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Original article

Histopathologic and immunologic effects of the itraconazole treatment in a murine model of chronic pulmonary paracoccidioidomycosis

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Abstract

A comparative study, based on histopathologic findings (inflammation, cellularity, and fibrosis) and immunologic parameters (pro-inflammatory and anti-inflammatory cytokines), was carried out in order to evaluate the effects of itraconazole (ITC) treatment and its starting time in a BALB/c murine model of chronic pulmonary paracoccidioidomycosis (PCM), induced by intranasal inoculation of *Paracoccidioides brasiliensis* (Pb) conidia. Two different groups of mice were exposed to ITC therapy beginning at the 4th or 8th week after Pb infection, respectively. ITC was administered daily, via gavage, for a period of sixty days. At weeks 0, 4, 8, 12 and 16 the animals were sacrificed and their lungs removed for histology staining with hematoxylin and eosin (H&E), Masson's trichromic and Gomori–Grocott; pulmonary levels of IL-1β, TNF-α, IFN-γ, IL-13 and TGF-β were also measured by ELISA. The development or absence of the principal pulmonary PCM sequela, lung fibrosis, was directly related to the therapy's starting time. This and other histopathologic findings were related to the behavior of cytokine levels.

Keywords: Paracoccidioidomycosis; Cytokines; Itraconazole; Lung fibrosis

1. Introduction

Invasive fungal infections are one of the major causes of morbidity and mortality, representing a serious and growing public health problem, not only for immunocompetent but also for immunocompromised patients, including those treated with corticosteroids, receiving organ transplants, or diagnosed with HIV [1–3].

Paracoccidioidomycosis (PCM) is one of the most important and prevalent human endemic and systemic fungal diseases in Latin America, mainly in Brazil, Colombia and Venezuela [4], where an estimated 10 million people are infected with the fungus. PCM is a progressive and chronic disorder initiated by the inhalation of infectious airborne propagules (conidia), which are produced by the mycelial form of the thermo-dimorphic fungus *Paracoccidioides brasiliensis*. These propagules change into the pathogenic yeast form when they reach body temperature [2,4].

PCM has three major clinical forms, an asymptomatic form (infection) observed in healthy individuals infected with Pb, and two progressive disease forms, the acute or sub-acute juvenile type, and the chronic or adult type. The chronic form represents 90% of all cases and is observed mostly in adult males in whom the disease may take months or years to fully develop [5,6].

The primary infection takes place in the lungs and subsequently disseminates to other organs and tissues, where it is recognized by the appearance of secondary lesions in mucous

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membranes, skin, lymph nodes, adrenal glands, and other tissues [2,5,6]. In the lungs, Pb causes chronic parenchymal damage leading to fibrosis, which severely restricts respiratory function; such pathology is observed in as many as 60% of the patients with this disease [2,7].

A number of studies have shown that the acute or juvenile form of the disease correlates highly with a Th2 type immune response, with high levels of cytokines IL-10, IL-4 and IL-5, as well as peripheral blood eosinophilia and low levels of Th1 cytokines. In patients with the chronic adult form, there is a similar tendency towards diminishing Th1 immune response, but without polarization towards a Th2 immune response [8–12].

Soares et al. [8] showed that in patients with the chronic form, lung lesions had high levels of both TNF-α and TGF-β; after 20 days of antifungal treatment, TNF-α levels decreased while those of TGF-β remained high, and these findings appeared to be associated with the presence of fibrotic areas in the lungs. It was also found that in healthy infected controls, the levels of some Th1 cytokines were high, including IL-12, IL-2, TNF-α and IFN-γ [8,9].

It is well known that cytokine profiles strongly influence the functioning of cells of the monocyte–macrophage lineage, which are the first to interact with both inhaled conidia and recently transformed yeast cells [10,12–15].

As it is impossible to detect the precise moment when the infection occurs in humans, because the fungal niche is unknown [4], our group developed a murine model of PCM two decades ago. In this model, BALB/c mice are inoculated intranasally with Pb conidia, imitating the natural occurrence in humans, with the objective to know and understand the total course of the developing disease [7,16–18].

Using this experimental model, we observed that during the early stages of infection, i.e., the first 72 h, an acute inflammatory process takes place, involving mainly polymorphonuclear (PMN) cells and macrophages. A decrease in PMN cells starts in the following weeks, preceding the formation of granulomas composed of macrophages, epithelioid cells, multinucleated giant cells, and lymphocytes, all of which surround the yeast cells of the fungus. At the end of the granulomatous process, the accumulation of connective tissue and the production of collagen lead to the establishment of fibrosis, generating structural and functional alterations to the lungs [7,18,19].

In clinical practice, systemic fungal infections are commonly treated with azolic derivates such as ITC, which is frequently used and constitutes the treatment of choice for PCM [20,21]. Its mechanism of action inhibits the fungal C-14α demethylase, a compound that is required for the biosynthesis of ergosterol, an essential component of the fungal cell wall membrane [22,23]. ITC effectively controls active PCM by restricting fungal proliferation. However, Tobón et al. [24] showed that over 30% of PCM patients treated with ITC had lung fibrosis at diagnosis and had not cleared at the end of the treatment period; during a follow-up done after therapy, the fibrosis even developed *de novo* in some patients [24].

So far, there are only few published studies using *in vivo* experimental models to evaluate ITC therapy, and most of them focused mainly on the antifungal effect rather than the fibrotic process. Furthermore, in those studies the experiments were performed using inoculum of yeast cells, which are not the naturally infecting *P. brasiliensis* particles [25–28].

In order to better understand the influence of azolic treatment during the establishment of pulmonary PCM and the development of lung fibrosis, we evaluated the effect of ITC treatment on immune responses (pro- and anti-inflammatory cytokines) and pulmonary histopathology (inflammation, cellularity, and fibrosis) in our animal model, starting at two different post-infection times.

2. Materials and methods

2.1. Animals

A total of 250 isogenic BALB/c mice, 6–7 weeks old and 18–20 g in weight, were obtained from the Corporación para Investigaciones Biológicas (CIB) breeding colony and used in all experiments. Mice were kept under controlled environmental conditions, temperatures of 24 °C and 12-h light/dark cycle; they were supplied, *ad libitum*, with sterile food and acidified water (pH 2.5–3.0) in sterilized bottles, as well as clean bedding. The Animal Experimentation Ethics Committee of our institution approved and verified the fulfillment of the policies concerning handling and caring of animals.

2.2. Trial design

The mice were divided into 5 groups of 50 animals to be sacrificed at 0, 4, 8, 12 and 16 weeks post-challenge respectively. Each group was subdivided by treatment options, as follows: 10 negative controls, 10 *P. brasiliensis* infected mice (positive controls), 10 *P. brasiliensis* infected mice treated with ITC starting at the 4th week post-infection, 10 *P. brasiliensis* infected mice treated with ITC starting at the 8th week post-infection and 10 uninfected mice treated with ITC. Each of these subgroups was divided equally (5 + 5 animals) for immunologic and histopathologic analysis.

2.3. Fungal culture, conidia production and inoculum preparation

P. brasiliensis American Type Culture Collection isolate 60855, known to produce abundant conidia, was used in all experiments [29]. For conidia production, the mycelial phase of the fungus was grown on water-agar plates at 18 °C for 8 weeks. For each experiment, the infectious conidia were collected as follows: The plates on which *P. brasiliensis* cultures had been grown in the mycelial form were flooded with physiologic saline containing 0.85% Tween 20. The growth was scraped from the agar surface with a gauged loop. The suspension was transferred to a capped Erlenmeyer flask containing three layers of 6 mm glass beads. The flask was