Comparative experimental infection of *Lacazia loboi* in BALB/c and B10.A mice

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Summary

Both hind foot pads of BALB/c and B10.A mice strains, were inoculated with a fungal suspension of *Lacazia loboi* obtained from a Jorge Lobo's disease patient. The suspension had 9 x 10^5 cells/ml and its viability index was 45%. The animals were sacrificed at different time periods varying from 24 h to 18 months after inoculation. The BALB/c mice developed an extensive granulomatous infiltrate, similar to the disease in humans, that progressively evolved. The number of fungal elements also increased as the disease progressed, and after the seventh month of inoculation, macroscopic changes of the foot pads were evident. Although the B10.A mice developed an exuberant granulomatous infiltrate, macroscopic changes were not detected. The number of fungal cells in the infected tissues increased in number, but they were lower then the numbers found in the BALB/c strain. The viability indexes were also lower for the B10.A strain. Considering the histopathological findings, the presence of macroscopic changes and the great amount of fungal cells in the infected tissues, the authors concluded that the BALB/c mice strain was more susceptible to *L. loboi* infection than the B10.A strain.

Key words Jorge Lobo's disease, *Lacazia loboi*, Mouse

Infección experimental con *Lacazia loboi* de ratones BALB/c y B10.A

Resumen

Ambos cojines plantares de las patas traseras en ratones de laboratorio BALB/c y B10.A, fueron inoculados con una suspensión de células de *Lacazia loboi*, obtenida de la lesión de un paciente portador de la enfermedad de Jorge Lobo, a una concentración de 9 x 10^5 células/ml y un índice de viabilidad del 45%. Los animales fueron sacrificados en períodos que variaron desde las 24 h a los 18 meses tras la inoculación. El grupo BALB/c un presentó extenso infiltrado de granulación, semejante al observado en la enfermedad humana, que evolucionó progresivamente. El número de células fúngicas en los ratones también aumentó, observándose a partir del séptimo mes de inoculación la presencia de alteraciones macroscópicas en los cojines de las patas traseras. El grupoB10.A también presentó un infiltrado con granulación exuberante, pero no se observaron alteraciones macroscópicas. El número de células fúngicas también aumentó en este grupo, pero fue menor que el obtenido en el grupo BALB/c. Los índices de viabilidad también fueron menores. Considerando los cambios histopatológicos, la presencia de alteraciones macroscópicas y la gran cantidad de células fúngicas obtenidos, los autores concluyeron que la especie BALB/c es más susceptible a la infección por *L. loboi* que la B10.A.

Palabras clave Enfermedad de Jorge Lobo, *Lacazia loboi*, Ratones

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Jorge Lobo's disease was first described in 1931 by Jorge de Oliveira Lobo in Recife, Brazil [1]. It is a cutaneous-subcutaneous mycosis of chronic evolution without visceral involvement, caused by *Lacazia loboi*, a fungal pathogen recently classified as the sister clade with *Paracoccidioides brasiliensis* in the dimorphic Onygenales [2,3].

The first studies of *L. loboi* inoculation in experimental animals yielded limited and isolated results [4-7]. In 1999, Opromolla *et al.* [8] first established a methodology for the experimental inoculation based on counts of fungal cells in a Neubauer chamber and determination of the viability index by the fluorescein diacetate-ethidium bromide technique (FD-EB) before and after inoculation [8]. Using Swiss mice, these investigators obtained good results by maintaining the infection for one year and six months. However, the number of retrieved fungal cells was always similar to the number of cells in the original inoculum and only some of them were viable. No macroscopic changes were observed in any animal.

Using the same methodology, Madeira *et al.* [9] were successful in reproducing the experimental disease in BALB/c mice. They obtained large number of fungi at the end of the experiment, many of them viable, and the formation of macroscopic lesions in the inoculated foot pads. The finding of an animal model for the experimental reproduction of fungal diseases has been of importance for the study of the pathogenic mechanisms involved in the infection, the host-parasite relationship, biochemical constitution of the pathogenic fungi and their antigenic properties, as well as new therapeutic approaches. Considering previous results obtained with the BALB/c and the Swiss strains, the objective of the present study was to determine whether the susceptibility of the BALB/c strain to *L. loboi* infection was not an isolated phenomenon. For this purpose, *L. loboi* was inoculated into the mouse B10.A strain, and the histopathological aspects of the lesions produced were evaluated together with the results obtained in the BALB/c strain. The number of fungal cells and the viability indices obtained at the different times of sacrifice were also evaluated in both strains.

Material and Methods

Biopsy. Fragment of a keloidal lesion was removed from a male patient with Jorge Lobo's disease from the State of Acre, Brazil, localized on his lower limbs. The fragment was immediately washed with physiological saline to remove excess blood and then macerated in a tissue homogenizer containing 7 ml 0.85% saline solution (SS).

Fungal suspension. The total number of fungal cells was determined with a Neubauer hemocytometry chamber and is reported as an absolute number. The final suspension contained 9×10^5 cells/ml or 2.7×10^4 cells/0.03 ml. Vital staining with FD-EB was used to determine the viability index [10] according to the technique standardized for *L. loboi* by Vilani-Moreno & Opromolla [11]. The value obtained was reported as percentage, and the viability index detected for the suspension was 45%.

Inoculation. The procedure was performed immediately after the determination of number of fungal cells and the viability index. Both hind foot pads of 44 BALB/c mice and 44 B10.A mice aged two months, were infected intradermally with 0.03 ml of the fungal suspension.

Sacrifice. Four BALB/c and four B10.A mice were picked up randomly to be sacrificed at the following time intervals: 24 and 72 h, 7 and 15 days, and 1, 4, 7, 10, 13, 16 and 18 months post-inoculation (p.i.).

Exeresis of the foot pad. Both foot pads were excised; one of them was sent for histopathological examination and the other was ground in a mortar with 1 ml 0.85% SS for the determination of viability index and number of fungal cells. The histological sections were stained with hematoxylin-eosin and methenamine silver.

Statistical analysis. The number of fungal cells and viability index, according to experimental group (BALB/c and B10.A strains), and time of sacrifice, were determined by a non-parametric test for a two factor model (experimental group and time of sacrifice), complemented with the respective multiple comparisons test, with the level of significance set at 5%. The Spearman correlation coefficient was used to calculate the correlation between viability index, number of fungal cells and the time of the duration of the macroscopic lesions [12].

Results

Histopathological analysis. The early phase of the infectious process observed in the two strains was characterized by the presence of a small granulomatous infiltrate consisting of isolated histiocytes containing fungal cells, a few diffusely scattered lymphocytes, polymorphonuclear cells (MSN), and some areas of necrosis. One month p.i., the cell constitution of the infiltrates was essentially the same in the two strains used in this study. Histopathologically, an inflammatory reaction consisting of histiocytes, many of them with a cytoplasm of lacy aspect; some plasmocytes were observed in the B10.A strain. Mice sacrificed four months p.i., were characterized by an increase in the number of fungal cells and in the size of the infiltrates. Histological sections of the tissues were found to be divided into blocks of varying sizes consisting of histiocytes, rare MSN, lymphocytes and foreign body-type giant cells, which presented a poorly delimited cytoplasm in the B10.A strain. In BALB/c animals, plasmocytes were observed for the first time. After seven months p.i., the infiltrates were larger, mainly consisting of giant cells. A small amount of lymphocytes, plasmocytes, MSN, fibroblasts, histiocytes with a lacy cytoplasm, and large amounts of fungal cells were observed in the BALB/c strain. In the B10.A strain the number of lymphocytes and MSN was slightly larger, with some necrotic areas. After 10 months of experimental infection, the size of the infiltrate and the amount of fungal cells continued to increase in both strains. The number of MSN was larger in strain B10.A, even forming small abscesses in some areas. After 13 months of infection, the infiltrate observed in the BALB/c strain showed a predominance of giant cells and large numbers of fungi which exceeded the number observed in B10.A animals. In the B10.A strain, the aspect of the infiltrate was similar to that observed previously, with many histiocytes that were present in an isolated manner or forming giant cells. Sixteen months after infection, the aspect of the infiltrate was the same, except for the number of fungal cells which was higher in the BALB/c strain. At the end of the experiment (18 months p.i.), BALB/c mice presented an extensive granulomatous infiltrate divided into blocks and septated by fibroblast bundles, with a predominance of giant cells containing many fungal elements. Sparse number plasmocytes and lymphocytes and moderate amounts of histiocytes perme-

ated the infiltrate. The presence of necrotic areas and an increased numbers of MSN were also observed (Figure 1). In B10.A mice there was a predominance of histiocytes with a lacy cytoplasm. Giant cells were present, but at a lower frequency than the one observed in the BALB/c mice. Small numbers of sparse lymphocytes and MSN were also observed (Figure 2).

Number of fungal cells at the different times of sacrifice. The values are reported in table 1 and were calculated as the median for the four animals sacrificed at each time point. Four months p.i., both strains showed a progressive increase in the values obtained, exceeding those in the inoculum; the number of fungal cells obtained 18 months p.i. was statistically significant from that obtained at other times ($p < 0.05$).

Viability indices at different times of sacrifice. The indices are presented in table 2 and were calculated as the median for the four animals sacrificed at each particular time. When the indices obtained for the two strains were compared, significant differences were observed only at 24 h, seven days, seven months and 16 months p.i. ($p < 0.05$). No viable fungi were observed in 17 (38.6%) B10.A mice and in four (9%) BALB/c mice.

Macroscopic lesions. Fifteen (34%) BALB/c mice presented macroscopic changes in their foot pads. The first changes were observed in two animals seven months after inoculation and were characterized by the presence of nodules that rapidly increased the size of the paw (keloidal lesions) (Figure 3). The time of onset of the lesions ranged from seven to 15 months p.i. A negative correlation was observed between viability index and time of lesion evolution ($r = -0.66$; $p < 0.01$) and a positive correlation was observed between number of fungi and time of lesion evolution ($r = 0.71$; $p < 0.01$). No statistically significant correlation was observed between viability index and number of fungi ($r = -0.25$; $p > 0.05$). No macroscopic lesions were observed in any B10.A mouse.

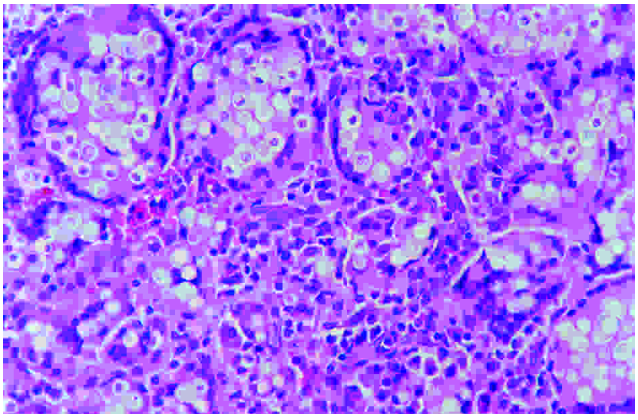

Figure 1. Granulomatous infiltrate with predominance of Langhans type giant cells filled with numerous fungal cells. BALB/c mouse 18 months after inoculation (hematoxylin-eosin, 400x).

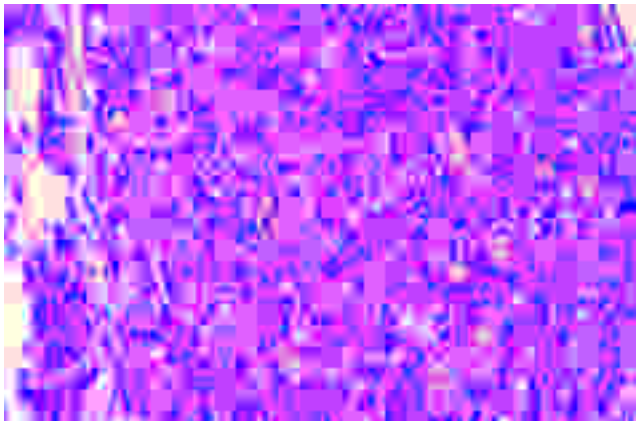

Figure 2. Granulomatous infiltrate constituted by macrophages with vacuolated cytoplasm, macrophages containing fungal cells and some foreign body giant cells. B10.A mouse 18 months after inoculation (hematoxylin-eosin, 400x).

Figure 3. Macroscopic aspect of the foot pad of a BALB/c mouse 18 months after inoculation with *Lacazia loboi*.

Discussion

The development of an animal model is very important to study the pathogenic microbes involved in human diseases. Although it is not always possible to reproduce in these models the same pathological situations occurring in man, the major objective of this type of experimentation is to obtain answers to specific questions about the pathogen under study.

Previous experimental studies using mice and *L. loboi* cells from patients with the disease, revealed that it was possible to reproduce the infection in mice [4]. In spite of this, there was not a standardized methodology for reproduction of *L. loboi* infection. The experimental data was always based on histopathological findings, and there were not studies evaluating the quantity of fungal cells inoculated and the one recovered after the sacrifice of the infected animals. There was also no mention of techniques to be used to assess the viability of fungi present in the suspensions, or of the animal strains used, especially mice.

From a histological viewpoint, the early phase of the infectious process observed in the present study on the two mouse strains was characterized by the presence of an acute inflammatory reaction rich in MSN, as is also the case for other infectious granulomatous diseases. One month after inoculation the cell infiltrate was practically the same in both strains, except for the presence of rare plasmocytes observed in the B10.A strain. Perhaps this suggest that the humoral immunity was activated in an attempt to contain the advance of the infection. Plasmocytes are also observed in human lesions where, together with MSN and lymphocytes, they are irregularly distributed through the infiltrate [13]. Many histiocytic cells present a lacy cytoplasm. According to some authors, these cells appear to be “xanthomized cells”.

Table 1. Median and total semi-amplitude of the number of fungal cells ($x10^3$) according to the experimental group (BALB/c and B10.A) and time of sacrifice, with the respective results of statistical analysis.

Time of sacrifice	G1 (BALB/c)	G2 (B10.A)	Group statistical test
24 h	22.50 ± 15.00	20.00 ± 12.50	0.01 (p >0.05)
72 h	20.00 ± 15.00	20.00 ± 5.00	0.01 (p > 0.05)
7 d	17.50 ± 7.50	12.50 ± 2.50	1.86 (p > 0.05)
15 d	23.50 ± 7.50	34.50 ± 11.50	0.59 (p > 0.05)
1 m	15.00 ± 10.00	10.00 ± 5.00	0.62 (p > 0.05)
4 m	100.00 ± 69.00	101.00 ± 85.00	0.73 (p > 0.05)
7 m	170.00 ± 160.00	112.50 ± 100.00	0.58 (p > 0.05)
10 m	660.00 ± 3515.00	140.00 ± 126.00	1.44 (p > 0.05)
13 m	1550.00 ± 3315.00	33.50 ± 168.50	2.02 (p < 0.05)
16 m	5445.00 ± 5270.00	65.00 ± 40.00	2.32 (p < 0.05)
18 m	7250.00 ± 4550.00	125.00 ± 65.00	2.31 (p < 0.05)
Statistical test time	36.82 (p < 0.05)	24.99 (p < 0.05)	

Table 2. Median and total semi-amplitude of the viability index (%) according to the experimental group (BALB/c and B10.A) and time of sacrifice, with the respective results of statistical analysis.

Time of sacrifice	G1 (BALB/c)	G2 (B10.A)	Group statistical test
24 h	15 ± 9	2 ± 3.5	2.32 (p < 0.05)
72 h	17 ± 11	7 ± 13	0.44 (p > 0.05)
7 d	30 ± 7.5	9 ± 4	2.31 (p < 0.05)
15 d	7 ± 6.5	8 ± 7.5	0.14 (p > 0.05)
1 m	13 ± 10	0 ± 3	1.56 (p > 0.05)
4 m	9 ± 8	3 ± 4.5	1.15 (p > 0.05)
7 m	6 ± 12	0 ± 0.5	2.48 (p < 0.05)
10 m	17 ± 15	5 ± 4	0.89 (p > 0.05)
13 m	9 ± 11	4 ± 5.5	0.44 (p > 0.05)
16 m	15 ± 10	1 ± 1	2.34 (p < 0.05)
18 m	4 ± 10	4 ± 3	0.29 (p > 0.05)
Statistical test time	36.82 (p < 0.05)	24.99 (p < 0.05)	

However, Opromolla *et al.*, using Sudan III staining, observed that the lipids visualized in the histological sections seem to be of fungal origin [14]. The lacy aspect appears to be related to fungal fragments digested by these cells. Four months after experimental inoculation, there was an increase in infiltrate size and in fungal cell numbers, which is in agreement with the data obtained with cell counts. Budding forms were frequent, suggesting that the fungi were indeed multiplying. Seven months p.i., the infiltrate was larger and basically consisted of giant cells and of large numbers of fungal cells, especially in the BALB/c strain. Some necrotic areas and increased in the number of lymphocyte were observed in the B10.A strain, suggesting a greater participation of the immune response in the attempt to control the infectious process, although the number of fungal cells was larger than in those mice sacrificed four months p.i. Ten, 13 and 16 months p.i., the infiltrate size increased in the BALB/c strain, with a predominance of giant cells and large numbers of fungal cells. The size of the infiltrate also increased in the B10.A mice, but isolated histiocytic cells were detected at higher frequency, although giant cells were also present. At the end of the experimental inoculation (18 months p.i.), both mice strains presented an extensive granulomatous infiltrate with necrotic areas and many MSN. Self-digestion by macrophage enzymes or enzymes of the fungus itself may probably be a cause of necrosis, in addition to the release of toxic metabolites after the death of these cells. In the BALB/c strain there was a predominance of enlarged giant cells containing large numbers of ingested fungal cells, many of them looking like empty capsules, suggesting that the fungal elements were already digested by these cells. The predominance of histiocytic cells with a lacy cytoplasm in B10.A mice indicates a greater competence of this strain in controlling the infectious process, with greater fungal destruction than in the BALB/c strain. The number of fungal cells was large in the B10.A strain, but clearly lower than the number observed in the BALB/c strain.

With respect to the number of fungal cells at the different times of sacrifice, a progressive increase in the values obtained for both strains was observed, suggesting the multiplication of *L. loboi* in the infected tissues. These values, however, were always smaller in the B10.A mice. This strain seems more competent in containing the infectious process. Although the elimination of dead *L. loboi* cells is a slow process, newly viable cells of *L. loboi* more likely keep the infection active. The major differences between the two strains were evident on 13 month p.i.

Macroscopic alterations of the inoculated foot pads were observed only in BALB/c mice. These alterations started seven months p.i. and were probably related to the increase in the number of fungal cells. Although the animals were isogenic and received an inoculum from the same fungal suspension, the time of onset of macroscopic lesions ranged from seven to 15 months after inoculation. This was probably due to the fact that the fungi clustered into clumps, with heterogeneous distribution in the suspension. When the number of fungal cells was counted in the suspension obtained from the patient's lesion, many of them were found in clusters, forming chains of up to five cells, but only 45% of them were viable. Thus, it is possible that not all animals received exactly the same number of viable fungi. These factors would better explain the variations in the onset of the lesions. Analysis of the association between number of fungal cells and time of the duration of the lesions revealed a positive correlation, i.e., the longer the time of lesion evolution, the larger the number of fungal cells. Possibly the elimination of dead fungal cells is a slow process, causing them to be mixed with those that are still alive and reproducing.

To estimate the percentage of viable fungal cells observed at different sacrifice times, the viability index was calculated. No significant differences in values were obtained along the experiment for either strain. When these values were compared between strains, significant differences were observed only at 24 h, seven days, seven months, and 16 months p.i. The relationship between the viability index and the number of fungal cells, in the animals that presented macroscopic lesions, did not show a significant correlation between these two variables, i.e., even when the number of fungal cells was larger, the viability index was not always high. On the other hand, a negative correlation was observed between viability index and time of evolution of the macroscopic lesion, i.e., the longer the evolution of the lesion, the lower the viability index. Probably, since the number of fungi was large, as this number increased the lesions also increased in size and the viability index would be diluted among the number of live and dead fungi, which would be slowly eliminated.

The determination of the viability index of microorganisms is important in the attempts to reproduce experimentally infectious diseases. Vilani-Moreno *et al.* [11] performed several tests with the cotton-blue, Trypan blue, Janus green and FD-EB stains in order to determine the best technique to be used in the detection of *L. loboi* viability. In their study, the cited investigators observed that

the technique based on the FD-EB stain proved to be more sensitive than the others. The viability index agrees with the morphological aspect of the fungus in parasitized tissues.

The number of fungal cells in the infected tissues, and the viability index obtained at the different times of sacrifice varied between strains. This finding suggests differences in their susceptibility to *L. loboi* infection, probably due to genotypic differences between the two mice strains studied. The BALB/c strain, which proved to be more susceptible to infection than the Swiss strain, also demonstrated higher susceptibility than the B10.A strain. As previously observed in paracoccidioidomycosis [16], genetic factors that determine the resistance pattern could also play a role in the susceptibility of the infected hosts by *L. loboi* [15]. However, other factors, such as cellular and humoral immunity, inoculum concentration, sex, age, and inoculation route should be studied to clarify which variables determine the differences in susceptibility. Successive passage of the fungi through different mice to determine changes in the behavior of *L. loboi* infection would also be of great importance for the study of the experimental aspect of this fascinating disease.

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