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Apoptosis in the intestines of mice infected with *Acanthamoeba* sp.

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ABSTRACT

Introduction: *Acanthamoeba* spp. are parasites that typically colonize the brain, cornea, and lungs. However, some studies suggest that amoebas may also be present in the gut microbiome, as they have been found in the stool of healthy humans and animals. Given research indicating that parasites in the small and/or large intestine may modulate apoptosis, this study aimed to assess apoptosis in the small intestines of mice infected with *Acanthamoeba* sp.

Materials and methods: The small intestines used in this study were collected from immunocompetent and immunosuppressed mice experimentally infected with *Acanthamoeba* sp. (AM22 strain, GenBank reference number: GQ342607). The intestines were homogenized, and the expression of pro-apoptotic Bax, anti-apoptotic Bcl-2, caspase 9 (Cas9) and caspase 3 (Cas3) proteins was determined using the Western blot method. The results were statistically analyzed.

Results: In immunocompetent mice infected with *Acanthamoeba* sp., there was an increase in the protein expression of the pro-apoptotic Bax. The level of the anti-apoptotic protein Bcl-2 was higher at each time point compared to the group of uninfected animals. The Bax/Bcl-2 ratio was similar in immunocompetent infected and uninfected mice, with no statistically significant difference. The levels of Cas9 and Cas3 in immunocompetent infected and uninfected mice were also similar. In immunosuppressed mice, increased Bax expression was found on days 16 and 24 post *Acanthamoeba* sp. infection. On the same days, a reduced level of Bcl-2 and statistically significant differences in the Bax/Bcl-2 ratio were observed compared to mice in the control group. Increased expressions of Cas9 and Cas3 were also observed at 16 and 24 days post-inoculation (dpi).

Conclusions: In immunocompetent hosts, systemic acanthamoebiasis does not affect every organ. However, infection with *Acanthamoeba* spp. in immunosuppressed hosts induces the apoptosis pathway in intestinal epithelial cells.

Keywords: *Acanthamoeba* spp.; immunological status; small intestine; systemic acanthamoebiasis.

INTRODUCTION

Acanthamoeba spp. are single-celled, opportunistic, free-living amoebas. They are found worldwide in natural habitats such as soil, freshwater, and seawater, as well as in artificial habitats like swimming pools and air conditioning systems. Thanks to their low nutritional requirements and highly resistant cysts, these amoebas can exist almost anywhere, making them one of the most frequently isolated species of amoebas globally [1]. They typically occur in 2 developmental forms – the vegetative form (trophozoite) and the resistant form (cyst). Both forms are invasive to humans [2, 3]. However, some authors suggest that only the trophozoite is the invasive form of *Acanthamoeba* spp. The cysts spread easily through the air and play a crucial role in disease progression because they can resist treatment in tissue and lead to reinvasion [4].

Acanthamoeba spp. primarily invade the corneal epithelium, destroying the corneal layers and leading to severe amoebic keratitis in immunocompetent individuals [5]. They can also enter the host's body through the upper respiratory tract or skin lesions, leading to systemic acanthamoebiasis. In immunosuppressed patients, amoebas can invade the central nervous system through the bloodstream, resulting in granulomatous amebic encephalitis (GAE). The exact route of *Acanthamoeba* spp. migration to the brain is not well understood, but the lungs and skin are believed to be the most critical portals of infection [3, 6]. Additionally, the literature indicates that *Acanthamoeba* spp. can access their host through the intestinal mucosa [7, 8]. Sadaka and Emam observed that mice with altered gastrointestinal microbiota due to receiving cimetidine or tetracycline and orally infected with 200–400 amoebae of the genus *Acanthamoeba* suffered from diarrhea. Histopathological examinations of the intestines revealed erosion of the intestinal mucosa along with the developmental forms of the parasites. Furthermore, 20% of infected normoacidic mice and all animals that received cimetidine or tetracycline before infection passed the parasite in their stools [7]. *Acanthamoeba* spp. have also been reisolated from the gastrointestinal tract of mammals and other animals [9, 10, 11, 12, 13, 14, 15].

It has been shown that protozoans in the host's gut can influence digestive processes in the host's intestines. Moreover, these parasites may impact the modulation of apoptosis pathways [16].

Apoptosis plays a crucial role in maintaining balance in the intestinal epithelial cells. The mammalian intestinal mucosa undergoes regular exfoliation, with epithelial cells being replaced by cells from deeper layers, which are pushed toward the surface. Cell proliferation takes place in the crypts of the intestinal villi, and exfoliation occurs in the intestinal lumen. This process takes 3–5 days [17]. Alterations in apoptosis, whether increased or decreased, can contribute to the development of pathological conditions in the gastrointestinal tract. Excessive apoptosis of enterocytes occurs in patients with celiac disease and may be responsible for the atrophy of intestinal villi [18]. Increased apoptosis is also observed in patients with ulcerative colitis [19]. Research indicates that apoptosis is a pathomechanism in Crohn's disease, where a high ratio of anti-apoptotic protein to pro-apoptotic protein (Bcl-2/Bax) causes T cell accumulation and chronic inflammation [20]. Increased self-destruction of intestinal epithelial cells leads to an accelerated rate of cell division, promoting mutations and cancer development. Consequently, patients with Crohn's disease or ulcerative colitis are at higher risk of developing intestinal cancer [21].

Given the presence of amoebae in the gut microbiome and the influence of intestinal protozoans on the apoptosis process, the aim of this study was to assess the protein expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) factors, as well as caspase 9 (Cas9) and caspase 3 (Cas3), in the intestines of mice infected with *Acanthamoeba* sp.

MATERIALS AND METHODS

Animal model

The study utilized the small intestines of mice infected with *Acanthamoeba* sp. (AM22 strain, GenBank reference number: GQ342607). Mice were divided into 4 groups based on immune status and *Acanthamoeba* sp. infection: hosts with normal levels of immune response infected with amoebas (group A), uninfected hosts with normal levels of immune response (group C), hosts with reduced levels of immune response infected with amoebas (group AS), uninfected hosts with reduced levels of immune response (group CS). The animals in groups AS and CS were administered intraperitoneal injections of methylprednisolone (MPS, Solu-Medrol; 10 mg/kg body weight) for 4 days to reduce their immune response. On the fifth day, animals in groups A and AS were intranasally inoculated with 10,000–20,000 trophozoites of *Acanthamoeba* sp. Animals in groups C and CS received 3 µL of physiological saline.

After 8, 16, and 24 days post-inoculation (dpi), mice were dissected following an intraperitoneal injection of an increased dose of pentobarbital sodium. The small intestines were collected from the mice and then stored at –80°C. Detailed experimental procedures are presented in the paper by Łanocha-Arendarczyk et al. [22]. All animal procedures were carried out in 2016 after obtaining consent from the Local Ethical Committee in Szczecin (Resolution No. 29/2015) and in Poznań (Resolution No. 64/2016).

Sample homogenization and protein concentration analysis

The collected tissues were stored in a refrigerator at –80°C until homogenization. The intestinal samples were homogenized using the hammer method. All equipment that had direct contact with the tissue, including the stand, head, tongs, paddles for transferring powdered tissues, and Eppendorf tubes, was cooled in liquid nitrogen beforehand. Each sample was placed separately into a cylindrical base with a hole for the tissue. Then, a head was inserted into the hole and struck with a hammer until the tissue was crushed into fine dust. The crushed tissue was transferred to 2 cooled Eppendorf tubes, and the equipment was wiped clean after each tissue to prevent contamination. The obtained homogenate was stored at –80°C until further analysis.

Lysis buffer with pH = 8.5 (50 mM Tris-HCl, 0.5% Triton X-100, and 150 mM NaCl) was added to the tissue homogenates, then mixed and incubated on ice. After 15 min, the samples were centrifuged for 20 min at 4°C (14,000 rpm). Total protein concentrations were determined in the resulting supernatant using the MicroBCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's methodology. Absorbance was read using an EZ Read 2000 spectrophotometer from Biochrom at a wavelength of 562 nm. The protein concentration was determined from a standard curve prepared from a set of known albumin concentrations.

The analysis of the expression of Bax, Bcl-2, Cas9, and Cas3 proteins by Western blot

Protein electrophoresis was performed in a polyacrylamide gel, which consisted of a separation layer (11%) and a stacking layer (3%) – Table 1.

TABLE 1. Composition of separating gel (11%) and stacking layers (3%)

Variables	Separating layer (11%)	Stacking layer (3%)
Double distilled water – dd$H_2$0	2.8 mL	3.58 mL
Acrylamide mix 30%	4 mL	670 µL
1.5 M Tris-HCl buffer	3 mL	625 µL
10% SDS	100 µL	50 µL
10% APS	100 µL	50 µL
TEMED	10 µL	5 µL

SDS – sodium dodecyl sulfate; APS –ammonium persulphate; TEMED – Thermo Scientific Pierce Tetramethylethylenediamine

Samples containing 30 µg of protein were prepared for separation. Samples were diluted with RIPA buffer and Laemmli buffer (Laemmli Sample Buffer, Bio-Rad) with 2-mercaptoethanol (Sigma-Aldrich). Each sample (15 µL) was incubated at 95°C for 5 min to remove sulfide bridges and denature proteins. Then, samples and a standard marker (a mixture of proteins ranging 10–250 kDa; Precision Plus Protein™ All Blue Prestained Protein Standards, Bio-Rad) were applied to the gel, which was placed

in an electrophoretic chamber filled with an electrode buffer consisting of distilled water, glycine, sodium dodecyl sulfate (SDS), and Trizma base. Electrophoresis was performed at 100 V for 15 min and then at 130 V for 60 min. Upon completion of electrophoresis, the gel and polyvinylidene fluoride (PVDF) membrane (ThermoFisher Scientific™) were placed between layers of tissue paper and sponges, forming a "sandwich" that was closed in a transfer cassette. The transfer was performed using a wet transfer method at 75 V for 60 min. The membrane was then blocked with 3% bovine serum albumin in blocking buffer for 60 min on a rocking platform at room temperature.

After blocking, the membrane was incubated with primary antibodies against Bax (cat. no. ab196494, Abcam), Bcl-2 (cat. no. ab196495, Abcam), Cas9 (cat. no. ab184786, Abcam), and Cas3 (cat. no. ab13585, Abcam) at a dilution of 1 : 400 at 2–8°C overnight. Following this, the membrane was incubated with secondary anti-mouse antibodies (cat. no. ab6789-1, Abcam) at a dilution of 1:4000 at room temperature for 60 min. Beta actin (cat. no. sc47778, Santa Cruz Biotechnology) was used as a reference protein.

The ECL Advance Western Blotting Detection Kit (GE Healthcare) was used to visualize the proteins, and images of the membrane were captured using a transilluminator (Molecular Imager ChemiDoc XRS+, Bio-Rad, USA). Densitometric analysis was performed using Image Lab Software 6.1.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis

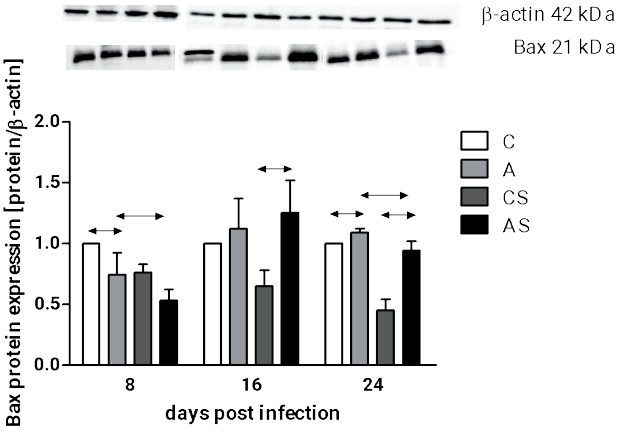
Statistical analysis of the results was performed using StatSoft Statistica 8.0. The Shapiro–Wilk test was employed to assess the normality of the data distribution. Since the data did not follow a normal distribution, non-parametric tests were used for further analysis. The Mann–Whitney U-test (U) was applied to compare 2 groups, while the Kruskal–Wallis test (H) was used for comparisons among 3 groups. Statistically significant differences were considered when the p-value was ≤0.05. Graphs were created using GraphPad Prism 4.0.

RESULTS

In group A the expression of the pro-apoptotic protein Bax was significantly different at 8, 16, and 24 dpi ($H = 11.90$; $p = 0.003$). A 26% reduction in Bax expression was observed in the group A at 8 dpi compared to the control group ($U = 0.00$; $p < 0.001$). However, on the last day of the experiment (24 dpi), immunocompetent mice infected with *Acanthamoeba* sp. showed an increase in Bax protein expression in the intestines by approx. 10% compared to mice from the control group ($U = 0.00$; $p < 0.001$) – Figure 1.

In group AS a statistically significant increase in Bax protein expression was found at 16 dpi, followed by a decrease at 24 dpi ($H = 19.38$; $p < 0.001$). The expression of Bax protein was almost 2 times higher in the group AS at 16 dpi compared to control mice and 2.1 times higher in the group AS at 24 dpi compared to control mice ($U = 0.00$; $p < 0.001$) – Figure 1.

The analysis also compared Bax protein expression between the groups A and AS, considering the immune status of the hosts. A statistically significant difference was found at 8 dpi ($U = 8.00$; $p = 0.01$) and 24 dpi ($U = 0.00$; $p < 0.001$), with higher expression in the group A of mice.



Arrows indicate statistically significant differences at $p < 0.05$. Analyses were performed on 6 mice per group. The figure shows a representative Western blot. A – mice with a normal immune response infected with amoebas; AS – mice with a reduced immune response infected with amoebas; C – control mice with a normal immune response; CS – control mice with a reduced immune response

FIGURE 1. The expression of the pro-apoptotic protein Bax in the small intestines of mice infected with *Acanthamoeba* sp.

In mice with a normal immune response infected with *Acanthamoeba* sp. (group A), the expression of the anti-apoptotic protein Bcl-2 showed statistically significant differences across different days of infection ($H = 16.16$; $p < 0.001$). Increased expression of Bcl-2 protein was observed in the group A at 8, 16, and 24 dpi by approx. 10%, 29%, and 8%, respectively, compared to the group C ($U = 0.00$; $p < 0.001$). The data are presented in Figure 2.

In the group AS (mice with a reduced immune response infected with *Acanthamoeba* sp.), Bcl-2 expression decreased at 16 dpi and further decreased at 24 dpi ($H = 20.58$; $p < 0.001$). Reduced Bcl-2 protein expression was observed at all time points in the group AS compared to the group CS, with statistically significant differences at 16 dpi (1.7-fold decrease vs. control mice) and 24 dpi (2.7-fold decrease vs. control mice; $U = 0.00$; $p < 0.001$) – Figure 2.

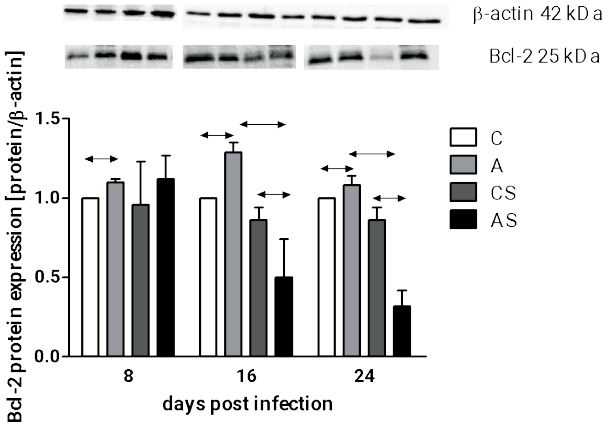
Comparing immunocompetent and immunosuppressed infected animals, a statistically higher Bcl-2 expression was found in the group A at 16 dpi ($U = 0.00$; $p < 0.001$) and 24 dpi ($U = 0.00$; $p < 0.001$).

The expression of Cas9 in the group A on different days of infection remained at a similar level. Analyzing the expression of Cas9 in the intestines of mice between the group A and the group C, a statistical difference was found only at 24 dpi, with 20% higher expression found in animals from the group C ($U = 0.00$; $p < 0.001$) – Figure 3.

In the group AS of mice, there was no statistically significant difference in Cas9 expression on different days of infection. Comparing the group AS to the group CS, increased levels

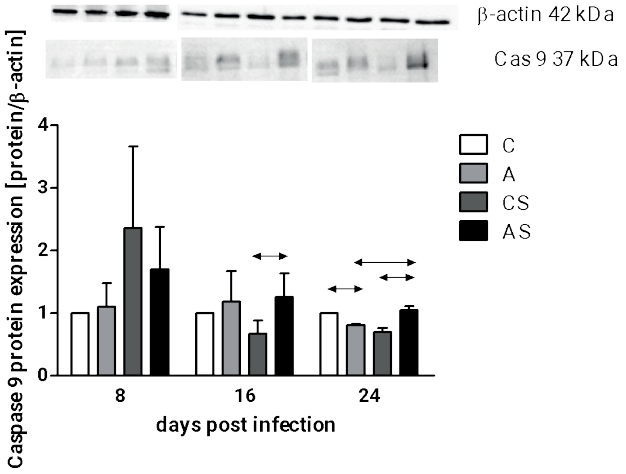
of Cas9 protein expression were noted at 16 dpi (U = 12.00; p = 0.04) and 24 dpi (U = 0.00; p < 0.001) in the group AS (Fig. 3). We observed 1.5-fold higher expression in the group AS at 16 dpi and 1.88-fold higher expression in the group AS at 24 dpi compared to control mice.

The statistical analysis also included the immune status of the hosts. A statistically significant difference was found only at 24 dpi (U = 36.00; p < 0.001), with higher expression observed in the group AS of mice (Fig. 3).



Arrows indicate statistically significant differences at p < 0.05. Analyses were performed on 6 mice per group. The figure shows a representative Western blot. A – mice with a normal immune response infected with amoebas; AS – mice with a reduced immune response infected with amoebas; C – control mice with a normal immune response; CS – control mice with a reduced immune response

FIGURE 2. The expression of the anti-apoptotic protein Bcl-2 in the small intestines of mice infected with *Acanthamoeba* sp.



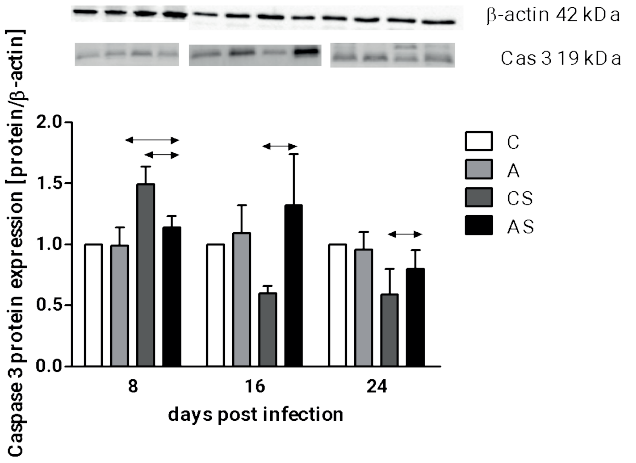
Arrows indicate statistically significant differences at p < 0.05. Analyses were performed on 6 mice per group. The figure shows a representative Western blot. A – mice with a normal immune response infected with amoebas; AS – mice with a reduced immune response infected with amoebas; C – control mice with a normal immune response; CS – control mice with a reduced immune response

FIGURE 3. The expression of the initiator caspase, Cas9, in the small intestines of mice infected with *Acanthamoeba* sp.

In mice with a normal level of immune response infected with *Acanthamoeba* sp., there was no statistically significant difference in Cas3 protein expression on different days of infection. Additionally, there were no differences in the expression of this protein between the groups A and C (Fig. 4).

In the group AS of mice, there was an increase in Cas3 expression at 16 dpi, followed by a decrease at 24 dpi (H = 11.82; p < 0.01). A 23.4% lower level of Cas3 expression was observed at 8 dpi compared to the group CS (U = 0.00; p < 0.001). At 16 dpi (U = 0.00; p < 0.001) and 24 dpi (U = 12.00; p = 0.04), higher levels of Cas3 expression were noted in the group AS than in the group CS. We observed 2 times higher expression in the group AS at 16 dpi and 1.35 times higher expression in the group AS at 24 dpi compared to the control group. The data are presented in Figure 4.

The statistical analysis also included the differences in Cas3 protein expression between the groups A and AS. A statistically significant difference was noted only on the eighth day after *Acanthamoeba* sp. infection (U = 8.00; p = 0.01).



Arrows indicate statistically significant differences at p < 0.05. Analyses were performed on 6 mice per group. The figure shows a representative Western blot. A – mice with a normal immune response infected with amoebas; AS – mice with a reduced immune response infected with amoebas; C – control mice with a normal immune response; CS – control mice with a reduced immune response

FIGURE 4. The expression of the effector caspase, Cas3, in the small intestines of mice infected with *Acanthamoeba* sp.

DISCUSSION

Scientific literature has shown that parasites colonizing the small and/or large intestine can affect the digestive processes in the host's digestive system, thereby limiting parasite elimination and the host's immune response. It has also been shown that parasitic protozoa modulate the process of apoptosis in host cells, thus facilitating their penetration into the intestinal epithelium. Depending on the species, the parasite can induce apoptosis through various pathways [16].

Bax and Bcl-2 are key regulators of the apoptosis process. They control the flow of ions and reactive oxygen species in the cell, the release of apoptogenic factors from mitochondria,

including cytochrome C and apoptosis-inducing factor (AIF), and the activation of caspases [23]. High Bax and/or low Bcl-2 levels, as well as high Bax/Bcl-2 ratios, have been found to promote apoptosis [24]. Increased Bax levels and/or calcium ions lead to the release of Cas9 from the mitochondria [25]. Caspase 9 activates effector Cas3, which is necessary for the effective execution of the apoptosis process [26].

Our study indicates an increase in the expression of the pro-apoptotic protein Bax in mice with a normal level of immune response infected with *Acanthamoeba* sp. The level of the anti-apoptotic protein Bcl-2 was also higher at each time point compared to the group of uninfected animals. The Bax/Bcl-2 ratio was at a similar level in the groups A and C. The levels of Cas9 and Cas3 in the groups A and C were also similar. To sum up, in the group of immunocompetent mice, there was no dysregulation of Bax/Bcl-2 and therefore no modulation of the apoptosis process was noted.

In immunosuppressed mice, increased Bax expression was found on days 16 and 24 post-*Acanthamoeba* sp. infection. On the same days, a reduced level of Bcl-2 and a statistically significant difference in the Bax/Bcl-2 ratio were observed compared to the control group. Increased expressions of Cas9 and Cas3 were also observed at 16 and 24 dpi. During long-term infection, changes in the levels of all studied proteins were observed in the group of immunosuppressed mice, which clearly suggest increased apoptosis of enterocytes. Due to the fact that the expression of Cas9 and Cas3 was increased, the extracellular parasite *Acanthamoeba* sp. can induce apoptosis in cells independently of membrane death receptors. It can activate the programmed cell death pathway through direct contact with the intestinal epithelium.

Similar results to those found in the immunosuppressed host were obtained by studying the apoptosis process in intestines infected with typically intestinal parasites. *Giardia intestinalis* activates the apoptosis pathway in intestinal epithelial cells [27]. The protozoan stimulates apoptosis by activating Cas3, increasing the expression of the pro-apoptotic protein Bax, and decreasing the expression of the anti-apoptotic protein Bcl-2 [28, 29, 30]. However, *G. intestinalis* has little effect on the apoptosis process in patients infected with this parasite; the cell self-destruction process was increased by only 0.2% compared to the control group [28]. Similar results apply to infection with opportunistic *Cryptosporidium* spp. [16, 31, 32]. Buret et al. used confocal microscopy to demonstrate signs of chromatin fragmentation and condensation typical of apoptosis in small intestinal epithelial cells [31]. Additionally, Chen et al. observed that cryptosporidiosis may induce apoptosis in human biliary epithelial cells [33]. Apoptosis has been suggested as an important defense mechanism against *Cryptosporidium parvum* infection because it helps stop the spread of infection by impeding the replication of the intracellular parasite [32, 34].

Parasites of the large intestine, including *Entamoeba histolytica* and *Blastocystis* sp., can also modulate apoptosis in intestinal cells. Studies on the relationship between *E. histolytica* infection and apoptosis have shown a strong induction of host cell death as a result of the presence of this parasite. In a mouse model, it was noticed that colon cells infected with this parasite showed rapid activation of Cas3, independent of caspase 8 and 9 [35]. Moreover, it has been shown that the activation of colon cell apoptosis in the presence of *E. histolytica* trophozoites is independent of the overexpression of the apoptotic mechanism Bcl-2 [36]. In the case of *Blastocystis* sp., it has recently been proven that this protozoan can induce the caspase-dependent apoptosis process without causing changes in the intestinal epithelium of rats, while maintaining proper functioning [37]. Puthia et al. showed that *Blastocystis ratti* induces apoptosis in cells of the rat intestinal epithelial cell line by significantly increasing the activity of Cas3 [38].

The mechanisms occurring in the intestines during acanthamoebiasis remain unknown. The present study shows that host immunological status plays a key role in the pathophysiology of acanthamoebiasis. In immunocompetent hosts, modulation of apoptosis in the intestine of mice infected with *Acanthamoeba* sp. did not occur. In the intestine of immunosuppressed mice, *Acanthamoeba* sp. induced increased apoptosis. Further analyses should include histopathological examination of the intestines and the examination of inflammatory markers. It is very possible that in immunocompetent hosts, systemic acanthamoebiasis does not affect every organ. While in immunosuppressed hosts, the induction of the apoptosis pathway in intestinal epithelial cells may be related to the host's protective mechanism of faster removal of damaged cells. So far, neither clinical cases nor mouse models have demonstrated a clearly negative effect of *Acanthamoeba* spp. on intestinal cells. Therefore, further research is necessary.

CONCLUSIONS

Infection with *Acanthamoeba* sp. in the immunosuppressed host: (i) activates the intrinsic apoptotic pathway in the host's small intestine, (ii) triggers apoptosis via the activation of a caspase cascade, including Cas3 and Cas9, (iii) enhances apoptosis by increasing the expression of the pro-apoptotic protein Bax and decreasing the expression of the anti-apoptotic protein Bcl-2. Infection with *Acanthamoeba* sp. in the immunocompetent host does not modulate apoptosis in the host's small intestine.

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