 May 2014/Accepted 17 October 2014/Published online in J-STAGE 14 November 2014)

ABSTRACT. Canine distemper virus (CDV) is a morbillivirus known to cause morbidity and mortality in a broad range of animals. Giant pandas (*Ailuropoda melanoleuca*), especially captive ones, are susceptible to natural infection with CDV. Interleukin-18 (IL-18) is a powerful adjuvant molecule that can enhance the development of antigen-specific immunity and vaccine efficacy. In this study, a giant panda IL-18 gene eukaryotic expression plasmid (pcAmIL-18) was constructed. Female BALB/c mice were muscularly inoculated with the plasmids pcAmIL-18, pcDNA3.1 and PBS, respectively. They were subsequently injected with an attenuated CDV vaccine for dogs, and the induced humoral and cellular responses were evaluated. The results showed that pcAmIL-18 remarkably improved the level of specific antibody, IFN-γ and IL-2 in mice sera, the T lymphocyte proliferation index and the percentage of $CD4^+$ and $CD8^+$ cells. These data indicated that pcAmIL-18 is a potential adjuvant that promotes specific immunity.
KEY WORDS: adjuvant, CDV, giant panda, giant panda IL-18

doi: 10.1292/jvms.14-0226; *J. Vet. Med. Sci.* 77(2): 187–192, 2015

Canine distemper (CD), which is caused by canine distemper virus (CDV), is a highly contagious and fatal disease in a wide range of mammals [1]. Giant pandas (*Ailuropoda melanoleuca*) are susceptible to natural infection with CDV [2]. It has been reported that CDV was implicated in disease and mortality in giant pandas. For instance, four pandas infected with CDV, died in Chongqing Zoo and Nanjing Zoo [6]. Recently, CDV has been isolated from captive giant pandas. Early studies showed that 94% of unvaccinated giant pandas had no antibodies against CDV [16]. To data, there is no standard vaccine strategy for captive giant pandas in China. Most giant pandas have not been vaccinated at the Wolong Conservation and Research Center, while bivalent attenuated live vaccines for dogs have been used at Chengdu Zoo and the Chengdu Research Base of Giant Panda Breeding (CPB). Loeffler *et al.* detected the concentrations of various antibodies against CDV among different individuals and times in 19 giant pandas at the CPB [12]. Wang *et al.* discovered high rates of low antibody titers and short sustaining times in 33 giant pandas vaccinated with a multivalent canine distemper attenuated live vaccines specialized for dogs [25]. It has been suggested that the CDV vaccine was inadequate to stimulate giant pandas to produce higher levels of antibody against CDV. These results indicated the CDV attenuated vaccine for dogs had poor efficacy in protecting giant pandas. Recently, injection of cytokine adjuvants has been considered as a candidate strategy for vaccination and has been widely applied to promote the induction of immune responses and immunoprotective effects of vaccines against viruses. Therefore, it is necessary to explore various vaccine adjuvants to produce adequate protective immunity with the CDV vaccine in giant pandas.

Interleukin-18 (IL-18), initially referred to interferon-γ (IFN-γ) inducing factor, is a cytokine secreted mainly by mononuclear macrophages [14]. IL-18 plays an important role in stimulating the differentiation of T helper 1 (Th1) cells and production of cytokines including INF-γ, IL-2, colony-stimulating factor (CSF) and tumor necrosis factor-α (TNF-α) [24]. As adjuvants, cytokines can enhance the immunogenicity of vaccines against infectious diseases [5, 21]. It has been demonstrated that IL-18 is a powerful adjuvant molecule that can effectively promote the development of antigen-specific immunity and vaccine potency in several mammalian species, such as mice [11, 26], pigs [23, 28] and chickens [4, 9, 20]. Co-immunization of plasmid IL-18 as an adjuvant enhanced immune response induction in pigs by strengthening $CD4^+$ and $CD8^+$ T-lymphocyte responses [28]. In addition, IL-18 not only induced the Th1 cytokines, but also reinforced mitogen-specific lymphocytes proliferative responses.

The objectives of this study were to determine the immune stimulatory effects of giant panda IL-18 (AmIL-18) on CDV vaccination. In mice, coadministration of pcAmIL-18 could improve both humoral and cellular immune responses.

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MATERIALS AND METHODS

Animals and vaccine: Specific-pathogen-free BALB/c female mice, 6–8 weeks old, were purchased from the Center of Experimental Animals, Sichuan University, PR China. All the animal experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Sichuan University. The vaccine was Nobivac puppy DP (Intervet, Nederland) for dogs, which was conventionally used to immunize giant pandas to prevent CD disease at Chengdu Zoo.

Expression plasmids construction: The plasmids pMD-AmIL-18 containing the AmIL-18 gene (GenBank accession number HQ874655.1) was stored in our lab. For construction of a recombinant expression plasmid, the AmIL-18 gene was amplified with the pMD-AmIL-18 plasmids as a template by using *Pfu* DNA polymerase (Fermentas, Burlington, ON, Canada) with forward primers containing *Hind*III restriction sites (5′-CGCCC*AAGCTT*GCCATGGCTGCTAACACAG-3′) and reverse primers containing *Eco*RI sites (5′-CCG*GAATTC*CTAGCTTTTGTTTTGAACAG-3′). The resulting fragments were purified, digested with *Hind*III and *Eco*RI, and ligated into the corresponding cloning sites of pcDNA3.1 vector (Invitrogen, Carlsbad, CA, U.S.A.). The recombinant plasmid was confirmed by sequencing and named pcAmIL-18. The plasmid's DNA was prepared using an Omega Endo-Free Plasmid Maxi Kit (Omega Bio-Tek, Inc., Norcross, GA, U.S.A.), as described in the manufacturer's protocol.

Transient transfection: Before animal vaccination, the expression of pcAmIL-18 was tested by transiently transfecting HeLa cells with pcAmIL-18 or pcDNA3.1 by using Lipofectamine 2000™ (Invitrogen). Total RNA was extracted from the cells, and the transcription of AmIL-18 was identified by reverse transcription-polymerase chain reaction (RT-PCR). The supernatants of the cells transfected were tested for the concentration of IL-18 protein by using a Human IL-18 ELISA Kit (Neobioscience, Shenzhen, PR China) after a 48 hr culture.

Animal vaccination: BALB/c mice were subcutaneously injected with 0.1 m*l* attenuated CDV vaccine. A total of 81 mice were divided randomly into 3 groups (n=27 per group). The mice in groups 1 and 2 were intramuscularly immunized with PBS and pcDNA3.1 (100 μg/mice) as controls on day 0, respectively. At the same time, the mice in group 3 were intramuscularly injected with pcAmIL-18 (100 μg/mice). The identical doses were given again after 3 weeks as booster immunizations.

Assay of anti-CDV antibodies: Peripheral blood was obtained from the mice via the orbital venous plexus after anesthetizing them on days 7, 14, 21, 28, 35 and 42 after booster immunization. A Sandwich ELISA Kit (Yanhuibioscience Tech, Shanghai, PR China) was used to assess the titer of anti-CDV mouse antibodies.

Assay of IFN-γ and IL-2 in mouse serum: The effects of the pcAmIL-18 adjuvant on IFN-γ and IL-2 production were investigated with Sandwich ELISA kits (R&D Systems, Minneapolis, MN, U.S.A.) using a serial dilution of the serum according to the instructions.

Splenocyte proliferation responses: Splenocytes were isolated and resuspended in RPMI-1640 supplemented with 10% FBS and seeded into 96-well plates at 2×10^5 cells per well. For the lymphocyte proliferation assay, the cultures were stimulated for 44 hr with either concanavalin A (ConA, Sigma, 5 μg/m*l*) or PBS as a negative control at 37°C with 5% CO_2. The proliferative response was measured by adding 10 μl Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) to each well with a further incubation for 4 hr. The optical density (OD) of each well was determined at 450 nm on a fluorescence microplate reader (BioTek, Winooski, VT, U.S.A.). The splenocyte proliferation stimulation index (S.I.) was calculated as the ratio of the average OD of antigen-treated cells to the average OD of untreated cells.

Analysis of $CD4^+$ and $CD8^+$ T lymphocytes: The isolated mouse splenocytes were adjusted to 1×10^6 cells/m*l*. The 100 μl of samples (1×10^5 cells) was stained for 30 min with PE-labeled anti-mouse CD4a and FITC-conjugated anti-mouse CD3e and then PE-labeled CD8a and FITC-conjugated CD3e (ebioscience, San Diego, CA, U.S.A.), respectively, at 4°C in the dark. After washing, the cells were analyzed with a FACSCalibur flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.). During analysis, T lymphocytes were gated based on forward and side scatter, and the percentages of $CD4^+CD3^+$ and $CD8^+CD3^+$ T lymphocytes were calculated.

Statistical analysis: All data are presented as the mean ± standard deviation (SD). Statistical analysis of the data was performed with the SPSS 13 software. One-way ANOVA was utilized to evaluate the statistical differences among groups. A value of $P<0.05$ was defined as significant.

RESULTS

Transient expression in HeLa cells: The PCR product containing an AmIL-18 gene with the size of 579 bp was amplified by RT-PCR, using cDNA derived from cells transfected with pcAmIL-18 (Fig. 1A). Moreover, no product could be observed from cells transfected with pcDNA3.1. Meanwhile, RNA was used as a template for PCR to monitor the possibility of contamination from the plasmid DNA, and no product was amplified. In the ELISA test, higher levels of IL-18 were observed in the culture medium of cells transfected with pcAmIL-18 than in the culture medium of the control pcDNA3.1-transfected cells (Fig. 1B). Thus, it was demonstrated that pcAmIL-18 could express in cells.

Splenocyte proliferation: Splenocyte proliferation was measured for six consecutive weeks from one week after the booster immunization. As shown in Fig. 2, the proliferation levels of spleen T lymphocytes from mice vaccinated with pcAmIL-18 as an adjuvant were highly significant, compared with the splenocyte proliferation rate of animals treated with pcDNA3.1 or PBS alone ($P<0.05$). The highest stimulation index was found on day 35 in mice inoculated with pcAmIL-18, while no remarkable differences were detected between the two control groups ($P>0.05$).

Specific antibody changes: After the second immuniza-

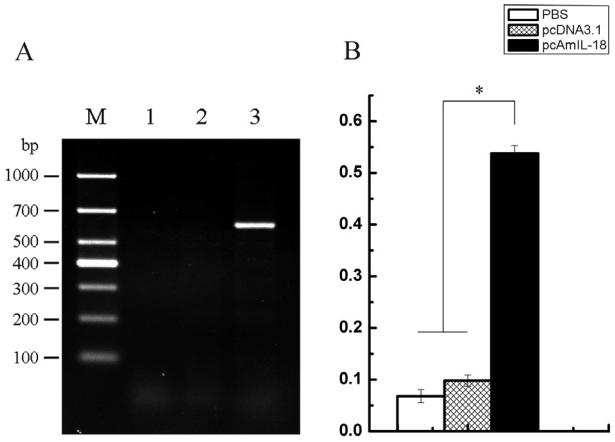

Fig. 1. Verification of AmIL-18 expression in Hela cells. (A) RT-PCR tests. Lane M, DL1000 DNA Marker. Lane 1, RNA template for PCR. Lane 2, no band from cDNA of cells transfected with pcDNA3.1. Lane 3, the AmIL-18 gene amplified from cDNA of cells transfected with pcAmIL-18. (B) The level of IL-18 observed in the culture medium of cells transfected with pcAmIL-18, pcDNA3.1 and PBS. *P<0.05.

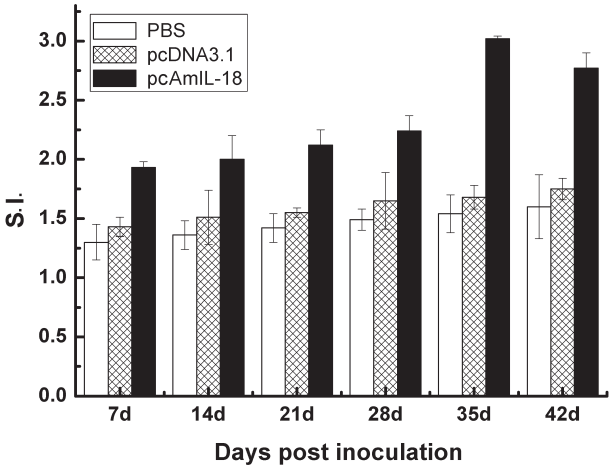

Fig. 2. The proliferation of T lymphocytes in the spleens of four mice was analyzed with a CCK-8 kit using ConA as a stimulating agent. The stimulation indices (S.I.) are shown on the vertical axis.

tion, specific antibodies to CDV antigens could be detected in the sera. The antibody levels in mice coadministered pcAmIL-18 were higher than those of mice inoculated with PBS and pcDNA3.1 from days 7 to 42 (P<0.05) (Fig. 3). The level of antibodies reached the peak at day 21 after vaccination. However, there was no obvious difference in the specific antibody titer in the PBS and pcDNA3.1 groups during the entire period of the experiment (P>0.05).

Cytokines production: Figure 4 demonstrates that the contents of IFN-γ and IL-2 in mice were enhanced by stimulation with AmIL-18, compared with those of the controls. These two main cytokines of the Th1-dominant immune response were increased from days 7 to 28 (P<0.05).

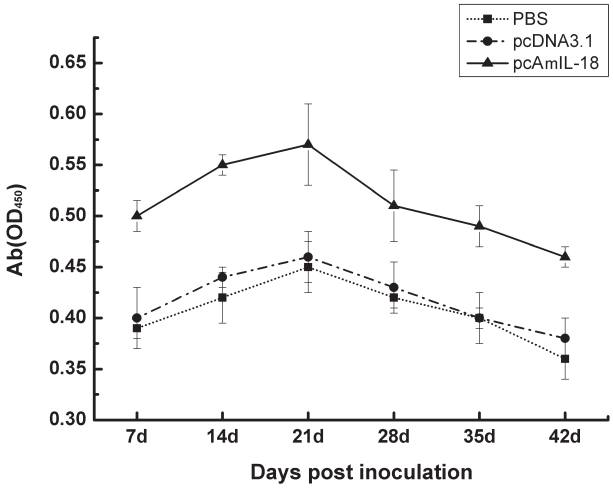

Fig. 3. Levels of specific antibodies to CDV from four experimental mice. The levels of antibodies of the pcAmIL-18 group were higher than those of the PBS and pcDNA3.1 groups at all times after vaccination (P<0.05).

$CD4^+$- and $CD8^+$-positive T cells: After immune selection, $CD4^+$ cells and $CD8^+$ cells in the spleens of mice administrated pcAmIL-18 also increased from days 14 to 42 (P<0.05). At day 42, the percentages of the $CD4^+$ $CD3^+$ and $CD8^+$ $CD3^+$ T lymphocytes subgroups in the pcAmIL-18 vaccinated group were all higher than those on days 14 and 28 (Fig. 5).

DISCUSSION

At present, there is no specific CDV vaccine for giant pandas. Although the use of live attenuated vaccines for dogs has helped to prevent CDV infections, it only stimulates limited immune responses. Hence, it is an urgent problem to enhance the immunizing effect of the CDV vaccine for giant pandas. It is important to find a safe and efficient adjuvant. IL-18 is a potent IFN-γ inducing factor and is expressed by a wide range of cells [15]. IFN-γ has an essential role in protection against virus infection by evoking effector pathways, such as nitric oxide (NO) [17]. Because of the obvious advantages of recombinant plasmids over proteins in vaccination, such as lower expenses to prepare, long-lived protective effectiveness and greater stability in storage, pcAmIL-18 was used as an adjuvant to promote immunity.

As one of the most precious species, it is difficult to get sufficient numbers of natural host cells for experiments. Besides, it is unimaginable to test any unverified vaccine on giant pandas. In our early trials, AmIL-18 was expressed in *E. coli*. *In vitro*, the purified protein showed excellent characteristics in stimulating IFN-γ in mouse splenocytes (unpublished). Thus, we selected a mouse model to evaluate the effect of AmIL-18 as an adjuvant to improve the immunogenicity of CDV attenuated live vaccines *in vivo*.

As the DNA vaccine or adjuvant, bovine or porcine IL-18 has been demonstrated to have antigen-specific immune-

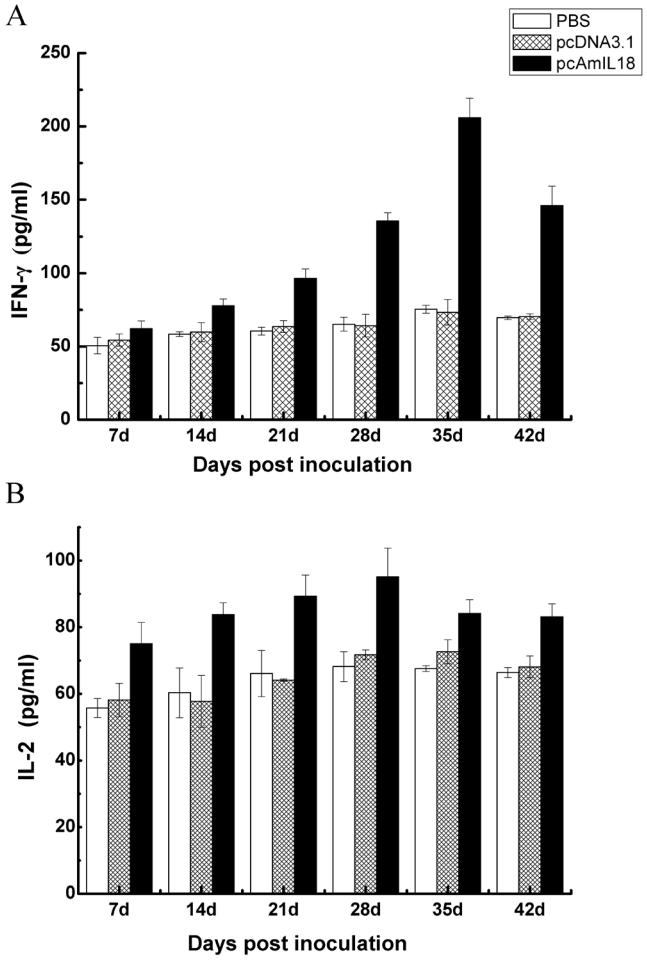

Fig. 4. Vaccination of mice (n=4) with pcAmIL-18 stimulated the production of Th1 type cytokines. (A) The content of IFN-γ in the pcAmIL-18-treated mice was higher than those of the control groups (P<0.05). There was no significant difference among the PBS and pcDNA3.1 groups (P>0.05). (B) The IL-2 levels in the sera differed significantly between the pcAmIL-18 and control groups beginning 7 days after inoculation (P<0.05).

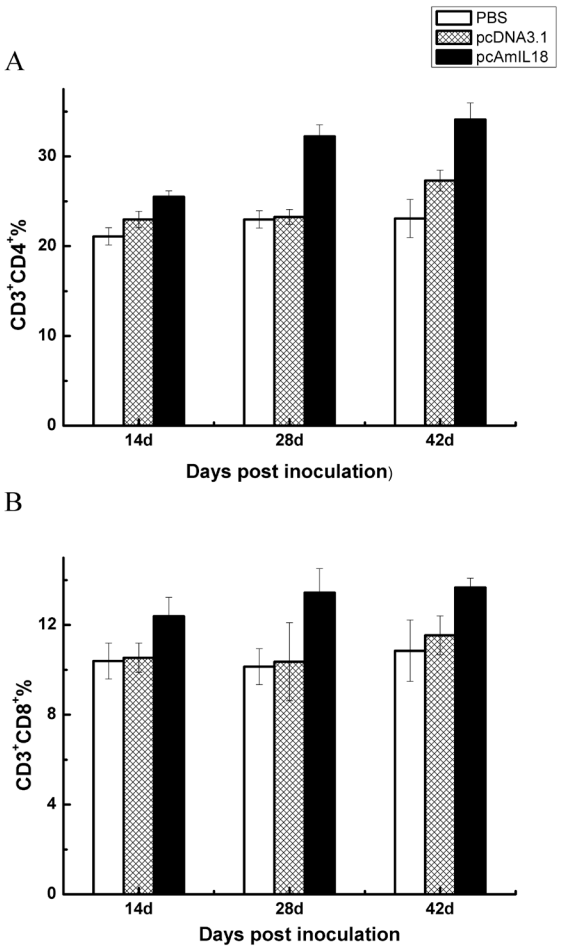

Fig. 5. Changes in the percentages of $CD4^+CD3^+$ and $CD8^+CD3^+$ in the spleens of four mice. (A) The enhancement of $CD4^+$ T cells from mice treated with pcAmIL-18 is distinctly different compared with the control groups (P<0.05) beginning at 14 days after inoculation, and there was no conspicuous difference in the PBS and pcDNA3.1 groups (P>0.05). (B) $CD8^+$ T cells increased more obviously than in the control groups (P<0.05).

enhancing effects in mice. Shi determined that bovine IL-18 could enhance the foot-and-mouth disease virus (FMDV) vaccine immune responses in BALB/c mice [19]. Suo found that coadministrator of a porcine IL-18 expression plasmid could induce high responses using a mouse model [23]. These experiments have verified the enhancing effect of exogenetic immunopotentiators, such as IL-18, in mice in preliminary trials [13, 27].

The T-lymphocyte proliferation response is closely related to cell-mediated immunity. CCK-8 analysis results demonstrated a significant difference (P<0.05) between the pcAmIL-18 vaccinated group and control groups. Because cytokines play a vital important role in the development of cellular immune responses and prevention of viral infections, cytokine production was also determined in this study. Higher levels of IL-2 and IFN-γ were observed in the pcAmIL-18 group. The profile of cytokine secretion and T-lymphocyte proliferation responses suggested that AmIL-18 enhanced the induction of immune responses by promoting a Th1-dominant response, which could contribute to virus clearance. The data are consistent with the results of other immune response studies of IL-18 plasmids administered as adjuvants in vaccines [7].

$CD4^+$ T lymphocytes can induce and enhance the immune response by secreting cytokines; $CD8^+$ T lymphocytes play a major role in cytotoxic killing of target cells [8, 22]. So, $CD4^+$ and $CD8^+$ lymphocytes represent critical functional subsets of adaptive cell-mediated immunity. The results concerning the detection of T-lymphocytes subgroups indicated that the percentages of the $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocytes subgroups in the pcAmIL-18 groups were significantly higher than those of the control groups. At

day 42, the percentages of the $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocyte subgroups were highest. The increased T cells would have confinement effects on replication of viruses and produce better protection. It was confirmed that the Nobivac vaccine combined with pcAmIL-18 might enhance cellular immunity by promoting the differentiation or proliferation of $CD4^+$ T-cells [3, 18]. Herein, $CD4^+$ T-cells appeared to be stimulated by AmIL-18 and were presumed to be IFN-γ-producing cells. IL-18 acted directly on activating $CD8^+$ T cells, which have a central role in viral clearance, suggesting a role for IL-18 in viral infection [10]. It is worth further study to determine whether antigen-nonspecific stimulation of $CD8^+$ T cells by cytokines, such as IL-2, IL-15 or IL-18, kills viruses via direct antiviral cytotoxicity or activation of accessory cells. In conclusion, we demonstrated that inoculation with pcAmIL-18 achieved a significant promotion of humoral and cellular immune responses in mice administered the CDV vaccine, which indicated that pcAmIL-18 could be a novel promising adjuvant in a future large-scale field trial. At this initial stage of research of the adjuvant, AmIL-18 might be considered an antigen in mice that could partially enhance lymphocytes immune responses. Further evaluation of robust immune responses efficacies for this adjuvant in a CDV challenge system and optimization for immunization of other animals will be the next areas we study. In order to save endangered giant pandas, more effective and specific immunological preparations should be developed.

ACKNOWLEDGMENTS. This study was financially supported by the Chengdu Research Fund for Giant Panda Breeding. We are grateful to Chengdu Zoo for providing experimental materials. The authors thank Prof. Yujun Zhang and Prof. Hongning Wang (Sichuan University) for their kindly assistances in the flow cytometer assays and mouse tests.

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