Ashdin Publishing
Journal of Neuroparasitology
Vol. 1 (2010), Article ID N100402, 6 pages
doi:10.4303/jnp/N100402

Research Article

Development of a Murine Model of Neuroparacoccidioidomycosis

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Received 08 April 2010; Accepted 21 July 2010

Abstract Paracoccidioidomycosis is the most important systemic mycosis in Latin America. In the last decades, it was verified that central nervous system involvement is frequent, occurring in 12.5% of the cases. Despite the relevance of this severe form of the disease, there are not experimental models for the study of the interactions established between the fungus and the central nervous system. We developed a murine model of neuroparacoccidioidomycosis with intracranial inoculation of 10^6 yeast cells of *Paracoccidioides brasiliensis* (strain PB18) in C57BL/6 mice. Animals developed lesions similar to those described in human patients and morbidity was evaluated by the SHIRPA behavioral battery, showing progressive and severe cognitive compromise. With the development of this model, future studies will be able to evaluate several pathogenic and therapeutic aspects of neuroparacoccidioidomycosis in order to improve survival or lessen morbidity of this severe disease.

Keywords paracoccidioidomycosis, neuroparacoccidioidomycosis, experimental model, behavior, histopathology

1 Introduction

Paracoccidioidomycosis (PCM), a systemic infection caused by the fungus *Paracoccidioides brasiliensis* (Pb), is the most important deep mycosis in Latin America [13]. It can be considered a public health problem due to elevated social and economic burden determined not only by the active disease but also by the frequent associated sequelae [14].

Although it can affect individuals of all ages, the disease is more common in patients between 30 and 50 years old. There are no differences in incidence between genders until puberty, after which there is a male:female ratio of 11:1 [7].

The infection occurs by the inhalation of fungal propagules, resulting in a primary pulmonary complex, usually asymptomatic, which tends to resolve spontaneously leaving residual lesions that may contain viable fungi for years [18]. In some cases, the primary infection develops, leading to a symptomatic pulmonary disease or spreading to other sites by hematological and/or lymphatic routes, affecting virtually any organ [16]. Lung is the most frequently affected site. In the last decades, with the development of new diagnostic methods, especially neuroimaging techniques, it was observed that secondary central nervous system (CNS) involvement is much more common than previously considered, taking place in approximately 12.5% of the cases [9].

The lesions of the CNS caused by the fungus may be located both in the meninges and in the nervous parenchyma, leading to two clinical forms of neuroparacoccidioidomycosis (NPCM): the meningeal/meningoencephalitic and the pseudotumoral forms, with the latter being the most common (89.4% of the cases) [17, 18].

Clinical manifestations of the meningeal form correspond to pictures of chronic meningitis, which can be diffused or localized, involving mainly the meninges at the base of the skull. Symptoms of the pseudotumoral form are usually polymorphic, depending on the location and the size of the lesions. The majority of the lesions (66.8%) are located in supratentorial compartment, especially in frontal and parietal lobes (13.1% and 17.0%, respectively). The progress of this form of the disease is slow, presenting with signs of intracranial hypertension (48.9% of the cases), motor deficits (49.8%), seizures (24.9%), cerebellar dysfunction (22.2%), cognitive compromise (21.3%), and sensory deficits (18.1%) [17].

Despite the growing relevance of this severe complication of the disease, there are not experimental models for the study of the interactions established between Pb and the CNS.

The aim of this study was to develop a murine model of NPCM and to characterize the clinical symptoms and histopathological changes caused by the experimental infection.

2 Materials and methods

Mice. Six- to eight-week-old male C57BL/6 mice were obtained from Animal Care Facilities of the Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Brazil. The local ethics committee for animal research approved all the experimental procedures described here.

Fungi. *P. brasiliensis* strain PB18, a highly virulent isolate, was maintained by weekly sub-cultivation in the solid YPD medium at 35°C and used on days 5 to 7 after culture. The yeast cells were washed in phosphate-buffered saline (PBS, pH 7.2) and adjusted to 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 yeast cells/10 μL.

Infection with Pb18. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg). The animals were divided into seven groups of five mice and they received, by intracranial injection, 10 μL containing 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, or 10^7 yeast cells of Pb18. Control mice received only PBS. The injection was made with a 29-gauge disposable needle, 1 to 2 mm to the right of bregma, in a depth of 3–5 mm. Mice were fully recovered within 5 to 10 minutes of the procedure, and no deaths resulted from the inoculation procedure. The standard inoculum concentration was obtained with the Reed-Muench median lethal dose (LD50) method [19].

Survival studies, weight, and food intake. All animals were observed up to 60 days post-infection (p.i.). Animals were inspected twice daily. Mice were weighted daily during 60 days p.i. Food intake was also quantified during the same period.

SHIRPA screen. The SmithKline/Harwell/Imperial College/Royal Hospital/Phenotype Assessment (SHIRPA) screen was conceived as a multi-test behavioral battery used for longitudinal studies with standardized guidelines and materials [20,21]. The primary SHIRPA screen consists of a series of observations of reflexes and basic sensorimotor functions and provides a behavioral and functional profile by assessment of individual performance [12]. The SHIRPA protocol was used to evaluate behavioral changes during the course of the infection with the standardized inoculum (10 mice/group). After a period of adaptation (2 days before infection), the procedure was carried out from the day of infection until death on daily basis. For analysis purpose, the individual parameters assessed by SHIRPA were grouped into five functional categories (neuropsychiatric state; motor behavior; autonomic function; muscle tone and strength, and reflex and sensory function) according to Lackner et al. [11] and Lacerda-Queiroz et al. [10], determining an overall score and five domain scores.

Histopathologic examination. Mice (20 mice/group) infected with the standardized inoculum were euthanized 4 and 8 weeks p.i. Coronal sections of the brain were fixed in 10% neutral buffered formalin for 24–48 h, processed for paraffin embedment, and stained with hematoxylin-eosin (HE) and Groccott's stain.

Statistical analysis. Data are shown as mean $\pm$ SEM. Kaplan-Meier test was used for survival studies. A one-way ANOVA with Tukey's correction was used for multiple comparisons. SHIRPA data were analyzed with Wilcoxon test. Statistical significance was set at $P < 0.05$.

3 Results

Survival studies and inoculum standardization. Mice were observed up to 60 days p.i. No deaths were registered in the animals infected with 10^1, 10^2, 10^3, and 10^4 yeast cells, as well as in control mice. The highest inoculum, 10^7 yeast cells/animal, determined a mortality of 80% after 60 days. Mice infected with 10^6 yeast cells presented a mortality of 60% and the group infected with 10^5 yeast cells presented a mortality of 40% at the end of the experiment (Figure 1). After Kaplan-Meier survival analyses, LD50 was calculated by Reed-Muench method, resulting in a dose of $10^{5.7}$ yeast cells/animal. This value was adjusted to the inoculum of 10^6 yeast cells/animal.

Weight and food intake. Body weight loss was observed in mice from groups infected with 10^5, 10^6 and 10^7 yeast cells in relation to all other groups ($P < 0.001$). The weight loss was greater in mice infected with 10^6 and 10^7 yeast cells comparing with the group infected with 10^5 yeast cells. No differences in weight were observed among control mice and mice infected with 10^1, 10^2, 10^3, and 10^4 yeast cells ($P > 0.05$) (Figure 1). Mean food intake after 60 days was 20.7 g/mouse/week for control mice and those infected with 10^1, 10^2, 10^3, and 10^4 yeast cells. Mice infected with 10^5, 10^6, and 10^7 yeast cells presented mean food intake of 12.5 g/mouse/week after 60 days. These three groups did not differ in relation to food intake, but mice which exhibited clinical signs of disease had significant reduction in food consumption ($P < 0.001$).

SHIRPA. To further analyze the clinical impact of disease in infected mice, we performed the SHIRPA behavioral battery on mice infected with the standardized inoculum of 10^6 yeast cells (LD50). SHIRPA analysis after 60 days confirmed that infected mice developed a wide range of behavioral changes during the course of the disease (Figure 2).

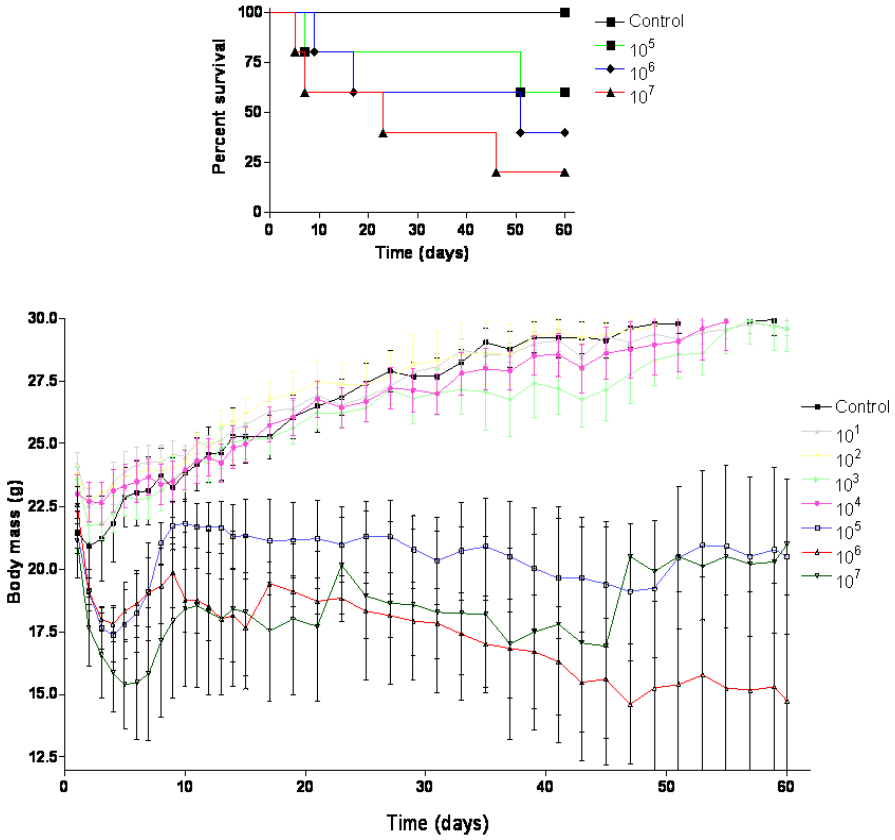

Figure 1: Survival curves (top) and body mass (bottom) of C57BL/6 mice infected with 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 yeast cells of Pb, strain PB18, and control mice inoculated with PBS after 60 days ($n = 5$ mice/group).

Muscle tone was the first altered domain, starting from day 26 p.i., followed by autonomic function, which was altered from day 29 p.i. Neuropsychiatric state was altered from day 34 p.i. and reflex and sensory function was altered from day 40 p.i. The last altered domain was motor behavior, which differed significantly between infected and control mice from day 39 p.i. The overall score, represented by the sum of the five domains, was altered from day 25 p.i.

Histopathology. Histopathological analyses were performed in brain sections from mice infected with the standardized dose and euthanized 4 and 8 weeks p.i. Lesions already present at 4 weeks p.i. had increased their volume, assuming the form of nodules and pseudotumoral aspect at 8 weeks p.i. (Figures 3(D), 3(E), and 3(H)). The nodular inflammation was mainly composed of macrophages, lymphocytes, and neutrophils. The appearance of large areas of necrosis (Figure 3(I*)), with and without deposition of fungal structures stained by Groccott (Figures 3(D), 3(E), and 3(H) insert) and by HE (Figures 3(H) and 3(I), fine arrows), contributed to the heterogeneous aspects of the lesions. The surrounding parenchyma was preserved but presented infiltration of mononuclear cells around some blood vessels. There was dissemination of the lesions which reached the meninges and also the cerebellum (Figure 3(B)). Control mice did not show any significant alteration (Figures 3(A) and 3(C)).

4 Discussion

Experimental models for the reproduction of the Pb infection in animals are well defined in the literature. Such models were induced by several artificial routes and they have been successfully used for the study of pulmonary or systemic forms of the disease [5]. However, these animals do not develop neurological symptoms and none of these models seem to reproduce Pb infection of the CNS. Thus, there are no experimental studies aiming to evaluate the interaction between Pb and the CNS.

In a systematic review of the literature [17], we observed a growing trend in the diagnosis of NPCM in the last two decades, with a mortality rate of 44.1%, and the development of potentially disabling sequelae in 50.1% of the survivors, especially motor impairment (28.6% of survivors). In addition, sensory deficits were registered in 8.3% of survivors and seizures in 4.8% [17]. However, there are only

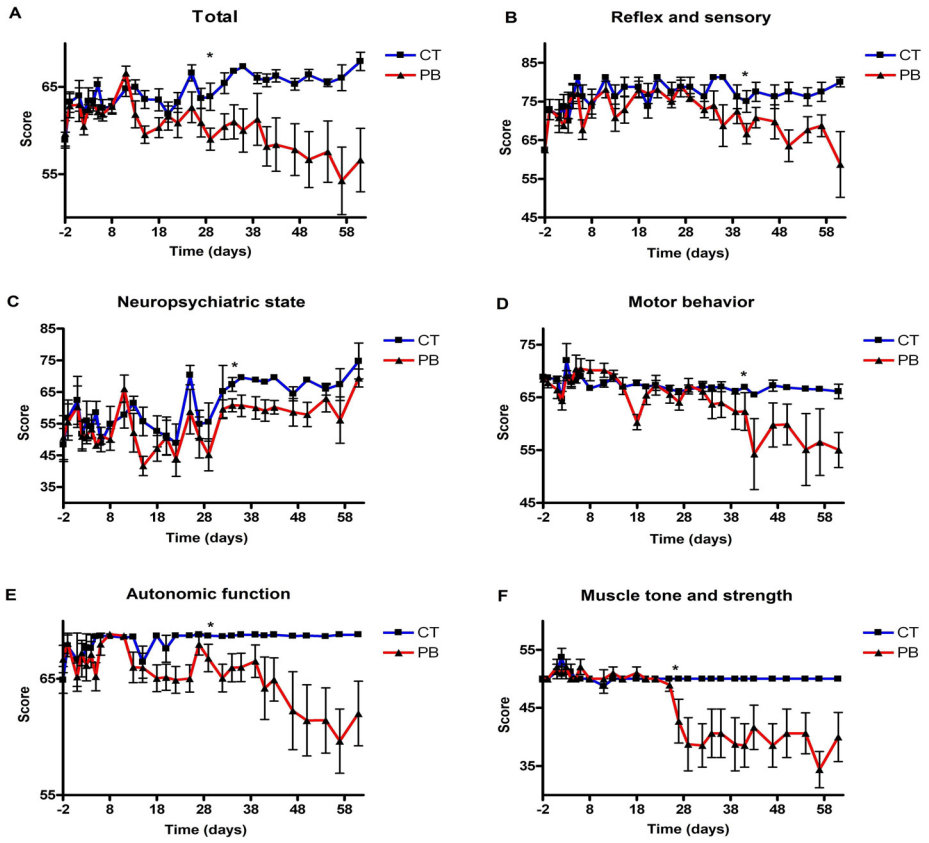

Figure 2: Visualization of performance of control mice (CT, $n = 10$) and Pb infected mice (PB, $n = 10$) in the five distinct functional categories of SHIRPA battery (B: reflex and sensory function; C: neuropsychiatric state; D: motor behavior; E: autonomic function; F: muscle tone and strength) up to 60 days of infection. Overall scores of the functional categories are shown in panel A.

few works focusing on the biology of this severe complication of PCM. In order to provide means for a better understanding of NPCM, we developed a murine model of the infection.

For this purpose, we infected, by intracranial route, seven groups of C57BL/6 mice with different inoculums of Pb (strain PB18) ranging from 10^1 to 10^7 yeast cells/animal. The intracranial route of inoculation is described for several murine models of mycotic (*Cryptococcus neoformans* [1], *Aspergillus fumigatus* [4], and *Scedosporium apiospermum* [3]) and non-mycotic infections of the CNS (*Streptococcus pneumoniae* [24] or HSV-1 [22,23]).

Mice were monitored for 60 days. Animals infected with 10^5, 10^6, and 10^7 yeast cells developed disease, expressed by weight loss, reduction in food intake, and behavioral changes. Survival curves demonstrated that mortality was related to the magnitude of the inoculum. Thus, the standardized dose was set to 10^6 yeast cells/animal, which represented the closest value to the one obtained by the Reed-Muench LD50 method. Interestingly, this is the same inoculum described for other experimental models of systemic or pulmonary PCM [2].

After the definition of the inoculum and the route of infection, we were able to set the experimental model and characterize aspects related to morbidity and pathology. Morbidity was evaluated by SHIRPA, a well established battery of behavioral tests designed for phenotypic characterization of mice [21]. The experimental infection was capable of inducing behavioral changes in mice that were classified in five functional categories, including neuropsychiatric state, motor behavior, autonomic function, muscle tone and strength, and reflex and sensory function. We were able to observe progressive and severe compromise of each of the categories over the course of the infection. Mice exhibited alterations in all the five domains, which is in accordance with the heterogeneous neurological symptoms observed in human patients with NPCM [17,18].

Intracranial Pb infection was also capable of inducing histopathological changes similar to the nodular lesions classically described for human patients with NPCM [15]. The presence of Pb with typical morphology was revealed

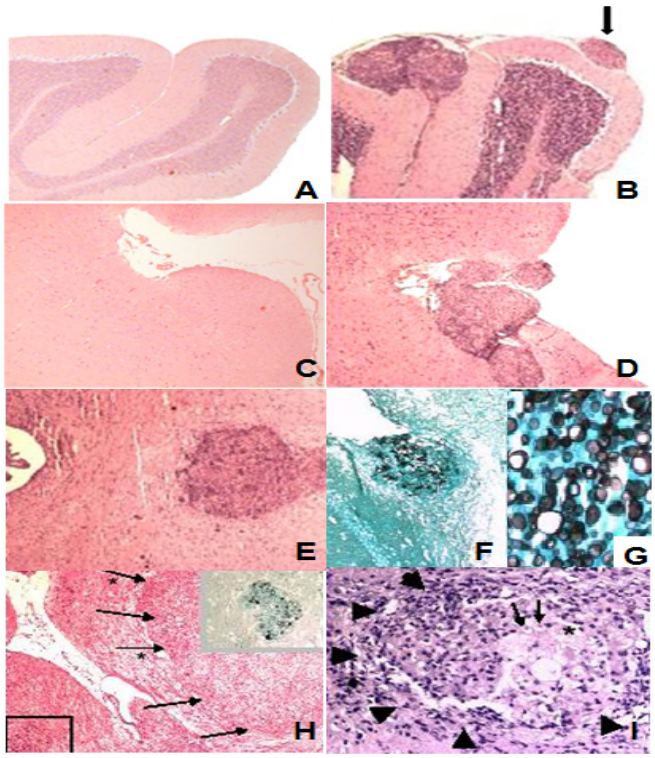

Figure 3: Histological observation of the brains of C57BL/6 mice infected with 10^6 yeast cells of Pb, strain Pb18, by intracranial route after 4 and 8 weeks. Tissue sections were stained with hematoxylin and eosin (A–E and H–I) and with Gomori's methenamine silver nitrate (F–G, insert H). Control tissue from cerebellum (A) and brainstem (C). Note the formation of nodule in the cerebellum (B) (arrow) and brainstem (D–E). Presence of nodular inflammation (fine arrows) containing *P. brasiliensis* (insert) (H). Presence of necrotic area with fungal yeast cells (asterisk) (I). Fungal cells with typical morphology (G). A–D, F–H (inset): ×40; E, H: ×100; I: ×400; G: ×1000.

by Groccott's staining. The lesions as well as severity of disease evolved over time. Dissemination of the lesions to other sites of the brain was also observed, including cerebellum and meninges. Cerebellum involvement is reported in human NPCM in 28.8% of the cases and meningeal compromise is observed in 10.6% of the cases [17].

There is evidence that Pb produces a melanin-like pigment in the presence of L-3, 4-dihydroxyphenylalanine (l-DOPA) *in vitro* [6,8]. Melanization protects the fungus from phagocytosis and increases its resistance to antifungal drugs, such as amphotericin B, ketoconazole, fluconazole, itraconazole, and sulfamethoxazole. The first choice in the treatment of NPCM is generally the association of sulfamethoxazole-trimethoprin while other drugs are reserved for intolerance or resistance to sulfonamides [18]. Thus, considering the great disponibility of l-DOPA in the CNS, this fact may play a role in the pathogenesis of NPCM and may influence its treatment. To our knowledge, there are no studies addressing this question.

The development of a murine model of NPCM is of great importance, since it provides the possibility to study the *in vivo* interactions of Pb and the CNS and the role of genetic, immunological, or therapeutic variables on the clinical outcome of NPCM.

5 Conclusion

The murine experimental model of NPCM was developed by the intracranial inoculation of 10^6 yeast cells of PB18/mice. Despite the limitations of experimental models, they may provide useful mechanistic and therapeutic insights. With the development of this model, future studies will be able to evaluate several aspects of the pathology and treatment of the disease in order to improve survival or lessen morbidity of this severe complication of PCM.

Acknowledgments This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Brazil.

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