Biomedicine & Pharmacotherapy 101 (2018) 563–571

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

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

Comparative study of effects of assemblages AII and BIV of *Giardia duodenalis* on mucosa and microbiota of the small intestine in mice

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ARTICLE INFO

Keywords:
Genotype
Duodenum
Giardiasis

ABSTRACT

Aims: Giardiasis is one of the major causes of diarrhea worldwide and its symptoms vary in intensity, which can be attributed to different parasite assemblages. The goal of the present study was to compare the effects of infection caused by assemblages AII and BIV of*Giardia duodenalis* on the response of the small intestine, microbiota, and behavioral parameters in mice.

Main methods: Swiss mice were infected with assemblages AII and BIV of *G. duodenalis* for 15 days. Leucometry, pain, intestinal microbiota and histological parameters of the duodenum and jejunum were evaluated in the experimental groups.

Key findings: Both assemblages modified the composition of the intestinal microbiota. Infection with assemblage AII promoted leukocytosis, reflected by increasing number of polymorphonuclear cells, intraepithelial lymphocytes and pain-related behavior, indicating that this was the more aggressive assemblage with regard to its effects on the intestinal mucosa and duodenum.

Significance: The specific assemblage of the parasite is an important parameter that affects symptomatology in the host.

1. Introduction

Giardiasis is caused by *Giardia* spp infection and is one of the major causes of diarrhea [1] in both developed and developing countries [2,3]. The pathogenesis of giardiasis involves the adherence of *Giardia duodenalis* trophozoites to the mucosa of the small intestine, which promotes villous atrophy and brush border shortening [4]. This parasite can cause epithelial barrier dysfunction, with consequent malabsorption [5]. Among the signs and symptoms of giardiasis are abdominal pain, nausea, vomiting, flatulence, and aqueous diarrhea [5,6]. This infection was recently associated with chronic manifestations, such as irritable bowel syndrome, which may arise even after the eradication of infection [6].

A balanced microbiota is capable of inhibiting the development of pathogenic microorganisms [7], but the presence of *G. duodenalis* can lead to dysbiosis with consequent intestinal disorders, referred to as "post-giardiasis" alterations [8,9]. Dysbiosis and other changes in the intestinal mucosa are responsible for dysfunction of the epithelial barrier and may contribute to microbial transposition and a local inflammatory response [8].

The symptoms of giardiasis present variable intensities that are attributable to both host and parasite factors. *G. duodenalis* can manifest as eight genetic assemblages (A–H). Assemblages A and B are the most prevalent in humans, and sub-assemblages AII and BIV are the most prevalent in southern Brazil [10–12]. Studies that have attempted to relate the symptomatology of giardiasis to specific parasite assemblages are scarce, and the results are controversial. The goal of the present study was to compare the effects of infection that was caused by assemblages AII and BIV of *Giardia duodenalis* on the response of the small intestine, microbiota, and behavioral parameters in mice.

2. Materials and methods

The study adhered to the guidelines of the Sociedade Brasileira de

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https://doi.org/10.1016/j.biopha.2018.02.141
Received 2 December 2017; Received in revised form 22 February 2018; Accepted 26 February 2018

Ciência em Animais de Laboratório and was approved by the Ethics Committee on Animal Experimentation of the Centro Universitário Integrado, Brazil (statement no. 1070).

2.1. Inoculum and experimental groups

Cysts of *G. duodenalis* were obtained from the genotyped sample database of the Laboratory of Environmental Parasitology and Food, Universidade Estadual de Maringá, Brazil (GenBank accession no. KJ741310 and KJ741313). The cysts were concentrated from freshly eliminated human feces that were positive for *G. duodenalis* and previously genotyped by sequencing the *glutamate dehydrogenase* (*gdh*) gene [11]. The cysts that were used to infect the animals were purified using the sucrose gradient technique [13–15]. The purified cysts were observed by optical microscopy to ensure material purity and determine inoculum counts.

Forty-two male Swiss specific-pathogen-free mice, 21 days old, were obtained from the animal facility of the Universidade Estadual de Maringá. The animals were randomly assigned to three groups: uninfected control group (CG) and animals infected with 1000 cysts of *G. duodenalis* assemblages AII and BIV by gavage (GIA and GIB groups, respectively). The infectious dose of 1000 cysts was chosen based on a previous study [16].

Three days postinfection (dpi) and on the day of euthanasia (15 dpi), parasitological examination was performed according to the method of Faust et al. [17]. *Giardia* genotyping was used to confirm infection by assemblages AII and BIV of *G. duodenalis*. Genotyping was performed using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) for the *gdh* gene [11]. For the PCR-RFLP assays, amplified products of the *gdh* gene were digested with two units of *Nla* IV endonuclease (New England Biolabs, Ipswich, MA, USA) for 3 h at 37 °C according to Colli et al. [11]. All of the analyses were performed in a blinded fashion.

All of the animals were given autoclaved commercial rodent chow and mineral water *ad libitum* to ensure that no other source of infection affected these animals over the experimental period. The groups of mice were housed in polypropylene cages with a wire grid floor to prevent contact with excreta. The animals were maintained on a 12 h/12 h light/dark cycle with controlled temperature and humidity.

2.2. Course of experimental infection

The experiment lasted 15 days. During this time period, the animals were observed daily for the presence or absence of diarrhea. At 7 and 14 dpi, tests of exploratory behavior were performed, and blood was collected to determine total and differential leukocyte counts. Intestinal mucus and feces were collected at the end of the experiment (15 dpi) to determine total leukocyte count. After removing the intestine, the duodenum mucus was gently scraped with a sterile spatula, and a pellet of freshly discarded feces was collected.

2.3. Leukocyte count

Blood was collected from the caudal vein and placed in tubes contained ethylenediaminetetraacetic acid. For total leukocyte count, the blood sample was diluted in hemolyzing solution in a 1:20 ratio, and leukocytes were quantified in a Neubauer chamber using four lateral quadrants. Differential leukocyte counts in blood and leukocyte counts in feces and mucus was made in smears with May-Grünwald-Giemsa staining [18].

2.4. Subjective pain assessment

The subjective pain assessment was performed using tests of exploratory behavior in the elevated plus maze (EPM) and open field test (OFT), previously validated by Pellow et al. [19] and Archer [20], respectively. These tests were used because pain can be defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (International Association for the Study of Pain) [21]. Additionally, animals with pain exhibit an increase in exploratory behavior [22]. The subjective pain assessments were always performed at the same time and by the same researcher.

2.4.1. Elevated plus maze

Each animal underwent a single 5-min session on each day of evaluation (0, 7, and 14 dpi). The session began when the researcher placed the animal in the center of the maze, facing one of the closed arms [22]. The time spent on the open and closed arms of the maze was recorded using X-Plo-Rat 3.3 software [23]. After each animal was tested, the apparatus was cleaned with 70% ethanol.

2.4.2. Open field test

The animals were placed in the center of the arena [24]. The sessions lasted 5-min each. After each session (0, 7, and 14 dpi), the apparatus was cleaned with 70% ethanol. The following parameters were analyzed: number of squares crossed (with all four paws), number of rears, time spent grooming (in seconds) and number of evacuations.

2.5. Euthanasia and material collection

The animals were intraperitoneally anesthetized with xylazine (10 mg/kg) and ketamine (110 mg/kg) and then euthanized by intracardial administration of KCl (2 mL) at 36 days of age (15 dpi). Necropsy was performed aseptically along the medial line to obtain the duodenum and proximal jejunum. A 1-cm ring was collected from each segment for histological processing. Jejunum was used for microbiological analysis, determination of myeloperoxidase (MPO) activity and determination of nitrite concentration. Duodenum fragment was collected for counting of leucocytes number in intestinal mucus.

2.6. Determination of myeloperoxidase activity

Myeloperoxidase activity was assayed using homogenate supernatants of the jejunum according to Bradley & Priebat [25]. The segment was weighed, and a 20× volume of potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (1 mL/50 mg of tissue) was added in a Potter homogenizer. The homogenate was shaken in a vortex mixer and centrifuged for 15 min at 5000 rotations per minute (rpm) at 25 °C. Aliquots (10 µL) of the supernatant were added to each well of a 96-well microplate with 200 µl of the buffer solution [*O*-dianisidine dihydrochloride (16.7 mg), double-distilled water (90 ml), potassium phosphate buffer (10 mL) and 1% H_2O_2 (50 µL)]. After 5 min, the reaction was stopped by the addition of sodium acetate. Myeloperoxidase activity was determined by reading absorbance at 450 nm using a microplate spectrophotometer (Spectra Max Plus), recorded at 15-s intervals for 2 min. All tests were performed in duplicate.

2.7. Determination of nitrite concentration

Nitrite concentration was determined in mucus and tissue fragments of the jejunum. Mucus samples that adhered to the mucosa of the jejunum were removed with a sterile spatula, and jejunum samples were macerated in sterile phosphate-buffered saline (PBS), both maintained at −20 °C until analysis.

The samples were centrifuged at 1500 rpm for 10 min, and the supernatant was used to determine the nitrite concentration using the Griess reaction [26], in which 50 µL of the supernatant was incubated with the same amount of Griess solution (phosphoric acid, sulfanilamide, and N-1-naphthalylethylenediamide). A 96-well microplate and enzyme-linked immunosorbent assay reader (570 nm absorbance) were