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Resistance against *Echinostoma caproni* (Trematoda) secondary infections in mice is not dependent on the ileal protein production

Alba Cortés [a,*], Javier Sotillo [a,b], Carla Muñoz-Antolí [a], Carla Martín-Grau [a], J. Guillermo Esteban [a], Rafael Toledo [a]

[a] Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain
[b] Centre for Biodiscovery and Molecular Development of Therapeutics, Australian Institute of Tropical Health and Medicine, James Cook University, Cairns, QLD, Australia

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ABSTRACT

Echinostoma caproni (Trematoda: Echinostomatidae) is an intestinal trematode, which has been widely employed to investigate the factors determining the rejection of intestinal helminths. Protein production patterns of intestinal epithelial cells are related to the infection-induced changes that determine the course of *E. caproni* infections. Herein, we compare the protein production profiles in the ileum of four experimental groups of mice: control; infected; dewormed and reinfected. Worm burdens were significantly lower in secondary infections, confirming the generation of partial resistance to homologous secondary infections in mice. However, quantitative comparison by 2D-DIGE showed that the protein production profile is similar in control and dewormed mice, and after primary and secondary *E. caproni* infections. These results showed that, unexpectedly, protein production changes in *E. caproni* infections are not responsible of resistance development. Fifty-one protein spots were differentially produced between control/treated and infected/reinfected mice and 37 of them were identified by mass spectrometry. The analysis of differentially abundant proteins indicate that cell metabolism and the regulation of proliferation and cell death are the most affected processes after primary and secondary *E. caproni* infections. These results provide new insights into the proteins involved in the regulation of tissue homeostasis after intestinal infection.

Significance: Intestinal helminthiases are highly prevalent parasitic infections with about 1 billion people infected worldwide. In this scenario, better understanding of host-parasite relationships is needed to elucidate the factors that determine intestinal helminth rejection. The intestinal trematode *Echinostoma caproni* has been broadly employed in this field, with resistance against secondary homologous infections reported in mice. In this paper, new insights are provided in the regulation of tissue homeostasis after intestinal infection. The unexpected lack of an altered pattern of ileal protein production associated to resistance development suggests that this resistance depends on rapid changes, affecting the early establishment of worms, rather than the activation of later effector mechanisms. These results may contribute to the development of new control tools for the management of these parasitic infections.

1. Introduction

Intestinal helminth infections are highly prevalent parasitic diseases affecting >1 billion people worldwide, especially in developing regions of Asia, Africa and Latin America [1]. These infections are causative of high morbidity, with most common symptoms related to effects on nutrition that may cause malabsorption syndrome, vitamin deficiencies, growth retardation or impaired cognitive function among other disorders [2,3]. Moreover, additional complications including intestinal obstruction, chronic dysentery, rectal prolapse, anemia or debilitating disease can appear [4,5]. Besides its interest in human health, helminth infections in livestock cause significant economic losses, directly by decreased productivity, or due to the indirect costs of antihelmintic treatment [1].

Echinostomatids are cosmopolitan intestinal trematodes that infect a wide variety of warm-blooded host species [6]. About 20 species belonging to nine genera of Echinostomatidae are known to cause human infections, thus constituting an important group of food-borne trematodes, mainly in Southeast Asia [7,8]. Additionally to their interest in public health, echinostomes, and particularly the species *Echinostoma caproni*, have been extensively used as experimental models to study the host-parasite relationships between food-borne trematodes and their vertebrate hosts [9]. Although *E. caproni* can infect a wide range of definitive hosts, host-parasite compatibility markedly differs between rodent species in terms of worm survival and development [10,11]. These features make the *E. caproni*-rodent systems highly suitable models to elucidate the host-dependent factors that determine the evolution of intestinal helminth infections [9].

* Corresponding author.
E-mail address: alba.cortes@uv.es (A. Cortés).

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Differences in host-parasite compatibility have been mainly attributed to the differential immune response generated by the host against the infection [12,13]. Moreover, differential proteomic analyses suggest that infection-induced intestinal alterations are associated with the intestinal changes that determine the course of the infection [14,15]. In rats, *E. caproni* infection induces the upregulation of proteins related with the cytoskeleton and the maintenance of the functional integrity of the epithelial barrier (e.g. actin, T-plastin, both 8 and 19 cytokeratins or annexin A4). Consequently, changes on the absorptive/secretory function of enterocytes and, especially, an increased regenerative capacity of the intestinal epithelium appear as effector mechanisms involved in the early rejection of worms. Over-production of the intestinal proliferation marker keratin19 (Krt19) and several chaperones, together with the downregulation of peroxiredoxin (Prdx) 3, prohibitin and 14-3-3 zeta isoform suggest that a strict control of proliferation and programmed cell death is essential to maintain the intestinal homeostasis, hence protecting the host against the injurious effects of the infection [14].

In mice, in contrast, *E. caproni* infection induces an early and intense remodeling in the protein production pattern of intestinal epithelial cells (IECs), primarily promoting the restoration of the epithelium and the control of homeostatic dysregulation. Upregulation of proteins like galectin 2 or Krt19, indicates that wound healing and crypt cell proliferation are constitutively active processes from the early stages of the infection. Concomitantly, mitochondrial dysfunction, cellular senescence and other pro-apoptotic events are induced. Augmented programmed cell death appears to be essential in this host to control the level of homeostatic dysregulation and eliminate potentially malignant damaged cells [15]. Proteomic data also indicates that cellular energy metabolism becomes differentially modified in the ileum of *E. caproni*-infected mice and rats. Whereas in mice the intestinal infection induces mitochondrial dysfunction and an increase in the anaerobic use of glucose to yield ATP, in rats the transition to a more aerobic and oxidative metabolism is suggested, leading to a reduced glycolytic flux and overall ATP production [14,15].

Recently, Muñoz-Antoli et al. [16] showed that *E. caproni* primary infections induce partial resistance against subsequent homologous infections in ICR mice, which evidences as a reduced infection rate, worm recovery and worm size. In the present paper, we compare the proteomes of IECs in mice, both before and after primary and secondary *E. caproni* infections, with the aim to elucidate the intestinal changes that may determine the establishment of secondary infections and the resistance observed.

2. Material and methods

2.1. Animals and infection procedures

The present study was performed using male ICR mice weighing 30–35 g. The strain of *E. caproni* and the infection procedures employed have been described previously [17]. Briefly, encysted metacercariae were removed from kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* snails and used for infection. A total of 48 mice were initially infected by gastric gavage with 50 metacercariae of *E. caproni* and randomly allocated in 3 groups (A to C) of 16 mice each. Additionally, 16 mice were left uninfected and used as controls (group D). Four weeks post primary infection (wppi) animals in group A, hereinafter infected, were necropsied to obtain tissue samples. At this time point (4 wppi), mice in groups B and C were all treated with a double dose of 100 mg/kg of praziquantel, orally administrated in alternate days. The elimination of the intestinal infection was confirmed by coprological examination as previously described [10].

Two weeks after antihelminthic treatment, animals in group B, hereinafter dewormed, were sacrificed and intestinal samples were recovered. The remaining 16 animals (group C) were secondarily infected with 50 metacercariae of *E. caproni* and maintained for 4 weeks post secondary infection (wpsi). Hereon, this group (C) will be referred as reinfected. Statistical comparison of the percentage of worms recovered from the intestine of infected and reinfected mice was performed by Student's *t*-test for independent samples.

Animals were maintained under conventional conditions with food and water ad libitum. This study has been approved by the Ethical Committee of Animal Welfare and Experimentation of the University of Valencia (Ref# A18348501775).

2.2. Cell collection and protein extraction

Ileal sections from mice in each group were removed at necropsy and IECs were isolated as described before [14]. In brief, the intestinal sections were opened longitudinally and rinsed by gentle shaking in washing buffer: ice-cold Hank's balance salt solution (HBSS) containing 2% of heat-inactivated fetal calf serum (FCS). Supernatant was then removed and fresh washing buffer was added to the ileal sections. This step was repeated at least 4 times, until the supernatant was clear. The tissue was then cut into small, 1 cm-long, segments and incubated for 20 min at 37 °C in HBSS containing 10% FCS, 1 nM EDTA, 1 mM DTT, 100 U/ml penicillin and 100 μg/ml streptomycin (dissociation buffer). The supernatant was collected and maintained on ice and the incubation was repeated a second time with fresh dissociation buffer. Supernatants were combined and filtered through a 100 nm cell strainer before IECs were pelleted out by centrifuging at 200*g* for 10 min at 4 °C and washed three times in PBS under the same centrifuge conditions to remove any residual medium.

Protein extraction was performed using M-PER Mammalian Protein Extraction Reagent containing Halt™ Protease Inhibitor Cocktail (both from Thermo Scientific) according to the manufacturer's instructions. Shortly, extraction reagent was added to the IECs pellet (20:1, v/v), mixed by vortex and incubated at room temperature (RT) for 20 min under continuous gentle agitation. The lysate was then clarified by centrifugation at 18,000*g* for 15 min at 4 °C, transferred into a new tube and stored at −80 °C until use.

2.3. Preparation of biological replicates and protein labeling

In order to increase the biological significance and avoid erroneous conclusions due to individual variations, four biological replicates were prepared for each experimental group (control, infected, dewormed and reinfected). Each biological replicate was obtained by pooling the same amount (20 μg) of protein extracted from the IECs isolated from four different mice. Then, 50 μg of protein from each biological replicate was cleaned and precipitated with 2D Clean-up Kit (GE Healthcare), pellets were resuspended in 18 μl of a proper buffer (25 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8,5) and proteins were fluorescently tagged with CyDye DIGE Fluor minimal dyes (GE Healthcare), following manufacturer's instructions. One microliter of dye (400 pmol) was added to each sample and maintained on ice for 30 min in the dark. The reaction was stopped by adding 1 μl of 10 mM lysine. To minimize any dye-specific labeling artefacts, two biological replicates of each experimental group were labeled with Cy3 and the other two were labeled with Cy5. The internal standard, prepared by mixing the same amount of protein of each sample included in the experiment, was always labeled with Cy2 (Supplementary Table S1).

2.4. Two-dimensional differential in gel electrophoresis (2D-DIGE)

Ileal protein extracts from control, infected, dewormed and reinfected mice were compared across 8 2D-DIGE gels to investigate changes in the intestinal production of proteins during the establishment of primary and secondary infections, as well as the restoration of intestinal tissue once the infection has been eliminated. The 8 pairs of Cy3- and Cy5-labeled biological replicates (50 μg of protein each) were combined with a 50 μg aliquot of the Cy2-labeled internal