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Experimental *Paracoccidioides brasiliensis* Infection Increases Apoptotic Rate in Thymus

Abstract

Many works have shown that immunosuppressive effects induced by systemic mycosis can be related with damage in primary lymphoid organs. Previous studies in our laboratory showed that *Paracoccidioides brasiliensis* was able to invade the thymus, inducing a severe atrophy. In this study, we evaluated the relationship between apoptosis and thymic alterations caused by P. brasiliensis in experimentally infected BALB/c mice. Histologically, it was observed a large number of cells showing nuclear condensation and karyorrhectic changes. By TUNEL technique, it was noticed an increase of apoptotic index during early stages of the infection. We believe that this augment could be involved in the immunosuppressive phenomenon frequently observed during the paracoccidioidomycotic infection in humans and experimental models.

Keywords: *Paracoccidioides brasiliensis*, thymus, thymic atrophy, TUNEL.

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Paracoccidioidomycosis, a disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*, is the most prevalent systemic mycosis in Brazil (Brummer *et al.*, 1993; Goldani & Sugar, 1995). As with other deep mycosis, cellular immune response is considered the main mechanism of defense against this fungus, but frequently a depression in this branch of the response is associated with disseminated forms of the disease (Castañeda *et al.*, 1988; Roblebo *et al.*, 1982; Silva *et al.*, 1981; Singer-Vermes *et al.*, 1993). Several mechanisms have been pointed out as responsible for this immunodeppression: deficiency in the antigen presentation and reduction of the T cells function (Teixeira *et al.*, 1987); presence of suppressor cells (Jimenez-Finkel & Murphy, 1988); a predominant Th2 response (Bernard *et al.*, 1997), and the tropism of this fungus for the lymphoid organs (Franco *et al.*, 1989).

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Considering that thymus is the local of development and maturation of T lymphocytes, the cells responsible for control of the immune response, it is plausible to suppose that an impaired function of this organ could contribute to the immunosuppression phenomenon. Previous works in our laboratory have shown that severe alterations occur in the thymus, mainly in the acute phase of *P. brasiliensis* infection, like a severe atrophy (Brito *et al.*, 2002). In this work, we investigated the apoptosis index in the thymus of experimentally infected mice, in order to aggregate new information to clarify the immunosuppression phenomenon frequently reported in paracoccidioidomycosis.

To further evaluate the effect of *P. brasiliensis* infection on the thymus, yeasts from 7 day-old cultures were collected. The fungal mass was suspended in phosphate-buffered saline (PBS), mixed twice for 10 s on a Vortex-mixer, centrifuged and double-washed in PBS. The concentration was adjusted based on hemocytometer counts. Specific Pathogen Free BALB/c male mice were injected intraperitonially with $5x10^6$ yeasts of *P. brasiliensis* or with PBS alone (control groups) and sacrificed at 1, 3, 5, 7, 14 and 28 days post-infection. For thymic index determination, mice were weighted and sacrificed. Thymus was removed, cleaned and also weighted. The organ index was calculated as the organ weight (g) x 100 / body weight (g). A severe thymic atrophy was detected in experimentally infected mice beginning at one-day post-fungal infection and peaking at day 7 (Figure 1). Although we could note the recovery of the thymus index in infected mice, it remained below the index observed in sham-inoculated animals.

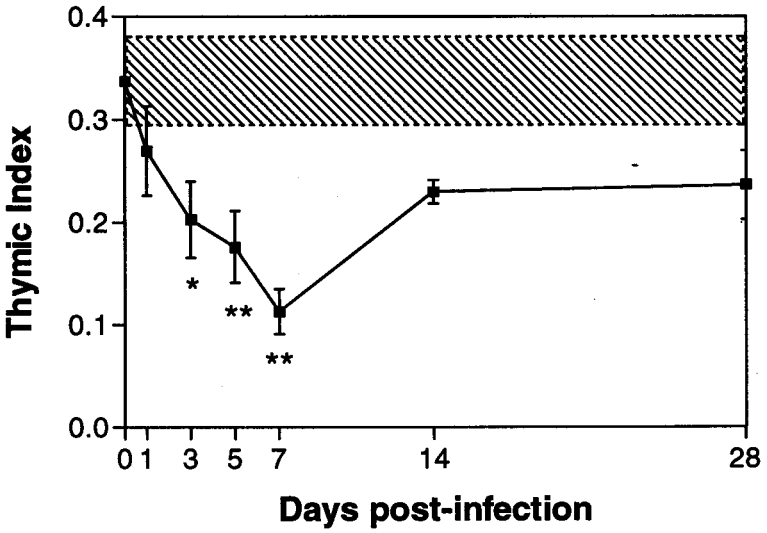

Figure 1. Kinetics of thymus index. Days 3, 5 and 7 in the infected animals are significantly different from the normal ones (*$p<0.05$, **$p<0.01$ by ANOVA test).

In order to analyze the cause of this atrophy, it was performed a histological study. Thymus was collected in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4μm, and stained with hematoxylin and eosin (HE) by routine histological techniques. Thymic morphological alterations run in parallel with weight loss. In cortical area, it was observed, as early as 24 h p.i., a huge number of cells showing nuclear condensation and karyorrhectic changes surrounded by histiocytes constituting a "starry-sky" pattern (Figure 2).

Since these alterations suggest the occurrence of apoptosis, this kind of cell death was investigated by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) technique. Sections were counterstained with methyl green and observed under a light microscope. Analysis by TUNEL showed an intense

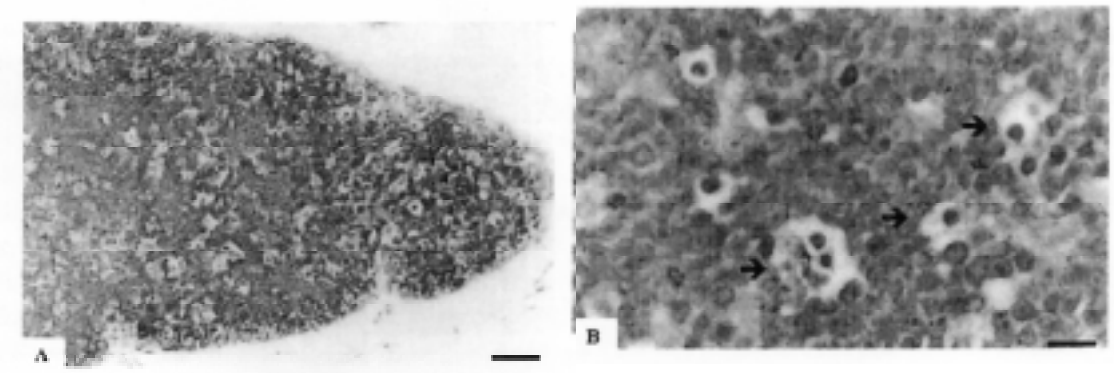

Figure 2. Thymus from *P. brasiliensis*-infected mice after 24 hours of infection. (A) Note thymic cortex with "starry sky" pattern. Bar =100μm. (B) Note cells showing nuclear condensation and karyorrhectic changes (➔). Bar = 10 μm. H&E stain.

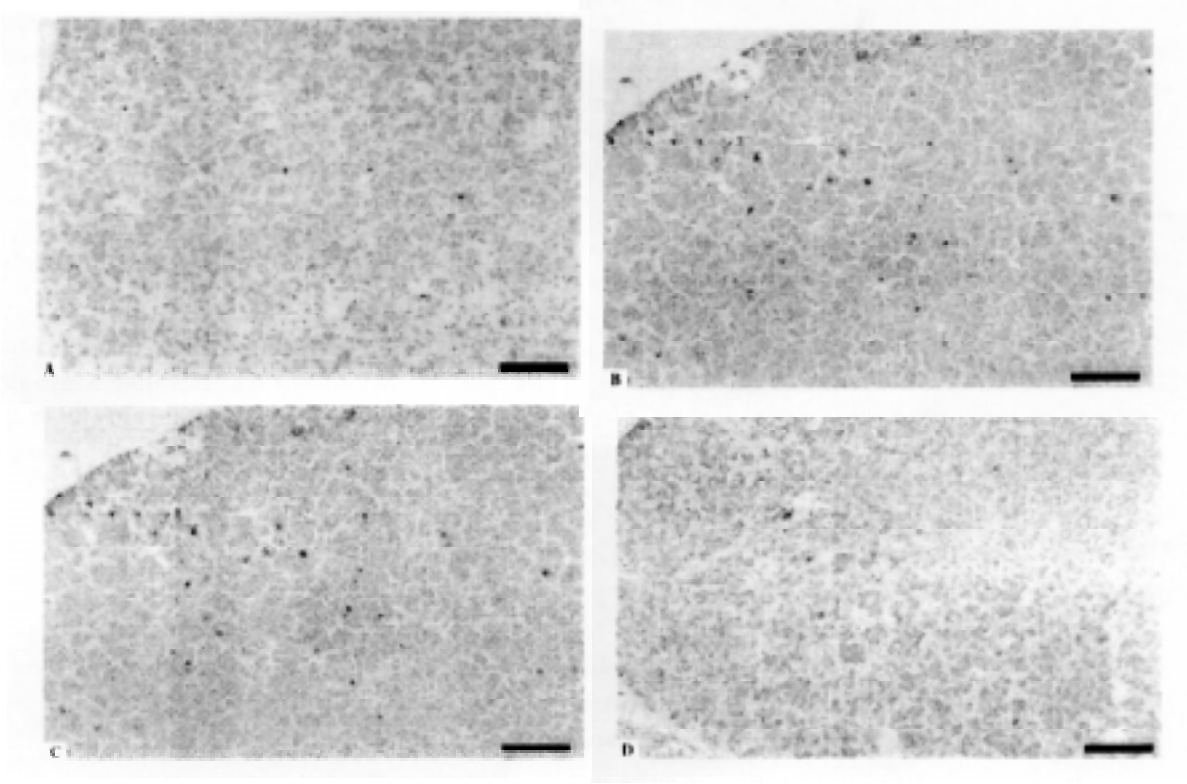

Figure 3. TUNEL performed on thymus. (A) Control. (B) 1 day after infection. (C) 7 days after infection. (D) 14 days after infection. Bar = 50μm.

relationship between thymic atrophy and apoptosis (Figure 3). It was observed an increase of apoptotic cells from 24 hours to 5 days post-infection.

In the last decade, some studies have disclosed that the thymus can suffer direct or indirect action from virus, fungi and bacteria that can harm the host immune response and contribute for the pathogenicity of these microorganisms (Price *et al.*, 1993; Sutton *et al.*, 1994; Alvarez *et al.*, 1995; Ozeki *et al.*, 1997; Islam *et al.*, 1998).

Some results gotten, as seem in previous work (Brito *et al.*, 2002), show that this fungus induces a severe thymic atrophy that takes place in the beginning of the infectious process. We assume that this atrophy is caused by apoptosis and could be related to the immunosuppression frequently observed during paracoccidioidomycosis (Silva *et al.*, 1981; Roblebo *et al.*, 1982; Brummer *et al.*, 1993; Singes-Vermes *et al.*, 1993).

Several mechanisms could lead to apoptosis in the course of paracoccidioidomycosis. Release of toxic substances by *P. brasiliensis* capable to modify the physiology of the thymus increasing the apoptotic rate could be a mechanism of atrophy as observed in other fungi infections. The gliotoxin, for example, a metabolite produced during pathogenic fungal infections is able to cause apoptosis in primary and secondary lymphoid organs (Sutton *et al.*, 1994). Another example is the toxin T-2, synthesized by Fusarium sp and other fungi, that present immunosuppressive properties inducing pronounced thymic atrophy and decreasing the numbers of T lymphocytes (Islam *et al.*, 1998).

On the other hand, thymic apoptosis caused by fungus-induced release of cytokines cannot be discarded. It has been demonstrated that TNFα is an important mediator during *P. brasiliensis* infection (Figueiredo *et al.*, 1993; Karhawi *et al.*, 2000; Souto *et al.*, 2000). Some reports, however, show that it acts directly on thymocytes as a potent inductor of apoptosis (Patiño *et al.*, 2000; Ozeki *et al.*, 1997; Isogai *et al.*, 1996). Ozeki *et al.* (1997), for instance, observed that mice inoculated with Mycobacterial Cord Factor presented profound thymic atrophy dependent of TNFα, since its absence reduces the atrophy and the apoptosis rate. Furthermore, Isogai *et al.* (1996) demonstrated that the administration of LPS induces apoptosis in thymus and raises the level of TNFα in the serum of experimental animals.

To summarize, our results show that the *P. brasiliensis* infection causes increase in the apoptotic rate. Since the literature has shown that this fungal infection causes an immunosuppression state in both humans and experimental models, it is reasonable to assume that the increase of apoptosis in thymus could be related to the immunosuppression. However, more studies are necessary in order to understand the precise mechanisms involved in this thymic atrophy and in attempt to explain the relationship among this process and the immunosupression phenomenon.

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