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The X-chromosome-linked immunodeficiency determines an improved course of murine lagochilascariosis

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Summary

Lagochilascaris minor is the causative agent of lagochilascariosis, a disease that affects the neck region causing exudative abscesses with eggs, adult parasites and L3/L4 larvae within purulent exudates. Nowadays, mice are considered intermediate hosts for the parasite. To determine the pattern of infection in B1 cell-defective mice, experimental lagochilascariosis was studied in BALB/c and X-chromosome-linked immunodeficient (Xid) mice. BALB.xid infected mice showed higher survival ratios and less intense lung lesions than BALB/c mice. Serum levels of IL-10 was higher in BALB/c infected mice when compared to BALB.xid animals; however, serum levels of IFNγ, in control and infected BALB.xid mice, were statistically different from that seen in BALB/c mice. We discuss the participation of B1 cells and their cytokines in the resistance to infection.

Keywords: B1 cells; BALB.xid mice; cytokine; *Lagochilascaris minor*; lagochilascariosis

Introduction

The genus *Lagochilascaris* includes five species comprising *L. minor, L. major, L. buckleyi, L. turgida and L. sprenti* (Leiper, 1909); with *L. minor* being the most important from a medical standpoint as it is the etiological agent of human lagochilascariosis in South America. Human lagochilascariosis is caused by *Lagochilascaris minor* and it is considered an emerging helmintosis limited to the Neotropical area (Mexico to Brazil), but infected cats have been found in Uruguay (Sakamoto *et al*., 2002). The parasitosis is not yet a public health problem, but it is prevalent in individuals of the lowest social-economic class, notably from rural areas. The majority of reported cases of infection have been in persons of both sexes, with lesions usually chronic, affecting the neck and head tissues with abscess formation. Sometimes the parasite invades the pulmonary tissue and central nervous system in fatal cases. Frequently, *L. minor* lesions contain different stages (eggs, larvae and adult worm) of the parasite which indicates autoinfection and favors the development of chronic disease (Fraiha *et al*., 1989).

The extraordinary capacity of *L. minor* to migrate across different human tissues can be also observed in animal models of the disease such as in mice and cats. In mice orally inoculated with infective parasite eggs, hatched larvae can be observed migrating in the intestinal tract, with third stage larvae (L3) migrating through intestinal mucosa reaching vessels and hepatic parenchyma and disseminating to other tissues such as lungs, skeletal muscles and subcutaneous tissues. In cats that eat infected mice, L3 migrate through the gullet, pharynx, trachea and cervical lymph nodes (Semerene *et al*., 2004).

As demonstrated in the literature, BALB.xid mice have an important impaired production of B1 lymphocytes, and in smaller proportion, of B2 cells. Although more than 20 years have passed since the discovery of B1 cells, a large number of studies have been conducted in order to characterize and determine their origin and function. It is well established that B1 cells constitute a minor fraction of the B cell population in the spleen and are not detected in the lymph nodes of mice. Nevertheless, they represent the main B cell population in the peritoneal and pleural cavities of these animals. B1 lymphocytes predominantly produce IL-10; however, it has been demonstrated that BALB.xid mice preferentially produce IFNγ (Popi *et al*., 2004). Based on these data we decided to investigate the mortality, tissue lesions and cytokine (IL10 and IFN-gamma) profile in the experimental lagochilascariosis in BALB/c and BALB.xid mice.

Materials and Methods

Animals and parasites

Six to eight-week-old Btk mutant X-linked immunodeficient (Xid) BALB.xid male mice, and their wild-type counterparts BALB/c male mice, were purchased from the University of São Paulo Animal Facility, and kept with food and water ad libidum and handled according to the local regulations. The Research Ethics Committee of the Federal University of Goiás approved the research protocols. Eggs from the parasite were collected from feces of *Felis domesticus* experimentally infected with a human isolate of *L. minor,* according Oliveira *et al.* (2002).

Experimental infection design

Fifty-one BALB/c and fifty-one BALB.xid mice were orally inoculated with a suspension of $10^3 \pm 200$ *L. minor* eggs per animal: twenty-six infected animals were followed for one year to determine the survival ratio; twenty infected animals were sacrificed at different time points (from 30, 60, 90 and 150 days post-infection) and subjected to necropsy for collection of organs for histopathology (five animals per point); and five infected mice of both strains were bled 150 days post-infection, for determination of serum INFγ and IL-10. Fifty-one uninfected control BALB/c and forty-five uninfected control BALB.xid mice received saline orally: twenty-six BALB/c and twenty BALB.xid were used as controls for mortality (followed for one year); twenty animals of each strain were used as control for histopathology (5 animals sacrificed at 30, 60, 90 and 150 days after the beginning of the experiments); and five uninfected control mice of both strains were bled 150 days after the beginning of the experiments, for determination of serum INFγ and IL-10.

Histopathological analysis

Sections of spleen, lung, lymph node, liver, muscle and subcutaneous nodules derived from groups of five uninfected and infected BALB/c and BALB.xid mice were collected from 30 to 150 days after infection, fixed in 10 % neutral buffered formalin embedded in paraffin, and subsequently stained with haematoxilin - eosin (H & E).

Cytokine detection

Cytokines were detected in serum samples of BALB/c (n = 5) and BALB.xid (n = 5) infected male mice sacrificed at day 150 post-infection. Sera from uninfected mice were used as controls (5 male BALB/c and 5 male Xid). Ninety-six-well plates were coated overnight with anti-INFγ or anti-IL-10 monoclonal antibody produced by hybridoma. Plates were washed and blocked with PBS-bovine serum albumin (BSA) 2 %. Dilutions of serum were incubated overnight at 4°C, and after washing, the wells were incubated with biotin-conjugated anti-IFNγ or IL-10 monoclonal IgG2a antibodies (mAb) (Sigma). After 1 hr incubation, plates were washed; horseradish peroxidase (HRP)-streptavidin conjugate was added, and the plates incubated for an additional hour. After this, the plates were washed and ortho-phenylenediamine (Sigma) and hydrogen peroxide were added. After developing, the optical density (OD) was determined at 492nm. The amount of cytokine in each sample was estimated from a standard curve.

Statistical analysis

Cytokines were expressed as mean and standard deviations. Data of two groups were analysed by the Mann-Whitney *U*-test; and the data from multiple groups were analysed by an ANOVA test followed by a multiple comparison test (Dunn's test). Survival curves were analysed using Kaplan & Meyer method and the differences between groups were tested using the Log-rank test (Program Prism 4.0).

Results

BALB/c mice began to die on day 34 of infection, reaching 57.6 % survival on day 343. On the other hand, BALB.xid mice began to die on day 53 of infection, reaching 80.7 % survival on day 343 (Fig. 1). BALB.xid mice displayed a greater survival rate than BALB/c mice throughout the whole period of infection with a significant difference (p = 0.01). In addition, only two animals of the BALB.xid and three of the BALB/c control groups died.

In BALB/c infected mice, granulomatous reactions of moderate intensity containing mononuclear, plasma cells, foamy macrophages, few polymorphonuclear cells, giant mononuclear cells and a few fibroblasts were found in lungs during the infection period studied (30 – 150 days of infection). Third stage larvae located at the centre of granulomas were present, and the lesions contained both whole and ruptured parasites, and some central tissue necrosis (Fig. 2A). Perivasculitis and interstitial inflammation were of moderate to deep intensity (Fig. 2B). In BALB.xid mice, granulomatous reactions of light intensity were observed in the lungs, containing concentric fibrosis interspersed with foamy macrophages, polymorphonuclear neutrophils and mononuclear cells, from 30 to 150 days of infection. Third-stage larvae located in the centres of the granulomas were detected, and several lesions contained

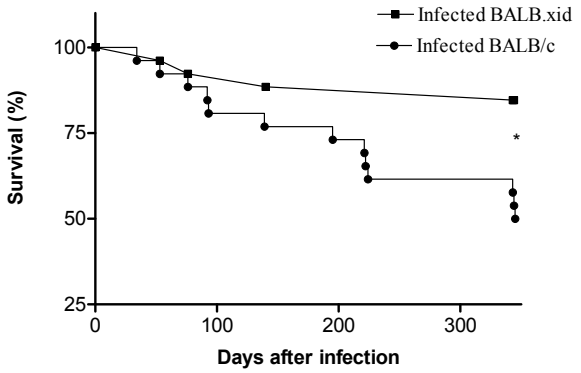

Fig. 1. Cumulative survival rates along period post infection of BALB/c (●) and BALB.xid (■) mice orally inoculated with viable eggs of *Lagochilascaris minor*. Survival ratio was determined using the Kaplan-Meier test (*p < 0.05).

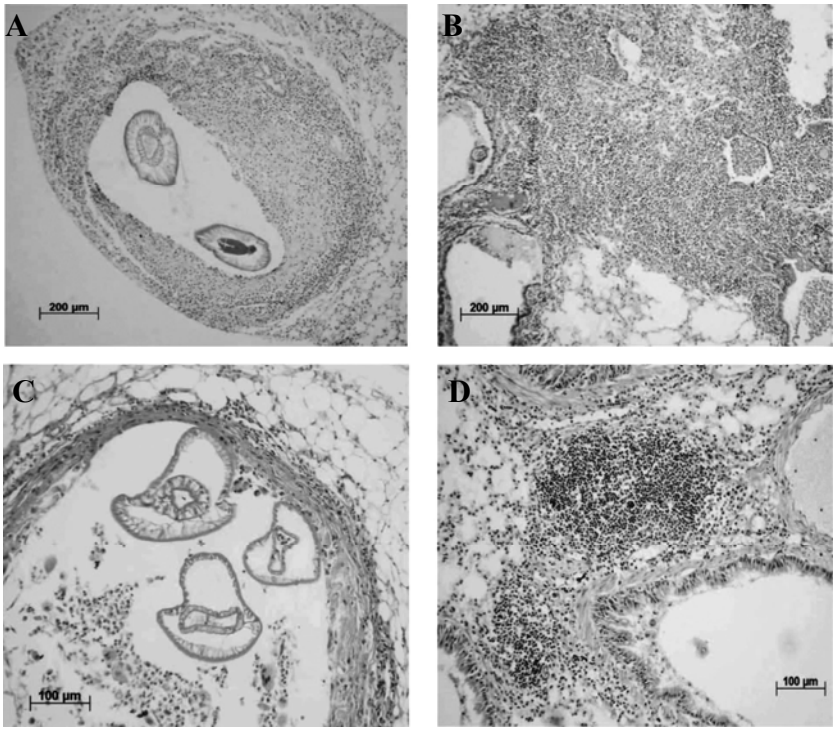

Fig. 2. BALB/c and Xid mice were infected with 2 x 10^3 eggs of *Lagochilascaris minor* and sacrificed 60 days after infection. Granulomatous reaction of moderate intensity with third stage larvae located at the centre of the granuloma (**A**) and interstitial inflammation of moderate to deep intensity (**B**) was seen in the lung. In BALB.xid mice, granulomatous reactions of light intensity were observed in the lungs with third-stage larvae located in the centres of the lesion (**C**) and interstitial inflammation were observed with prevail of mononuclear cells (**D**), H&E staining.

ruptured parasites (Fig. 2C). Little perivasculitis and interstitial inflammation were observed with prevalence of mononuclear cells in lung lesions (Fig. 2D). In both lineages the liver had large hepatocytes with bilobular nuclei and congestion with an occasional perivascular inflammatory infiltrate. Spleen and lymph nodes had lymphoid follicles with expanded germinative centres.

Cytokines were analysed in the serum of animals at 150 days post-infection because we demonstrated in a previous work that in this period it exists statistical differences in the number of larvae recovered and in the antibody levels of specific IgM, IgG and IgA between the two strains of mice (Freitas *et al.*, 2009). Non-infected BALB/c (42.6 pg/ml) and BALB.xid (167 pg/ml) mice presented low level of sera IFNγ, though it was higher in the BALB.xid uninfected control animals (p = 0.01). For both strains, infected animals produced a large amount of IFNγ when compared to uninfected control mice ($p \leq 0.05$). On the other hand, infected BALB.xid mice presented higher IFNγ serum levels (1516 pg/ml) compared to BALB/c infected mice (526.1 pg/ml, p = 0.03) (Fig. 3A). Non-infected BALB/c (29 pg/ml) and BALB.xid (20 pg/ml) mice presented similar levels of serum IL-10. For both strains, infected animals produced larger amounts of IL-10 when compared to control uninfected mice, but it was statistically significant only for the BALB/c strain ($p \leq 0.05$). BALB.xid infected mice presented lower IL-10 serum levels (26 pg/ml) compared to BALB/c infected mice (44 pg/ml, p = 0.008) (Fig. 3B).

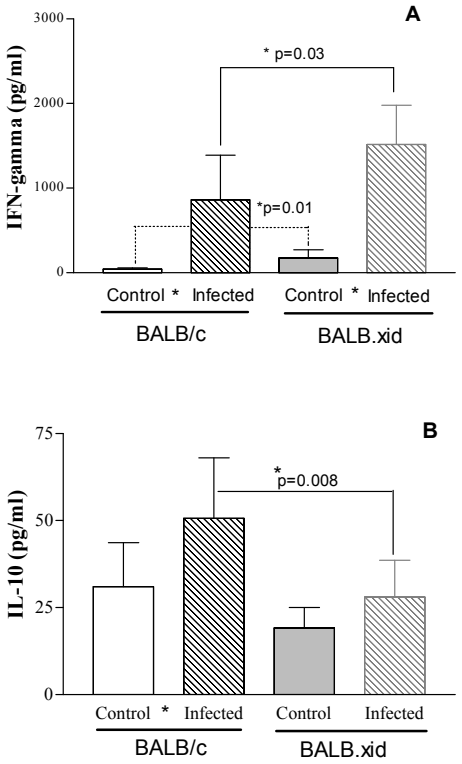

Fig. 3. Serum levels of IFNγ (**A**) and IL-10 (**B**) in uninfected and infected BALB/c and Xid mice, 150 days post orally administration of eggs of *Lagochilascaris minor*. Results are expressed as the mean (ng/ml) of cytokine from each group of mice (n = 5 animals per group) (*$p \leq 0.05$).

Discussion

This report presents evidence suggesting that the absence of B1 cells, along with low production of antibodies, IL-10, and the over production of INFγ in BALB.xid mice infected with *L. minor,* influences survival during experimental infection. The survival rate of infected BALB.xid mice was higher and statistically different from the survival rate of infected BALB/c mice. The difference in susceptibility to *L. minor* infection may be due to the difference in cytokine and antibody production, as the animals present the same genetic background and the same Major Histocompatibility Complex locus ($H\text{-}2^d$), differing only in the Btk mutation.

Experimentally *L. minor* infected BALB/c and BALB.xid mice developed a non-robust pathogenic infection, different from the one that we can see in C57BL/6 infected mice (Freitas *et al*., 2008), but lesions presented by BALB/c mice were more serious than that in the BALB.xid animals. In infected BALB/c mice, granulomatous reactions in lungs were of moderate intensity with third stage larvae (generally undamaged) located at the centre of granulomas, accompanied for perivasculitis and interstitial inflammation of moderate to deep intensity with the prevail of neutrophils. In BALB.xid mice, granulomatous reactions of light intensity were observed in the lungs, with third-stage larvae (generally ruptured) located in the centre of the granulomas, accompanied for little perivasculitis and interstitial inflammation with prevail of mononuclear cells.

A key feature of helminth infections is the induction of strong Th2 immune responses in their hosts (Holland *et al*., 2006). Th2-like responses mediate susceptibility to *Taenia crassiceps*, probably by inhibiting Th1 responses required for the development of protective immunity against this parasite. In experimental murine filariosis, the lack of IFNγ confers impaired neutrophil granulocyte function and prolongs the survival of larvae (Saeftel *et al*., 2001). Nevertheless, it has been demonstrated that BALB.xid mice are significantly more resistant to *Trypanosoma cruzi, Paracoccidioides brasiliensis* and lymphatic filarial parasite infections (Popi *et al*., 2004).

C57BL/6 mice present a severe pathology after infection with *L. minor*, with high mortality, and almost no production of IFNγ (Freitas *et al*., 2008). Although low levels of serum IL-10 in BALB.xid mice due to the lack of B1 cells may be important, *L. minor* infected BALB.xid mice presented higher IFNγ serum level compared to controls or to infected BALB/c. High IFNγ production in infected BALB.xid mice may have a function in the evolution of lagochilascariosis, controlling the number of L3 larvae and permitting better survival of the animals.

We consider these initial studies very significant but it will be important to study the participation of other cytokines and to look into the population of cells that are involved in the development of lesions and infection at the systemic and local levels in this experimental model of infection, and others, like interleukin-KO animals.

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