Acta Tropica 164 (2016) 395–401

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

CpG oligodeoxynucleotides with crude parasite antigens reduce worm recovery in *Opisthorchis viverrini* infected hamsters

Chamraj Kaewraemruaen[a,c], Rasana W. Sermswan[b,c], Surasakdi Wongratanacheewin[a,c,*]

[a] Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand
[b] Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand
[c] Melioidosis Research Center, Khon Kaen University, Khon Kaen, 40002, Thailand

ARTICLE INFO

Article history:
Received 25 April 2016
Received in revised form 4 October 2016
Accepted 9 October 2016
Available online 11 October 2016

Keywords:
CpG ODN
Opisthorchis viverrini
Protection
Hamster
T cells
IFN-γ

ABSTRACT

Opisthorchis viverrini, a human liver fluke, is still an endemic parasitic infection in Thailand and nearly all countries in Southeast Asia. *O. viverrini* induces a chronic stage of infection in hamsters. During the first 2 weeks of infection, Th1 inducing cytokine, IL-12, increased but was down regulated in chronic infection. In this study it was found that unmethylated-CpG ODN (oligodeoxynucleotides) 1826 increased hamster mononuclear cell proliferation and stimulated IFN-γ production *in vitro*. The IFN-γ levels in hamster sera were significantly increased in hamsters injected with CpG ODN 1826 alone or plus crude somatic antigens (CSAg). Further investigation using the flow cytometer found that $CD4^+$T cells and IFN-γ^+ $CD4^+$T cells (Th1-like cells) in the hamster blood were significantly increased. The role of these cells in the protective responses in hamsters was evaluated by challenging with 25 metacercaria and observation for 3 months. The number of worms recovered was significantly reduced in the hamsters injected with CpG ODN 1826 with CSAg, but not in CpG ODN 1826 alone groups when compared to PBS control. The percent of reduction in hamsters against this parasite were 32.95% and 21.49% in the CpG ODN 1826 with CSAg and CpG ODN 1826 alone. This study indicates that CpG ODN 1826 plus parasite antigens elicit a Th1-like response that leads to the enhancement of worm reduction.

1. Introduction

Opisthorchis viverrini is a helminth parasite that causes a public health problem in the Southeastern Asia region. Opisthorchiasis is endemic in the northeast (Thaewnongiew et al., 2014) and north (Wongsawad et al., 2012) of Thailand, and other neighboring countries i.e. Laos, Cambodia, and Vietnam (Sithithaworn et al., 2012; Sripa et al., 2011). *O. viverrini* infection causes chronic infection and can be a risk factor for cholangiocarcinoma (CCA) in patients (Srivatanakul et al., 1991a, 1991b). Humans or animals, for example dogs, cats, pigs, minks, weasels, civets, and house rats are the hosts of this parasite, and the hamster is the suitable animal model for *O. viverrini* infection (Boonmars et al., 2009). In the chronic stage of *O. viverrini* infection in hamsters, IL-10 and TGF-β were the regulatory cytokines that were found significantly increased. In contrast, the IL-12 responses (Th1 induced cytokine) were highly expressed during the first two weeks of infection but were significantly lower in the chronic stage. This indicated that IL-12 responses could be found during the stage of juvenile parasite development (Jittimanee et al., 2007). Similarly, Th1- IFN-γ levels were found increased in rats infected with a trematode, *F. hepatica,* on day 7 post infection (Tliba et al., 2002). Moreover, Th2 cytokine (IL-4) and Treg cytokine responses such as IL-10 and TGF-β, were found significantly expressed more than Th1 responses in spleens and mesenteric lymph nodes (MLN) in the chronic stage (Jittimanee et al., 2007). It was then hypothesized that since the Th1 cytokine responses were upregulated during the early stage of infection, this cytokine might play a role in the protection against this infection. Moreover, the parasites from infected hamsters have been shown to induce significantly high levels of regulatory cytokines (IL-10 and TGF-β) (Jittimanee et al., 2007) and such TGF-β levels were stimulated by CSAg. It was found that when hamsters were immunized to protect against *O. viverrini* infection, such protection was correlated with the reduction of TGF-β and IL-10, but not IFN-γ expression. (Jittimanee et al., 2012). To determine whether the stimulation of Th1 responses could provide increased protection against *O. viverrini* infection by reduction of worm revovery, the Th1 inducing

* Corresponding author at: Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand.
E-mail address: sura_wng@kku.ac.th (S. Wongratanacheewin).

http://dx.doi.org/10.1016/j.actatropica.2016.10.005
0001-706X/

adjuvant, unmethylated-CpG oligodeoxynucleotide (CpG ODN) has been used in this study. The CpG ODN stimulates the immune cells via TLR9 (Toll-like receptor 9) (Hemmi et al., 2000), and induces a strong inflammatory response to activate the immune system. CpG ODN was used as the adjuvant of immune activation in the vaccination of bacterial infections (Liu et al., 2011), viral infections (Davis et al., 1998), and parasitic infections (Aldridge et al., 2010; Cervi et al., 2004; Gupta et al., 2011; Pinzon-Charry et al., 2010; Ribeiro et al., 2009; Sanchez et al., 2011; Sane et al., 2010). BALB/c mice treated with CpG ODN reduced *Taenia crassiceps* burden (Aldridge et al., 2010). Moreover, CpG ODN was used as an adjuvant to enhance the partial reduction of *Schistosoma mansoni* (Teixeira de Melo et al., 2013). In this study, the effect of CpG ODN on Th1-like cytokine activation in hamsters was determined. It was demonstrated that CpG ODN could stimulate significant levels of $CD4^+$ T cells producing IFN-γ in hamster blood. It was demonstrated herein that these cells could increase the resistance to *O. viverrini* infection in hamsters.

2. Materials and methods

2.1. Metacercarial preparation

O. viverrini metacercaria were prepared as previously described (Sirisinha et al., 1984). Briefly, infected cyprinid fish, obtained from a fresh water lake in the endemic area of the northeast Thailand, were homogenized and placed in a digesting solution (0.25% Pepsin, Sigma, MO), normal saline and 15% hydrochloric acid (BDH, Poole England) at a ratio of 1:3 and then incubated at 37 °C for one hour. The digested solution was sieved through 1000, 300, and 106 μm meshes. The debris obtained by filtering on 106 μm mesh was washed repeatedly many times with normal saline until clear. Cleared sediments were investigated for metacercarial isolation under a dissecting microscope. These metacercaria were stored in normal saline at 4 °C until infection.

2.2. Antigen preparations

Crude somatic antigens (CSAg) were prepared from adult *O. viverrini* worms as described by Wongratanacheewin and colleagues (Wongratanacheewin et al., 1988). The worms, aged approximately 3 months, were collected from bile ducts of *O. viverrini* infected hamsters and washed with normal saline 3 times. The worms then were transferred into a grinder tube containing complete protease inhibitor cocktails (Roche Diagnostic GmbH, Mannheim Germany) and homogenized at 4 °C. The homogenate was sonicated in an ultrasonic disintegrator set to operate at 1 min intervals for 10 min at 4 °C. The sonicated homogenate was stored overnight at 4 °C and centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant was transferred into new tubes, and sterilized with filter membranes with a pore size of 0.2 μm (Whatman, Buckinghamshire England) and kept at −20 °C. The protein concentration was determined in the supernatant by Bradford's reagent (Bio-Rad, CA), and protein patterns (Supplement Fig. 1) were checked by the silver staining method.

2.3. Oligodeoxynucleotides

Oligodeoxynucleotides (ODNs) were used with the following sequences: CpG ODN 1826, 5′TCCATGACG TTCCTGACGTT3′ and non-CpG ODN 2138, 5′TCCATGAGCTTCCTG AGCTT3′. All ODNs were purchased from Invivogen (InvivoGen, CA).

2.4. Animal infections

Male Syrian golden hamsters, 6–8 weeks old were orally infected with either 25 (for challenging) or 50 (for CSAg preparation) *O. viverrini* metacercaria (Mc). They were housed in the animal care unit at Faculty of Medicine, Khon Kaen University on a 12 h light/dark cycle and fed a commercial feed. Uninfected hamsters were used as controls. These *O. viverrini* infected hamsters were housed and taken care of for 3 months. Animal experiments were approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 7/2558).

2.5. Experimental design for study of CpG ODN stimulation in hamsters

Hamsters were divided into five groups (5/group). The animals were injected either with PBS (Group 1), 100 μg CpG ODN 1826 (Group 2), 100 μg CSAg (Group 3), 100 μg non-CpG-ODN 2138 (Group 4) or 100 μg CpG ODN 1826 plus 100 μg CSAg (Group 5). Blood was collected via lateral saphenous vein from hamsters 2 days before and 2 days after CpG ODN injections. The injections were administrated via the intraperitoneal (i.p.) route. Each hamster's blood sample was prepared as serum for cytokine detection while whole blood was prepared for cell staining for investigation of T cell populations by flow cytometry.

2.6. CpG ODN stimulation

2.6.1. Cell proliferation in vitro

Normal hamster spleens collected from 4 to 6 week naïve hamsters were used for the *in vitro* study of CpG ODN stimulation. Mononuclear cells were prepared and isolated by ficoll-paque solution (GE Healthcare, Uppsala Sweden) then suspended in RPMI medium supplemented with 10% inactivated fetal bovine serum (FBS) and 100 U/100 μg/ml penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY). The 2×10^6 cells/ml in 96 well plates (Corning Costar, Corning, NY) were cultured either with CpG ODN 1826 at 10 μg/ml, 10 μg/ml non-CpG ODN 2138 or 10 μg/ml CSAg. The 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Enzo, Lausen Switzerland) with 1 μg/ml ionomycin (Enzo, Lausen Switzerland) was used as a positive control. The cell suspensions were incubated at 37 °C, 5% CO_2 for 72 h. The XTT (sodium 3′-[1-(phenyl-aminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (Sigma, MO), that is a tetrazolium salt reagent, was used to measure mitochondrial dehydrogenase activity of viable cells. The XTT solution was added to the final concentration of 20% for at least 4–6 h before harvesting. Finally, cell culture plates were gently shaken and measured using a spectrophotometer at 450 nm.

2.6.2. IFN-γ production assay

Mononuclear cells were prepared from naïve hamster spleens and adjusted to be 2×10^6 cells/ml. These cells were added into 24 well plates (Corning Costar, Corning, NY) containing 1 ml complete medium. Cell suspensions (2×10^6 cells/ml) were cultured either with medium alone, 10 μg/ml CpG ODN 1826, 10 μg/ml non-CpG ODN 2138, 10 μg/ml CSAg or 50 ng/ml PMA plus 1 μg/ml ionomycin, and incubated at 37 °C, 5%CO_2. After 72 h incubation, supernatants were harvested for detection of IFN-γ production levels. Culture supernatants or sera from hamsters were collected for determination of IFN-γ levels by Enzyme-Linked Immunosorbent Assay (ELISA) (BioLegend, CA). The protocols were performed according to the manufacturer's recommended protocols.