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The oral route in the pathogenesis of paracoccidioidomycosis: An experimental study in BALB/c mice infected with *P. brasiliensis* Conidia *

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Abstract

Due to the high frequency of oral mucosal lesions observed in paracoccidioidomycosis patients, it was advocated that the infection was acquired by the traumatic implantation of the etiologic agent *Paracoccidioides brasiliensis.* Although at present this theory is considered invalid, it has not yet been excluded in experimental studies. In order to determine if intra-oral inoculation could explain the pathogenesis of paracoccidioidomycosis, 64 BALB/c mice were inoculated intra-orally with 850.000 viable *P. brasiliensis* conidia into the mandibular body. Animals were sacrificed at various time intervals up to 20 weeks and cultures were made from gingiva, lungs, spleen, and liver. Additionally, histopathological studies of the mandibular body were also performed. *P. brasiliensis* was isolated from all gingival tissues during the interval 24–72 h, indicating that the infection was active. During the 5–10 week period, the infection appeared to have been controlled at the inoculation site as cultures showed a significant reduction in colony forming units (CFU); however, at the 15–20 week period such control was lost and the fungus was recovered once more. Dissemination to other body sites was rare; thus, the lungs were involved in just one animal (2%), the liver in two (3%) and the spleen in seven (11%).

The infection became established as proven by positive organ cultures, but the dissemination pattern did not correspond to the one observed in humans. Based on these findings, the intra-oral traumatic route does not appear to mimic the natural history of paracoccidioidomycosis.

Key words: experimental paracoccidioidomycosis, pathogenesis, intra-oral route

Introduction

Paracoccidioidomycosis (PCM) is a systemic disease, manifested mainly by pulmonary involvement and lesions of the oral mucosa, and less frequently by pathological manifestations of the lymphatic nodules, the adrenal glands, the skin, the spleen and the liver [1–4]. The mycosis is restricted to Latin American countries, from Mexico to Argentina [1, 4]. The etiologic agent, *Paracoccidioides brasiliensis*, is a thermically dimorphic fungus with an as yet undefined habitat [1, 4]. Since the disease was first described in 1908, Lutz placed special emphasis in the oral manifestations [5]; further studies described involvement of the mucosa in 63% of the cases [1]. These lesions are usually ulcerated, have a mulberry-like appearance, and often produce dental exfoliation [1, 6–8]. Although pulmonary involvement is observed in a larger proportion (85%) of the cases [9, 10], symptoms such as cough and dyspnea, are nonspecific and do not suggest the diagnosis [9, 10]. Infection by *P. brasiliensis* is characterised by long latency periods; this has been especially evident in patients reported in non-endemic areas [1, 4], in whom the disease appeared 30–40 years after visiting the endemic regions [1, 11, 12]. The frequent and notorious involvement of the oral mucosa, as well as the lack of knowledge on *P. brasiliensis*' habitat, has led to postulate a series of hypothesis on the possible route of infection. By the late 1940's, the traumatic route was advocated as the origin of infection [4, 13]; it was believed that inoculation occurred because the patient

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had the habit of chewing vegetable residues and used plant fragments as toothpicks [2, 4, 5]. However, early in the 1950's, researchers began to consider the aerogenous route of infection. González-Ochoa based on the study of the Mexican cases who had a high frequency of pulmonary involvement, as well as on the fact that in several patients there was initially lung involvement followed by extrapulmonary lesions, he proposed the aerogenous route as the natural form to acquire paracoccidioidomycosis [13–15].

Experimental animal models allow a rational approach for the understanding of the basic mechanisms of fungal pathogenesis. Additionally, in 1986, Restrepo et al. succeeded in releasing the conidia produced by *P. brasiliensis* mycelia and this permitted the use of the most likely infectious particle for animal experimentation [16]. Furthermore, conidia can be quantified, and the viability determined. On the other hand, the mycelium is poorly adapted to standardization.

McEwen et al. inoculated BALB/c mice via the aerogenous route with conidia, and demonstrated that *in vivo* conidia transformed into yeast cells. Pulmonary involvement was demonstrated in 98.8% of the animals by means of cultures; dissemination to the spleen and liver occurred in 35% and 8% of the cases, respectively [17]. These figures are quite similar to those found in human autopsy cases [4, 8], and correspond to clinical findings [9, 10]. Presently, the aerogenous route is accepted as the most likely route of infection; however, the oral-traumatic route has not been totally discarded [3, 6]. Recent studies on the results of oral trauma in the dissemination, have focused on immune response within the tissues rather than in the pathogenetic aspects of the mycosis [18–20].

The objective of this study was to evaluate the role of the intra-oral traumatic route in the pathogenesis of this mycosis by inoculating viable *P. brasiliensis* conidia into the mandibular body, and observing the occurrence of dissemination in various deep organs at different times post-challenge.

Materials and methods

Animals

64 BALB/c mice 8–12 week old, of either sex, and from the Corporación para Investigaciones Biológicas' breeding colony were used. Animals were fed *ad libitum* with a commercial sterile concentrate and with acidified water [21].

Production of conidia and preparation of the inoculum

Conidia were dislodged from the parental mycelium of ATCC 60855 *P. brasiliensis* isolate, according to techniques already described [16, 17]. All the procedures were carried out in a safety hood used exclusively for this fungus. The total number of conidia was determined by counts in a Neubauer chamber. The viability of the inoculum was established by the fluoresceine diacetate ethidium bromide test [22]. In this experiment, a viability of at least 90% was required. The inoculum was adjusted to 100% viability at the expense of increasing the suspension volume, in order to obtain an inoculum with 850.000 viable conidia suspended in 0.1 mL of PBS buffered solution.

Anaesthesia

Prior to the inoculation of conidia, intraperitoneal anesthesia was administered with a mixture of 2.3 mg of ketamine (Ketalar, Park Davis) and 0.3 mg of xilazine (Rompun 2%, Bayer) diluted in 0.1 mL of saline solution.

Intra-oral inoculation

The intra-oral inoculation was done in the mandibular body at the mesial aspect of the first right molar, approximately 4 mm from the gingival margin. The inoculum was injected with a syringe equipped with a 26 gauge needle assembled in a microdispenser (Tridek stepper, Chaney adapter, Thomas Scientific, N.Y). All inoculations were done within a safety chamber and by the same researcher.

Sacrifice

Animals were sacrificed under deep anesthesia with ether, at 24, 48, and 72 h, and then, at weeks 2, 5, 10, 15, 20. For each period, 8 mice were used and their liver, spleen, and lungs were obtained for cultures, as well as Gingiva in 3 animals of each group. Likewise, right hemimandibulectomy was practiced in 5 mice per group for the histophatologic evaluation.

Histopathology

The mandibular bone was decalcified in 5% nitric acid for 36 h and then washed with sterile water for 24 h. Coronal cuts of the mandibular body were performed just at the inoculation site in order to evaluate the external cortical bone, cancellous bone, and periodontal

space. Sections were stained with hematoxylin-eosin and silver methenamine.

Cultures

The various organs (gingiva, lung, spleen and liver) were weighed and homogenized in a tissue blender in 2000 μL of PBS solution; this suspension was then transferred to a test tube and used to prepare further dilutions (10^{-1}, 10^{-2}). One hundred μL of each dilution were used to seed duplicate Petri dishes with Sabouraud dextrose agar medium (Mycosel, BBL). Cultures were incubated at 18 °C for four weeks. After this period, the number of colonies were counted and expressed as $\log^{10}$ of colony forming units (CFU) per gram of tissue.

Statistics

Results were analyzed using the *Statistica* software (StatSoft^{TM}). One way multifactorial variance tests (Anova/Manova) and the Scheffé tests were used.

Results

Gingiva

As shown in Table 1, *P. brasiliensis* was isolated from the gingiva of all mice at intervals of 24–48 h (3.05 $\log^{10}$ CFU/g). Macroscopically, friable tumors involving all the mandibular body space were observed at 72 h in all animals. At later periods, variations in the number of CFU/g occurred not at random, but in a time-dependent manner ($P = 0.000063$). At week 2, partial control of the oral focus began to be noticed as CFUs had diminished (1.88 $\log^{10}$ CFU/g), and at the same time, a rubber-like tumoral lesion that deformed the mouse's cheek was found in all mice. During the 5–10 week interval, the infection continued to recede with significantly lower counts (0.48 $\log^{10}$ CFU/g) (P =0.0008); no tumor was found macroscopically, and the gingiva lesions had, at this point, a cicatricial aspect in all the animals. However, during the 15–20 week interval, CFUs increased again (1.51 $\log^{10}$ CFU/g) and no statistically significant difference was found with those figures recorded during the initial experimental period (24, 72 h). The gingiva was again macroscopically friable in 10 of 16 animals.

Table 1. Isolation of *P. brasiliensis* from the gingiva in 24 mice inoculated with *P. brasiliensis* conidia by the intra-oral route

Time of sacrifice post-inoculation	No. of animals sacrificed	Mean CFU/g ($\log^{10}$)
24 to 72 h	9	3.05
2 weeks	3	1.88
5–10 weeks	6	0.48
15–20 weeks	6	1.51

Dissemination

Systemic dissemination from the oral focus was present in 7 animals (11%) (Table 2). At 2 weeks post-challenge, the lung and also the spleen were involved in one mouse. Additionally, the spleen was involved in other 6 animals (11%). The dissemination occurred at 72 h in one mouse, at weeks 2 in two mice and in week 10 in, again, one animal. At week 20, spleen dissemination was noticed in another 3 animals. Dissemination to the liver occurred in 2 cases (3%), concurrently with the presence of fungal lesions in other organs, such as the spleen.

Histopathology

The transformation of conidia to yeast cells could be observed in tissues up to 48 h after inoculation (Figure 1). In the first 24 h post-inoculation the inflammatory infiltrate was predominantly compound by polymorphonuclear leucocytes. 72 h after inoculation, microabcesses with polymorphonuclear leucocytes in the center surrounded by fibrotic tissue infiltrated with inflammatory mixed cells (lymphocytes, histiocytes and plasmocytes) were found (Figure 2). By week 2 formation of epithelioid granulomata was seen (Figure 3). By week 10, the tissue reaction had diminished; however, it increased again in weeks 15–20. The presence of osteoclasts was observed only in 1 of the 40 animals (2.5%) that were studied histopathologically.

Discussion

Due to the fact that *P. brasiliensis*' natural habitat has not been precisely defined, the natural route of infection continues to be a matter of debate. Moreover, the role of trauma in the pathogenesis of this mycosis remains incompletely understood [9, 10, 13, 14, 23].

Table 2. Internal Organ Dissemination in BALB/c mice after intra-oral inoculation of *P. brasiliensis* conidia

Time of sacrifice Post-inoculation	No. of animals sacrificed	Extra-oral dissemination		No. (%) organs involved		
		Absent	Present	Lung	Spleen	Liver*
24 to 72 h	24	23	1	0	1	1
2 weeks	8	6	2	1	2	1
5 to 10 weeks	16	13	3		1	0
15 to 20 weeks	16	13	3	0	7	2
Total (%)	64 (100)	57 (89)	7 (11)	1 (2)	7 (11)	3 (3)

*Dissemination occurred simultaneously with that of the spleen.

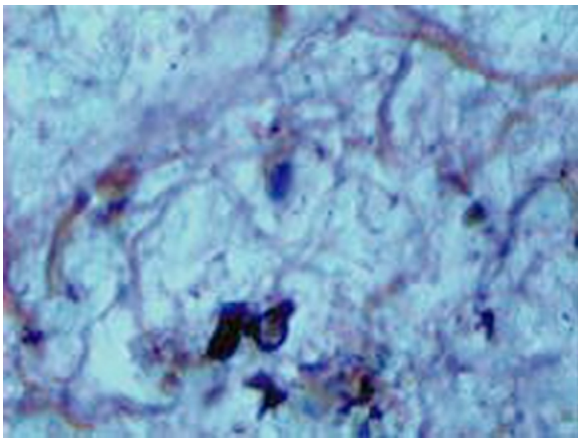

Figure 1. Presence of yeast cells 48 h after inoculation with *P. brasiliensis* conidia (silver methenamine; 100×).

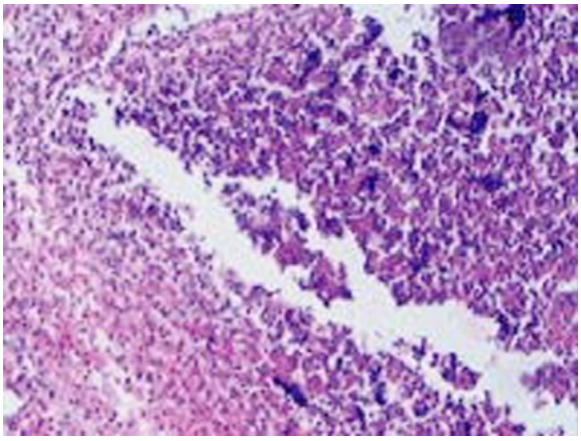

Figure 2. Gingiva 72 h after inoculation with *P. brasiliensis* conidia. Microabcess with polymorphonuclear leucocytes in the center surrounded by fibrotic tissue infiltrated with inflammatory mixed cells (hematoxylin and eosin; 100×).

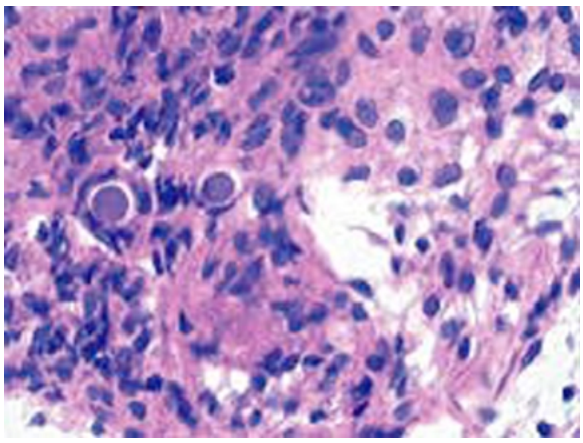

Figure 3. Gingiva 2 weeks after inoculation with *P. brasiliensis* conidia. Epithelioid granulomata with ingested balstoconidia (hematoxylin and eosin; 400×).

In former studies, *P. brasiliensis* yeast cells had been injected traumatically [20]; however, by doing so, the natural history of paracoccidioidomycosis was not reproduced as the conidium-to-yeast transformation was avoided, thus favoring proliferation of the tissue form. In the present study, the most likely infectious propagules, the conidia, were inoculated into the oral mucosa of BALB/c mice. This type of inoculum allows to both assess their viability and to determine their quantity so as to deliver the same number to each animal, something that is difficult to achieve with mycelial fragments [17]. During the first 72 h post-infection, the inoculum not only remained viable at the inoculation site but multiplied as shown by the recovery of an important number of CFU per g of tissue (3.05 $\log^{10}$), thus showing that the experimental infection had been successful. After these earlier periods, from weeks 5 to 10, there

was a tendency towards eradication of the fungus as shown by a significant ($p = 0.0008$) decrease in CFU/g (0.48 $\log^{10}$). However, the infection flared up in the gingiva at weeks 15–20 reaching figures (1.51 $\log^{10}$ CFU/g) which were similar to those observed during the initial post-inoculation periods. A similar behavior, this is, initial control of fungal multiplication and re-activation at later periods, has been recorded in experiments with conidia given by the aerogenous route [17]. The nadir was at weeks 1–2 post-inoculation, with counts within the same range at week 4, but with statistically significant increases up to 1000-fold at week 20 [17]. Somaglia-Albino [20] also observed the same phenomenon when inoculating *P. brasiliensis* yeast cells intratesticular in hamsters; indeed, 85% of the animals showed adequate cellular immune responses up to week 14, that resulted in control of the infection. However, at week 20, only 33% of the animals had retained the capacity to mount an immune response [20]. This particular deficit was thought to give rise to unrestricted fungal multiplication.

The significance of the traumatic route in the pathogenesis of paracoccidioidomycosis has not been clearly defined. In this study, dissemination to other organs occurred in 11% of the animals infected intraorally; the lung was involved in just one animal (2%), the spleen in 7 animals (11%), and the liver in 2 cases (3%). Arruda et al (1994), reported similar findings when studying the immune response to *P. brasiliensis* yeast cells inoculated in the hamster's cheek pouch. Dissemination occurred in 10% of animals but no indication was given concerning the organs involved during the process [18]. These researchers also inoculated animals in the footpad and found that by this route, dissemination was present in 36%. The explanation for this difference was attributed to the lack of lymphatics in the pouch in comparison to the rich lymphatic net of the footpad [18]. It was also suggested that probably dissemination could be due to the disruption of the in-site capillaries [18]. In this report, no reference to the role of the oral trauma in the pathogenesis of the disease was mentioned, as the main purpose was to evaluate the immunological parameters.

Back in 1959 [14], Mackinnon inoculated Swiss mice by different routes with *P. brasiliensis* yeast cells, and observed that after intramuscular inoculation in the extremities, dissemination occurred in one animal (6%), while inoculating the fungus in the tongue caused no dissemination. Recently, Somaglia-Albino [20] inoculated hamsters with *P. brasiliensis* yeast cells in the dental alveolus, 4 days post-extraction. Dissemination was observed in the liver (36%) and the spleen (2%). These results were comparable to the dissemination pattern obtained in the control group after intratesticular inoculation, as the liver was affected in 39% of the animals. The author concluded that disseminated paracoccidioidomycosis had been reproduced by the intra-alveolar inoculation of the fungus [20]. Although this author was indeed able to evoke the disease, the observed pattern of dissemination did not copy the one seen in humans. In contrast to our model, in the former study, yeast cells were used and the route employed was rather unusual and, furthermore, in the healing alveoli there was a rich-capillary bed that would probably make this model akin to those that use the intravenous or the intravascular routes.

The study of autopsies in humans has permitted a more realistic approach to the frequency of organic involvement in paracoccidioidomycosis. In Brazil, Montenegro et al. studied autopsies for 20 years, and reported the following rate of involvement: lungs 89%, oropharyngeal mucosa 54%, spleen 22%, and liver 28% [8]. This sequence is similar to the one observed in clinical studies. Brummer et al. reviewed three series of patients for a total of 352 and found that the lung was affected in 77%, the mucosa in 66% and the spleen and liver in 5% [1]. As it can be observed, patients exhibit a different pattern to the one found in animals infected traumatically.

Intranasal inoculation of *P. brasiliensis* in mice allows a more rational approach to the study of the pathogenesis of this mycosis. Mackinnon [14], when evaluating the different routes of inoculation, found that following intranasal infection, there was involvement of various organs quite like in human disease. This led him to propose that extra-pulmonary lesions corresponded to metastatic foci occurring after hematogenous dissemination. McEwen et al. [17], inoculated *P. brasiliensis* conidia in mice by the aerogenous route, and showed that pulmonary involvement occurred in more than 85% of the animals by histopathology , and in almost all of them (98.8%) by culture. Dissemination to the spleen and liver occurred in 35% and 8%, respectively [17]. This model mimicked human disease, and since then, the aerogenous route has been more widely accepted.

In the present study, presence of osteoclasts was observed in just one animal (2.5%). It has been shown that this fungus does not have a strong osteolytic potential and when bone involvement occurs, it is in highly irrigated zones such as the metaphysis, epi-

physis, and medulla [4, 24]. However, it is known that *P. brasiliensis* has a predilection for the periodontal and periapical tissues leading to dental exfoliation [3, 4, 6]. The various publications on the subject and the results obtained in this study, seem to indicate that periodontal lesions do not occur by direct inoculation of the fungus. However, reaching the bone by the hematogenous route may allow formation of an adequate niche where the fungus would then be able to thrive.

Traumatic inoculation of *P. brasiliensis* conidia into the gingiva resulted in sporadic dissemination to internal organs. Although paracoccidioidomycosis was established as a direct consequence of fungal inoculation in the oral region, the corresponding pattern of dissemination does not match the pathogenic traits commonly observed in human disease. Consequently, oral traumatic implantation does not seem to represent the natural route of infection by *P. brasiliensis.*

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