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Immune protection conferred by recombinant MRLC (myosin regulatory light chain) antigen in TiterMax Gold® adjuvant against experimental fasciolosis in rats

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ABSTRACT

Protection against experimental fasciolosis in rats immunized with recombinant myosin regulatory light chain (MRLC) in TiterMax Gold® adjuvant was assessed. The experimental trial consisted of four groups of 15 animals; group 1 was unimmunized and infected, group 2 was immunized with MRLC in adjuvant and infected, group 3 was infected and immunized with adjuvant only and group 4 was unimmunized and uninfected. Immunization with MRLC in TiterMax Gold® adjuvant (group 2) induced a reduction in fluke burdens of 51.0% ($p < 0.001$) when compared with the adjuvant control group, and 61.5% ($p < 0.001$) when compared with the unimmunized infected controls. There was a reduction in fecal egg output in group 2 of 44.8% and 37.3% compared with group 1 and group 3, respectively; although this difference was not statistically significant. Measurement of cytokine levels revealed higher levels of TNF-alpha and IL-2 as well as lower levels of IL-4 in group 2 during the chronic stage of infection ($p < 0.05$), along with higher levels of IFN-gamma during early stages of infection ($p < 0.05$). These results suggest a mixed Th1/Th2 phenotype immune response; however predominance of Th1 cytokines was observed. Levels of anti-MRLC serum IgG in group 2 were significantly higher than controls at the time of euthanasia ($p < 0.05$). This is the first report of immunization with recombinant MRLC in rats, demonstrating that this antigen significantly reduces fluke burdens, increases the Th1 immune response and encourages further studies to improve the vaccine's efficacy.

1. Introduction

The trematode *Fasciola hepatica* is the causative agent of fasciolosis, a parasitic disease responsible for significant economic losses in animal production worldwide [1,2]. *F. hepatica* affects mainly domestic and wild ruminants; however, it can cause disease in a wide range of species, including humans [3,4]. Subclinical infections in dairy cattle reduce milk production by 8%, and weight gain between 8% and 22% [5]; moreover, clinical infections frequently lead to mortality, mainly in young livestock and small ruminants. Estimated losses are US$ 3000 million annually [6].

F. hepatica infection is an important neglected foodborne trematodiasis in humans [7–9]. This parasite has been recognized by the World Health Organization as an emerging human pathogen [10], and the number of infected people worldwide is estimated to be up to 17 million [3].

The control of liver fluke is usually conducted through chemotherapy of parasitized animals with anthelmintic drugs [2,6]. However, most commercially available drugs are not effective against immature stages of *F. hepatica*, and multidrug resistance has been increasingly reported [11,12]. Furthermore, anthelmintic therapy does not represent an efficient method to

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control fasciolosis, since there is no significant reduction in the transmission rate in endemic areas due to continuous reinfection [11]. There is an increasing interest in the development of a vaccine against *F. hepatica* due to the elevated costs of anthelmintic treatments, along with increased parasite resistance to anthelmintic drugs, as well as concerns with chemical residues in food [2,6]. In the recent years, several molecules have been tested and demonstrated protective potential against this parasite experimentally; however, most immunization trials have shown variable results which makes the production of a commercially available vaccine currently unfeasible [2,13,14].

Myosin regulatory light chain (MRLC) is involved in smooth muscle contraction. Phosphorylation of MRLC in the presence of Ca2+ and calmodulin activates myosin, allowing its interaction with actin [15]. Previous studies with *Schistosoma mansoni* have indicated that praziquantel, one of the drugs commonly used to treat fasciolosis, acts by phosphorylating MRLC, which leads to calcium influx. This event allows the action of eosinophils; thus leading to the parasite's death [16]. Furthermore, another antigen from the same class (MLC) has been successfully used to immunize sheep against ticks [17].

To date, no immunization trial against fasciolosis with MRLC antigen has been reported in animals. Therefore, we believe that MRLC properties represent a great potential as an antigen against fasciolosis aiming to induce an efficient immune response in order to diminish parasitism as well as tissue damage, and consequently disease.

The aim of this study was to evaluate the protection conferred by the MRLC antigen in TiterMax Gold® adjuvant in rats experimentally infected by *F. hepatica*. The fecal egg count, fluke burdens and the immune response were determined as well as the hepatic damage by histopathology.

2. Methods

2.1. Antigen preparation

2.1.1. Cloning antigen

The gene encoding *Fasciola hepatica* myosin regulatory light chain (MRLC) was amplified from larval stages of *F. hepatica* cDNA using the primers MyosinF46 (5′ AAATCCGCACGCTAAACCAG) and MyosinR664 (5′CCTAAATATCTTGGTTGCCACGTTTG) using *Taq* polymerase, and ligated into the pGEM®-T Easy Vector (Promega) followed by cloning in *E. coli* XL-1 blue. The gene was sequenced and deposited in GenBank (Acession No. GU059940).

The primers MyosinFNcoI (5′ GCGCCATGGGTGAAGAAAAA-GAAAAG) and MyosinRXhoI (5′ GGCGCTCGAGAATATCTTGGTTGC CACGTTTG) were used to facilitate subcloning into the expression vector pET28α (Novagen) using the restriction enzymes *Nco*I and *Xho*I. This resulted in the plasmid encoding a recombinant protein with a C-terminal His-tag (6 histidines) allowing purification of recombinant MRLC using nickel resin.

2.1.2. Expression and purification

An overnight culture of *E. coli* BL21 containing the expression plasmid was used to inoculate LB medium containing kanamycin (50 μg/ml). When the culture reached OD600 = 0.6, expression was induced using IPTG (1 mM). After 4 h the bacteria were pelleted, resuspended in 10 mM Tris, 1 mg/ml lysozyme, pH 8, incubated on ice for 30 min, and sonicated. Cellular debris was removed by centrifugation and the sample was diluted in 5 volumes of wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, pH 7.8). Endotoxin removal by Triton X-114 phase separation was then undertaken as outlined by Liu et al. [18]. The recombinant MRLC was then batch purified by affinity chromatography using Protino Ni-NTA Agarose (Macherey-Nagel, Düren, Germany) under native conditions. Briefly, the sample was mixed with the resin overnight at 4 °C. The resin was then washed with 6 column volumes (CV) of wash buffer containing 20 mM imidazole, 50 CV of wash buffer containing 15 mM imidazole, 1% Triton X-114 for endotoxin removal, 5 CV of wash buffer containing 20 mM imidazole, 2 M NaCl, 60 CV of wash buffer containing 20 mM imidazole to remove residual Triton X-114, then eluted with 250 mM imidazole. Protein concentration and purity was determined by Bradford assay and SDS–PAGE, respectively. The protein was subsequently dialyzed against 10 mM Tris, pH 8, lyophilized and stored until used.

2.2. Experimental design

This study consisted of a 7 week period of immunization, followed by experimental infection and the euthanasia of animals (Table 1). Wistar male rats, 90 days-old and 420 g ± 90 were first acclimatized for 45 days to the experimental environment; the animals were kept in cages housed in a room with controlled temperature and humidity (25 °C; 70%). The diet was composed of commercial rations and the animals received water *ad libitum*. The rats were divided into four groups (G1, G2, G3 and G4); each one consisted of 15 animals free from specific pathogens. From each group, five animals were euthanized one week post infection (PI) and ten animals 15 weeks PI, which represented a total experimental period of 22 weeks. Groups 1, 2 and 3 received an inoculum (infective doses) composed of 20 viable metacercariae orally at week seven. Group 1 (G1) was represented by unimmunized infected animals. Group 2 (G2) consisted of infected animals immunized with MRLC antigen (100 μg) and 900 μL of water mixed with 1 mL of TiterMax Gold® adjuvant (Sigma-Aldrich, St. Louis, USA) on weeks 0 and 4, by subcutaneous injection in the shoulder using a 27 gauge (20 mm) hypodermic needle. Group 3 (G3) consisted of infected animals immunized with water (1 mL) mixed with 1 mL of TiterMax Gold® adjuvant on weeks 0 and 4, by subcutaneous injection in the shoulder using a 27 gauge (20 mm) hypodermic needle. Finally, group 4 (G4) was represented by animals uninfected and unimmunized. The Animal Welfare Committee of the *Instituto Federal Catarinense* (IFC) approved the procedure, under protocol number 001/2013.

2.3. Isolation of Fasciola hepatica and inoculum preparation

In this study the Weybridge strain (*F. hepatica*) was used. First, in order to obtain parasite eggs one sheep was infected, and feces were collected directly from the rectum of the animal once per

Table 1
Details of experimental design showing time of immunization, infection and euthanasia.

Group	Weeks 0	4	7	8	22
1			Infection	Euthanasia	Euthanasia
2	Immunization	Immunization	Infection	Euthanasia	Euthanasia
3	Immunization	Immunization	Infection	Euthanasia	Euthanasia
4				Euthanasia	Euthanasia

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