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Alterations in the pathogenicity of one *Paracoccidioides brasiliensis* isolate do not correlative with its *in vitro* growth

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Abstract

The *in vitro* subcultivation of some microorganisms for long periods causes measurable loss of their pathogenicity, which can be reverted by reisolation from infected hosts. We compared the pathogenicity and the *in vitro* growth pattern of one *P. brasiliensis* isolate (Pb 18) in its yeast phase, using the following samples: 1) The original pathogenic Pb 18 (OP). 2) Pb 18 attenuated by continuous *in vitro* subcultivation (AT). 3) Pb 18 (AT) reisolated from susceptible B10.A mice (RS). 4) Pb 18 (AT) reisolated from resistant A/SN mice (RR). Pathogenicity was evaluated by anatomopathology and mortality of mice infected i.p. with 5×10^6 fungi. Median survival times of mice infected with OP ranged from 74 to 117 days during the first 51 months of subculturing; with more cycles of subculturing the median survival time increased, reaching 250 days at the 64^{th} month. This indicated decreasing virulence of OP during this period of subculturing. Survival of mice infected with RS and RR was respectively 112 and 123 days, which is similar to the behavior of the OP variant. The *in vitro* growth curve profile of RR showed significantly higher numbers of total and viable yeasts than the other studied variant. These results show that: 1) Pb 18 isolate loses its pathogenicity by continuous subcultivation. This phenomenon is reverted by reisolation from mice, independently from their susceptibility to the fungus; 2) the *in vitro* growth patterns of Pb 18 do not correlate with alterations in pathogenicity but are influenced by the host's environment.

Introduction

Paracoccidioidomycosis is a deep mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The disease is endemic in Latin America, being the most prevalent deep mycosis in this geographic area [1].

Epidemiologic studies show that only a very small percentage of the exposed individuals develop symptomatic paracoccidioidomycosis [2]. When the mycosis is established, distinct clinical forms are observed: acute or subacute forms (juvenile type) or chronic forms (adult type) [3].

The wide range of clinical forms observed was ascribed mostly to characteristics of the patients that may reflect varying degrees of impairment of the immune system; conversely this can also be attributed to factors which are intrinsic to the fungus, since differences in physiological and biochemical characteristics among various *P. brasiliensis* isolates have been demonstrated [4, 5, 6].

Recently, we reported that different isolates of

P. brasiliensis in their yeast phase, showing roughly the same morphological and ultrastructural aspects displayed different *in vitro* growth curves patterns [7]. We also observed a dissociation between the *in vitro* behavior and the pathogenicity of these isolates, assayed in sensitive inbred mice [8].

A murine model of paracoccidioidomycosis was developed, in which polar forms of the disease were obtained by using inbred mouse strains infected intraperitoneally with a virulent *isolate (Pb 18) of Paracoccidioides brasiliensis.* Mice of the B10. A strain were found to be highly susceptible to the infection, since they presented progressive disseminated lesions in different organs and tissues, leading to the death of the animals after a relatively short time. On the other hand, mice of the A/SN strain were highly resistant to the infection, presenting benign, localised lesions and their survival times were similar to those of the non-infected controls [9]. This pattern was characteristic and reproducible, but after several months of *in vitro* subcultivation, a remarkable loss in the virulence of *P. brasiliensis* Pb 18 isolate was observed, to such an extent that this fungus isolate was almost non-pathogenic even to the susceptible mouse strain. In the present work we report a systematic follow-up of the decrease in the virulence of one Pb 18 isolate, assayed in B10. A mice, after 64 months of continuous *in vitro* subcultivation. This phenomenon was reverted after one passage either in susceptible or in resistant mice. We also analyse the *in vitro* behavior of the Pb 18 isolates subjected to these manipulations in order to verify if there is a parallelism between the *in vivo* degree of virulence and the *in vitro* growth characteristics.

Materials and methods

In vivo studies

Mice

Nine groups of 8–12, male 9–11 weeks old mice from the susceptible B10.A strain were used for the pathogenicity studies; another nine groups of mice were used as controls. Resistant A/SN mice of the same sex and age were used for the anatomopathological studies.

Fungi

Pb 18 *Paracoccidioides brasiliensis*, originally isolated from a patient in 1929, was a gift from Prof. C. Fava Netto. The isolate was maintained by weekly sub-cultivation in our laboratory since 1981, in the conditions described below.

Growth conditions

Semisolid Fava Netto's culture medium [10] was used to cultivate the fungi in their yeast phase, at 35 °C. The fungi were used on the 7th day in culture.

Infection of mice

The fungal cells were washed in saline, counted in an hemocytometer and the concentration was adjusted to 5×10^6 fungi in 0.5 ml. Mice were infected intraperitoneally (i.p.) with this dose. Viability of the fungic suspensions, determined using Janus Green B vital dye [11], was always higher than 80%. Saline was injected as controls.

Reisolation of Pb 18 from infected mice

Yeast cells obtained after 58–62 months of *in vitro* subcultivation were inoculated i.p. in either the susceptible B10.A or in the A/SN mice. Seven to eight months later the animals were sacrificed, autopsied under sterile conditions and various organs from the peritoneal cavity were collected, manually disrupted and seeded in semisolid culture medium containing antibiotics (Mycosel, BBL). After 2–3 passages in this medium the cultures were passaged in Fava Netto's medium four times at 7 days intervals before used in the pathogenicity and in the growth curves studies.

Pathogenicity studies

B10.A mice were infected with *P. brasiliensis* at months 0, 32, 35, 51, 55, 61 and 64 of *in vitro* subcultivation. Pb 18 variants reisolated from susceptible mice (RS) and from resistant mice (RR) were inoculated i.p. one month after reisolation. Pathogenicity was assayed by mortality

data. Deaths were registered daily for over 300 days. The medians of survival days post-infection were calculated.

Anatomopathology

A group of 30 resistant, A/SN mice was inoculated with *P. brasiliensis* obtained after 60 months of *in vitro* subcultivation. Another 30 A/SN mice were inoculated i.p. with Pb 18 yeasts obtained one month after reisolation. Ten mice from each group were sacrificed at 2, 8 and 16 weeks after the infection and their organs examined for gross paracoccidioidomycotic granulomata. The severity of the lesions was recorded using a semi-quantitative scale of 0 to 4 +, taking in account the number and size of granulomata found in each organ examined.

In vitro studies

Growth curves determination

The studies were carried out as described previously [7], using *P. brasiliensis* variants at months 23 and 70 of *in vitro* subcultivation and at one month after reisolation from B10.A or from A/SN mice. Briefly, tubes containing 6 ml of medium were seeded with 2×10^6 fungal units, that correspond approximately to the 5×10^6 fungal cells inoculated in the pathogenicity studies. The total and viable fungal units counts were determined from 3 randomly selected tubes. The mean counts of triplicates were used to graph the curves.

Preparation for light microscopy

Morphological studies under light microscopy were performed with yeasts of each Pb 18 variant at the point of maximum percentage of cell viability in the growth curve.

Statistical analysis

The median scores of the survival times, as well as of the peritoneal lesions were compared by a multisample median test followed by a multiple comparison test [12].

Results

Pathogenicity studies

The alterations in the virulence of Pb 18 *P. brasiliensis* isolate during continuous *in vitro* subcultivation as well as the recovery of virulence after one passage *in vivo* either in susceptible or in resistant mouse strain are shown in Fig. 1.

During the initial 51 months of *in vitro* cultivation, the median survival times of the infected B10.A mice ranged from 74 to 117 days post-infection. Virulence of Pb 18 was roughly constant during this period and the sample behaved as the original pathogenic isolate (OP).

After 51 months, the median survival times sharply increased, reaching 250 days or longer at the 64th month of *in vitro* maintenance. These data clearly indicate that a marked decrease or even loss of virulence had occurred ($P < 0.05$). This Pb 18 sample is referred to as attenuated Pb 18 (AT).

One passage of AT in either susceptible or resistant mice resulted in the recovery of the virulence at similar levels to that of the OP sample, the median survival times being 112 and 122 days, respectively.

Anatomopathology

Table 1 shows the cumulative percentage of mice presenting granulomata in different organs and

Table 1. Cumulative percentage of A/SN mice presenting granulomata after infection with two Pb 18 *P. brasiliensis* variants.

Organs/tissues examined	AT[a]	RS[b]
Intestinal mesentery	62.5	92.3
Spleen	54.2	92.3
Oementum	58.3	88.5
Diaphragm	29.2	92.3
Liver	29.2	46.2
Lungs	0.0	0.0
Abdominal muscles	0.0	61.5
Kidney	0.0	57.7

[a] AT: Pb 18 attenuated by continuous *in vitro* subcultivation.
[b] RS: Pb 18 reisolated from susceptible B10.A mice.

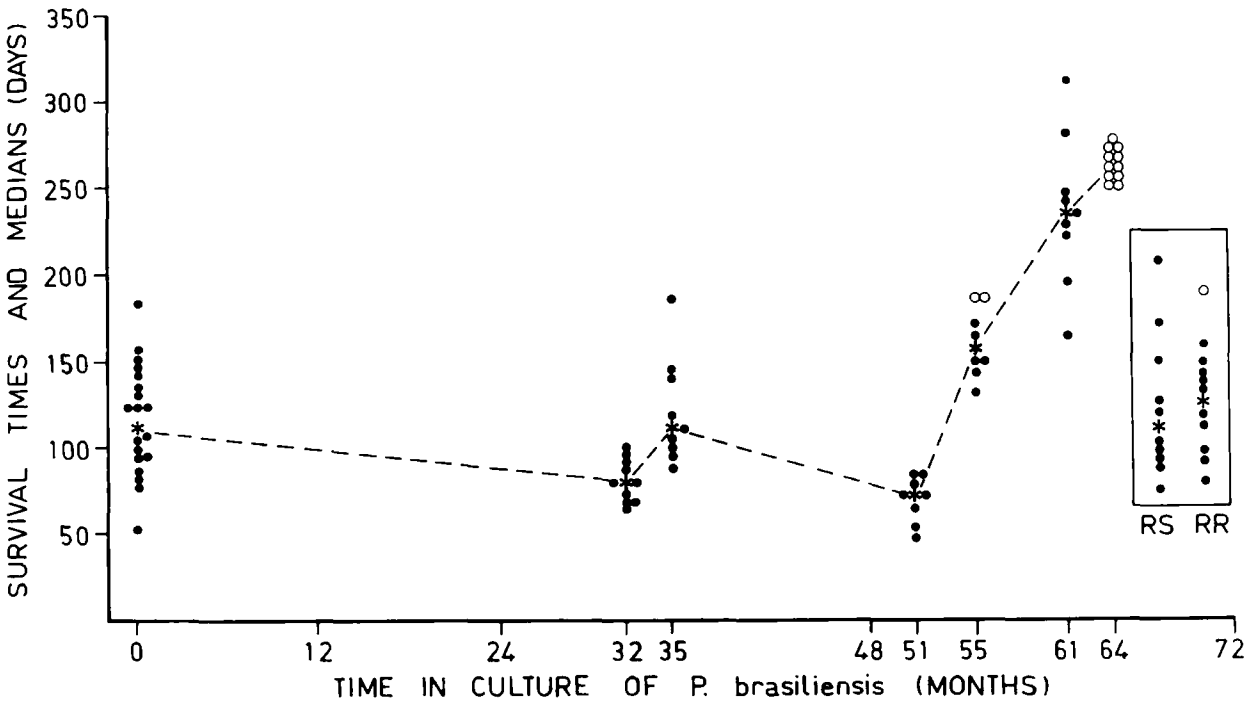

Fig. 1. Pathogenicity studies of mice injected with Pb 18 *Paracoccidioides brasiliensis* variants during continuous *in vitro* subcultivation and after reisolation from A/SN (RR) or from B10.A (RS) mice. *, median of survival days; ●, death; o, sacrifice.

tissues examined 2, 8 and 16 weeks after inoculation with Pb 18 *P. brasiliensis* variants. The percentage of animals displaying specific granulomata was higher in the RS infected mice when compared to the AT inoculated animals, in all organs examined except the lungs. Here, no lesions were observed. These data show that the infectivity of Pb 18 increased significantly ($P < 0.05$) after one passage in mice.

Table 2 shows the median scores of the intensity of lesions found in different organs and tissues of mice at 2, 8 and 16 weeks after infection with Pb 18 variants. The intensity of lesions of AT inoculated mice were always very weak or absent, independently from the time of the infection or the examined organ. On the other hand, the intensity of the lesions caused by the RS variant increased significantly in the course of the infection. When AT and RS infected mice were compared, there was no significant difference between the two groups up to the second week of infection; from the 8th week on, the lesions found in almost all the organs of the RS infected mice were significantly more intense than those of the AT groups. The exceptions were the lungs, that were unaffected in both groups and the liver, that seems to be affected later (16th week) in mice infected with the RS variant. These data show that RS infected mice have a progressive increase in the severity of lesions, in contrast to those infected with the AT variant.

Growth curves

Figure 2 shows the total and viable cell counts of the OP, AT, RS and RR variants. Both cell counts lead to similar results: the OP, AT and RS variants displayed similar growth curve profiles. In these variants we observed a slow increase in the number of cells; the average maximum cell counts ranging from approximately 19.5 to 37.9 $\times$ 10^6 fungal units, observed from the 12th to the 18th days in culture. The highest number of

Table 2. Median scores of the intensity of lesions found on different organs/tissues of A/SN mice infected with two Pb 18 *P. brasiliensis* variants.

Fungal isolate	Weeks after infection	Intestinal mesentery	Spleen	Oementum	Diaphragm	Liver	Lungs	Abdominal muscles	Kidney
AT[a]	2	1	1	1	0	1	0	0	0
	8	0*	0*	0*	0*	0	0	0*	0*
	16	1*	1*	0.5*	0*	0*	0	0*	0*
RS[b]	2	1	1	1	1	0	0	0	0
	8	3	3	3	2	0	0	1.5	1.5
	16	4	4	3	4	4	0	3	3

[a] AT: Pb 18 attenuated by continuous *in vitro* subcultivation.
[b] RS: Pb 18 reisolated from susceptible B10.A mice.
* Significantly lower than the RS data ($P < 0.05$).

viable cells was observed from the 8th to the 16th days and a progressive decrease in viability thereafter until the 20th day in culture.

The growth curve of the RR variant, on the other hand, had a completely different pattern: a rapid increase in total cell numbers reaching 142 x 10^6 fungal units at 16 days in culture was observed. The highest number of viable cells was reached at the 12th day, falling abruptely from day 18 on. The growth curves patterns seem not to be altered by the time of *in vitro* subculturing; on the other hand, there is an influence of the mouse strain used for reisolation on the growth curve profiles.

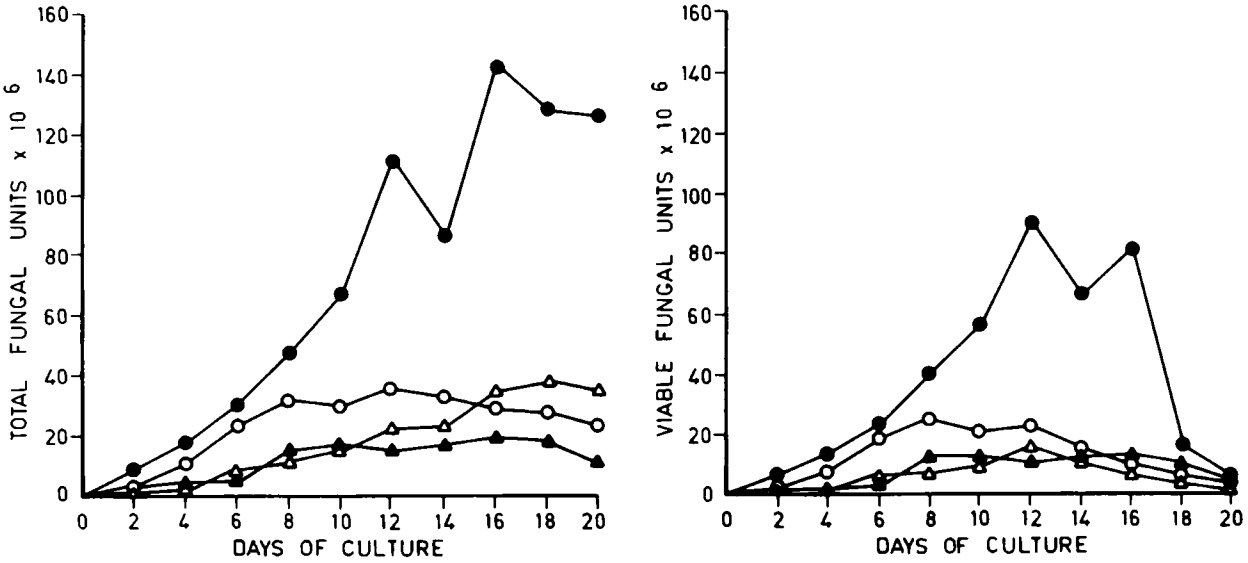

Fig. 2. Growth curves of Pb 18 *Paracoccidioides brasiliensis* variants during continuous *in vitro* subcultivation and after reisolation from mice. ▲, original pathogenic variant (OP); △, Pb 18 attenuated by continuous *in vitro* subcultivation (AT); ○, Pb 18 reisolated from susceptible B10.A mice (RS); ●, Pb 18 reisolated from resistant A/SN mice (RR).

Morphological studies

The OP, RS, and RR variants were studied on the 8th day in culture, while the AT variant was studied on day 12.

Examination under light microscopy of the Pb 18 variants revealed that all shared the basic characteristics already described for the Pb 18 isolate: the yeast cells were spherical, oval or pear-shaped, measuring 25–50 μ (short axis) and presenting one or multiple buds. No noticeable morphological alteration could be detected and associated with alterations in the virulence to inbred mice.

Discussion

After many years employing *Paracoccidioides brasiliensis* Pb 18 isolate to study the resistance and susceptibility of isogenic mice, we noticed a marked decrease in its virulence throughout continuous *in vitro* subculturing.

Similar events have been described with several microorganisms; it is likely that most such observations were made during the routine of laboratory work, not receiving a thorough analysis. The present paper reports a systematic follow-up of the variation in the virulence of Pb 18 isolate, and the study of its *in vitro* characteristics, in order to determine whether any correlation exists between these parameters.

The phenomenon of marked loss in virulence after *in vitro* maintainance has also been described in fungi causing deep mycosis. Isolates of *Coccidioides immitis* that have been subcultured *in vitro* for long time became less virulent to animals than the original virulent sample. The virulence of those attenuated isolates could be recovered by culturing in complex culture media or by a passage *in vitro* [13]. It was also observed that the maintainance of cultures of *Blastomyces dermatitidis* over long periods of time resulted in changes in fungal cell wall composition as well as in decreased virulence [14]. This has also been reported with the yeast form of *P. brasiliensis* [15, 16].

We report here a remarkably diminished virulence of Pb 18 *P. brasiliensis* isolate, assessed by the few numbers of paracoccidioidomycotic granulomata and much longer survival times of mice infected with this isolate.

The progressive loss in the virulence of Pb 18 reached such an extent that at the 64th month of *in vitro* subcultivation, this variant was non-lethal to susceptible mice. After reisolation, which was possible even though the sample was attenuated, Pb 18 recovered its lethality in levels comparable to those of the original isolate.

In order to confirm the recovery of virulence, resistant mice were also assayed. Since this mouse strain takes approximately 380 days to develop lethal disease, the presence of granulomata was used as a parameter. The anatomopathological findings were parallel to the mortality data. The reisolated variant caused a progressive and intensive dissemination of the lesions, in contrast to the attenuated variant. In spite of these different behaviors, both variants had similar target organs. These results are in agreement with those of Castaneda *et al.* [16] who showed that an animal-passaged *P. brasiliensis* isolate was more infective than the same isolate which displayed reduced infectivity after serial passage in media.

Concomitantly with our *in vitro* results, marked alterations in the behavior of Pb 18 were observed in *in vitro* culture. No correlation was observed between the *in vitro* growth profiles and the degree of virulence displayed by the Pb 18 variants.

These data are in agreement with those previously obtained [8] in which no correlation was found between the growth curves patterns of several *P. brasiliensis* isolates and their pathogenicity to inbred mice. This phenomenon occurs both when comparing different *P. brasiliensis* isolates and within a same isolate submitted to different environmental conditions.

The observations of the present work may be explained either by the selection of variants which possessed some competitive advantage or by the occurrence of certain modifications in the metabolic activity of the yeast cells; in both alternatives, the different environments to which the fungal isolates were exposed would play an im-

portant role. Phenotypic changes of microorganisms, resulting from changes in media composition are frequently described in the literature. It has been reported that *P. brasiliensis* grown in media supplemented with fetal calf serum suffered biochemical alterations (expressed as an increased synthesis of a cell-wall component) analogous to those induced by its inoculation in animals [17].

The effect of the host environment on the yeast cells metabolism must be overwhelming, but it is quite difficult to determine such influence experimentally *in vivo*. The gamut of physiological adaptations involved in the successful establishment of a deep mycosis should be extremely complex. In the present work there are indications that some factors of the host play a role in either selecting a variant or in inducing modifications in the *P. brasiliensis* yeast cells. When the attenuated Pb 18 variant was inoculated in A/SN and in B10.A mice for reisolation, the variants obtained from each one of the mouse strains had similarly recovered the original virulence. Such modifications had somewhat different effects over the fungus, since the *in vitro* growth curves of the two reisolated variants showed different profiles: the variant reisolated from A/SN mice presented a luxuriant *in vitro* growth, not observed in the variant reisolated from B10.A mice. These findings suggest that in order to survive within a host that is resistant to the infection, the yeast cells underwent metabolic modifications which also favored its *in vitro* growth.

Our data also suggest that good *in vitro* growth has no correlation with long time in culture, during which the fungus would adapt to these conditions, confirming previous results, obtained when we compared several *P. brasiliensis* isolates [7]. The best *in vitro* growth herein observed was attained by a Pb 18 variant very recently isolated from mice.

The fact that this particular *P. brasiliensis* isolate shows a significant variation in its *in vivo* and *in vitro* behavior has some interesting implications. The investigations which focus on immunogenicity, pathogenicity and fungal physiology will be affected by alterations such as those here reported; moreover, antigenic preparations of this *P. brasiliensis* isolate may vary in composition during such metabolic alterations. It remains to be elucidated what kind of interactions of the fungus with the external environment are involved in such modifications.

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