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In vivo and *in vitro* characteristics of six *Paracoccidioides brasiliensis* strains

Suely Sanae Kashino, Vera Lúcia Garcia Calich, Eva Burger & Lúcia Mary Singer-Vermes
Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 2415, CEP 05508, São Paulo – SP, Brasil

Abstract

The yeast-like forms of six *P. brasiliensis* strains were characterized and compared using *in vitro* (growth curve determination) and *in vivo* (pathogenicity to sensitive inbred mice) criteria. Strains Pb 18 and Pb 265 which behaved similarly *in vitro*, showing low counts of fungi and long mean generation times, were respectively the most and the least pathogenic strains. Strains Pb 2052 and IVIC Pb 267, which grow abundantly *in vitro* were, respectively virulent and avirulent. Strains Pb SN and IVIC Pb 9 behaved similarly both *in vitro* and *in vivo* displaying an intermediate pattern of virulence and growing conditions.

Introduction

Paracoccidioidomycosis presents multiple clinical aspects, ranging from benign to severe forms. The fungal agent, *Paracoccidioides brasiliensis*, disseminated by the bloodstream or the lymphatic system, can establish itself in any part of the host organism and the disease can be localized or disseminated. Healthy subjects living in endemic areas and having opportunity of contact with *P. brasiliensis* may develop clinically-active disease or paracoccidioidomycosis-infection (without disease) (1, 8).

The different clinical forms of the disease, or the occurrence of paracoccidioidomycosis-infection, may be due to host-related factors (e.g. sex, age, immunological status) as well as to characteristics of the infecting agent, mainly its virulence (1, 17).

Comparative studies of some strains of *P. brasiliensis* were made *in vitro* by several authors, by determining the growth curves of mycelial and yeast-like forms of the fungus in many culture media (2, 12–14, 16). Although these studies elucidated some biological aspects of different strains of *P. brasiliensis*, further investigations are necessary to better characterize the causal agent of paracoccidioidomycosis.

Using a murine model of intraperitoneal paracoccidioidomycosis, we showed that there are significant differences in susceptibility of inbred strains: the A/SN mice were found to be the most resistant while the $B10D_2$/nSn, B10.A and $B10D_2$/oSn mice, the most susceptible to *P. brasiliensis* (strain Pb 18) infection (4).

In the present report, genetically homogenous mice, sensitive to paracoccidioidomycosis (B10.A) were used to determine the virulence and the organ distribution of lesions elicited by six *P. brasiliensis* strains. The growth curves of the yeast-like forms of these strains were also established. In order to investigate fungal growth characteristics which might provide clues to the mechanism of pathogenicity in paracoccidioidomycosis, a comparison of the *in vivo* and *in vitro* patterns was made.

Materials and methods

Strains and growth conditions

Six strains of *P. brasiliensis* were used: Pb 18, Pb SN and Pb 265 are human isolates, obtained

from the fungal culture collection of the Departamento de Microbiologia, Universidade de São Paulo, Brasil; Pb 2052, recently isolated from a patient was a gift from Dr Arminda de Jesus Machado (Goiás, Brasil); IVIC Pb 9 and IVIC Pb 267 were kindly supplied by Drs Gioconda and Felipe San-Blas (Venezuela). Strain IVIC Pb 9 is a human isolate (15) and IVIC Pb 267 a chemical mutant of IVIC Pb 9 obtained after treatment with nitrosoguanidine (G. & F. San-Blas, personal communication).

The yeast cells of *P. brasiliensis* strains Pb 18, Pb 2052, Pb 265 and Pb SN were maintained in semisolid Fava Netto's medium (6) at 35 °C. IVIC Pb 9 and IVIC Pb 267 cells were grown in semisolid peptone-yeast-glucose medium (PYG) at 35 °C and 23 °C, respectively; the mutant strain IVIC Pb 267 grows well at this temperature, and not at 35 °C (G. & F. San-Blas, personal communication).

The cultures of each fungus strain were grown for eight days. The cell suspensions were washed three times in phosphate buffered saline (PBS), pH 7.2 and counted in an hemocytometer. Each cell was considered individually for the determination of pathogenicity, and for the *in vitro* studies fungal units (F.U.: mother cell plus attached buds) were considered.

The viability of all fungi preparations was determined using the Janus Green vital stain (3) and was always higher than 80%.

Growth curves determination

Each of the thirty tubes containing 6 ml of culture medium was inoculated with 0.1 ml of *P. brasiliensis* suspensions adjusted to 20.0×10^6 F.U. yeast cells/ml. This procedure was repeated for each *P. brasiliensis* strain. Three tubes were taken at 2 days intervals during 20 days after the initial inoculation. The entire growth of each tube was removed, washed three times, properly diluted, and total and viable counts were made. The mean value of each determination was recorded and the mean generation times were calculated on the basis of increment in the number of cells for all strains studied.

Mice

Eleven-week-old male B10.A mice, originally obtained from Jackson Laboratory (Bar Harbor, Maine) were used in all experiments. These mice were housed in the Departamento de Imunologia facilities and were given lab chow and water *ad libitum.*

Pathogenicity studies

Mice were infected intraperitoneally (i.p.) with the appropriate dose of *P. brasiliensis* contained in 0.5 ml of PBS.

For the fifty percent lethal dosis (LD 50%) estimations of each *P. brasiliensis* strain, groups of 8–10 mice were infected with at least five different doses of yeast cells. Concentrations ranging from 0.2×10^6 cells/ml to 34.0×10^6 cells/ml were used. A PBS inoculated control group was included in each experiment. The mice were kept in adequate housing conditions and deaths were registered daily over 200 days. The LD 50% was estimated by the probit method (7) taking in account the number of viable cells inoculated.

For the anatomopathological studies with each *P. brasiliensis* strain, groups of at least 20 mice were inoculated i.p. with 1.0×10^6 yeast cells. Six mice from each group were sacrificed monthly, the animals were autopsied and their organs examined for gross paracoccidioidomycosis granulomata.

Results

Growth curves

Growth curves showing total and viable cell counts of the six *P. brasiliensis* strains studied are depicted in Fig. 1. The growth curve patterns (total and viable cells) were parallel within each strain, with the exception of IVIC Pb 267 which presented a low number of viable cells from the 12th day on. Differences in the kinetics of growth among strains were observed and the mean generation times were markedly distinct, ranging from 21.27 h for Pb 2052 to 102.65 h for Pb 265 (Table 1).

The lag in growth is apparent during the first 48 h. All strains propagated actively from the 4th day of incubation on, when the exponential phase of growth started. This phase lasted for approximately 4–10 days, the maximum growth varying from 10.2×10^6 F.U. for strain Pb 265 to

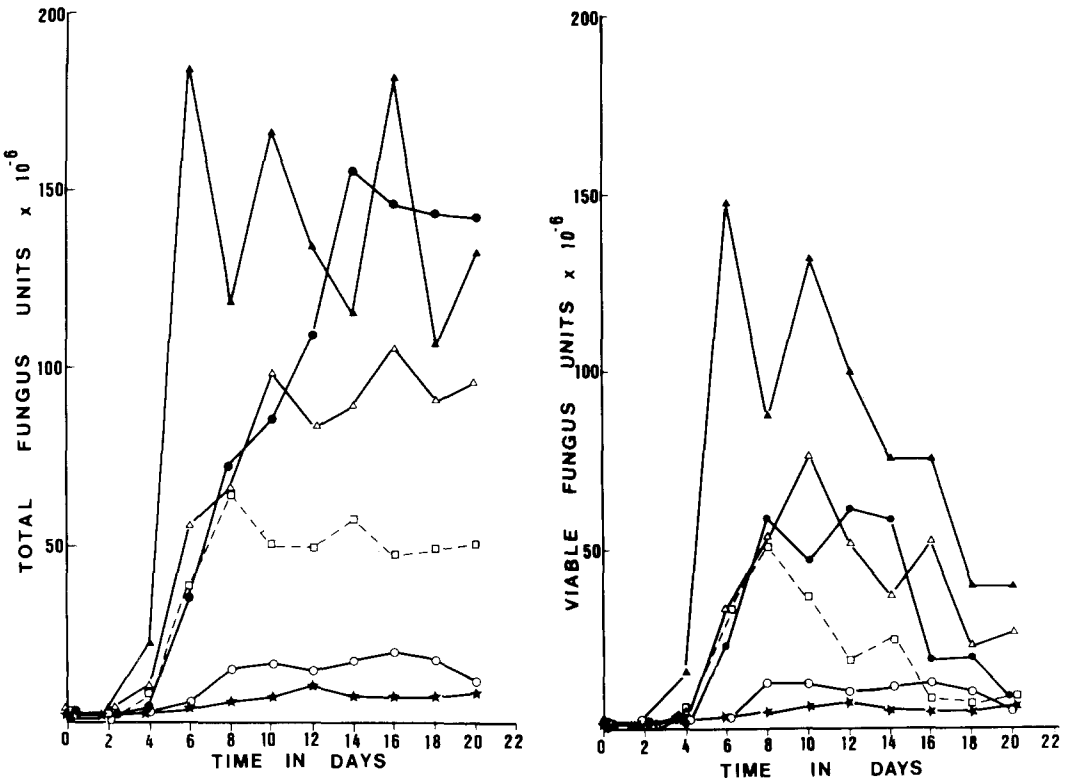

Fig. 1. Growth curves of the yeast forms of six strains of *P. brasiliensis:* Pb 265 (★———★), IVIC Pb 267 (●———●), IVIC Pb 9 (□ – – – □), Pb SN (△———△), Pb 18 (○———○) and Pb 2052 (▲———▲). Total and viable counts are shown, respectively on left and right side.

Table 1. Comparison among six *P. brasiliensis* strains in relation to mean generation times (MGT), 50% lethal dosis (LD 50%) and cumulated number of granulomata observed in infected B10.A mice.

P. brasiliensis strains	MGT (h)	LD 50%[a]	Cumulated number of granulomata[b]
Pb 265	102.65	no dose-response	1
IVIC Pb 267	48.50	no dose-response	12
IVIC Pb 9	35.87	19.87×10^6	18
Pb SN	39.98	9.43×10^6	34
Pb 2052	21.27	1.93×10^6	169
Pb 18	58.38	0.58×10^6	132

[a] LD 50% was estimated 200 days after inoculation.
[b] Six mice were autopsied monthly for 3 months and all their organs were examined for gross paracoccidioidomycotic granulomata.

184.7×10^6 F.U. for strain Pb 2052. Maximum growth and the beginning of the stationary phase was observed on the 6th day for Pb 2052, on the 8th day for IVIC Pb 9, on the 10th day for Pb SN and Pb 18, on the 12th day for Pb 265 and on the 14th day for IVIC Pb 267. Maximum percentage of viable cells was found on the 6th day for Pb 2052 and IVIC Pb 9, on the 8th day for Pb SN, Pb 18 and IVIC Pb 267 and on the 10th day for Pb 265. On the 20th day of incubation, the percentage of viable cells was over 28% for those strains maintained in Fava Netto's medium but was 15.9% and 6.1% for IVIC Pb 9 and IVIC Pb 267, respectively, both maintained in the poorer PYG medium.

Determination of pathogenicity

The pathogenicity of each fungus strain was evaluated by mortality and anatomopathological survey.

Intraperitoneal challenge with increasing doses of the six *P. brasiliensis* strains revealed that Pb 18 and Pb 2052 are highly lethal for B10.A mice, while Pb 265 and IVIC Pb 267 are the least pathogenic; Pb SN and IVIC Pb 9 induce an intermediate pattern of mortality (Fig. 2). For instance, all mice infected with 9.0×10^6 viable yeasts of strains Pb 18

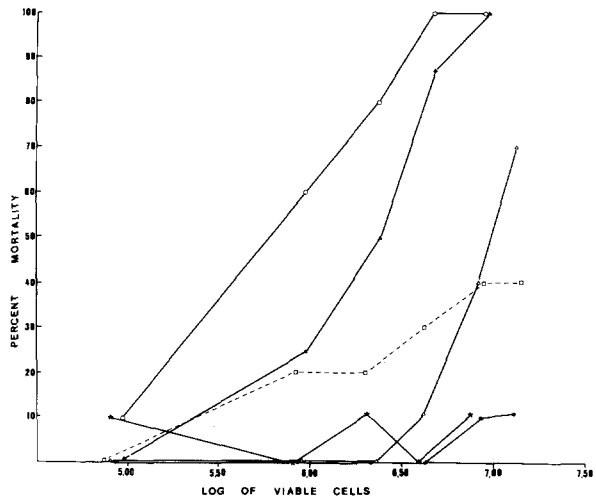

Fig. 2. Percent mortality observed 200 days after i.p. challenge of B10.A mice with different doses of *P. brasiliensis:* Pb 265 (★———★), IVIC Pb 267 (●———●), IVIC Pb 9 (□ – – – □), Pb SN (△———△), Pb 18 (○———○) and Pb 2052 (▲———▲). Each point corresponds to percent mortality of 8–10 mice.

and Pb 2052 were dead 200 days after inoculation while the percentage of deaths of the animals infected with approximately the same dosis of IVIC Pb 267 or Pb 265 was less than 11%. Pb SN and IVIC Pb 9, under the same conditions induced 40% mortality.

The LD 50% estimated for the animals infected with IVIC Pb 9, Pb SN, Pb 2052 and Pb 18, 200 days after inoculation, ranged from 0.6×10^6 (Pb 18) to 19.9×10^6 (IVIC Pb 9) viable cells. Strains Pb 265 and IVIC Pb 267 were non-lethal and no dose-response relation was observed (Table 1).

The cumulative number of paracoccidioidomycotic granulomata observed in the course of the infection of mice challenged with the different *P. brasiliensis* strains are shown in Table 1.

The anatomopathological survey of mice infected with Pb 265 revealed that this strain is avirulent: only one animal presented a discrete lesion on the spleen. Mice infected with IVIC Pb 267 showed also a small number of paracoccidioidomycotic granulomata and the liver was the most affected organ. The animals challenged with IVIC Pb 9 and Pb SN showed a limited cumulated number of lesions (18 and 34, respectively); these lesions were found mainly on the liver and intestinal mesentery for the IVIC Pb 9 infected animals and on the diaphragm of those inoculated with strain Pb SN.

Strains Pb 18 and Pb 2052 induced very high number of granulomata, disseminated on many organs, especially on the oementum, intestinal mesentery, diaphragm, spleen and pancreatic lymph nodes, characterizing a general peritonitis.

Comparison of the in vitro *and* in vivo *characteristics of the* P. brasiliensis *strains*

The mean generation times and the LD 50% of the six *P. brasiliensis* strains, as well as the cumulated number of lesions elicited by them on B10.A mice, are shown in Table 1.

A correlation between lethality and number of paracoccidioidomycotic granulomata was observed: Pb 18 and Pb 2052, the most lethal strains evoked the highest number of lesions while the non-lethal strains, Pb 265 and IVIC Pb 267, the lowest. On the other hand, no correlation was found when the *in vivo* and *in vitro* results were compared: the apathogenic Pb 265 strain as well as the pathogenic Pb 18 strain presented the longest mean generation times, with very similar growth curves; furthermore, the virulent Pb 2052 strain had the shortest mean generation time, with a growth curve pattern different from the above mentioned strains.

Discussion

In 1979, Smith (20) pointed out that 'one of the main blocks to research on fungi seems to be the lack of well-characterized virulent and attenuated strains and, therefore, the virtual absence of the classical comparisons between them that could lead to recognition and identification of virulence determinants'. The present investigation was undertaken to contribute to the enlightening of these points. The *in vitro* growth characteristics and the *in vivo* pattern of pathogenicity were here established and compared.

The mycelial and yeast-like forms of *P. brasiliensis* have been the subject of several comparative *in vitro* studies, using different conditions of: inocula (size and viability), culture media (physical and chemical characteristics), evaluation of fungal growth, time intervals for the counts, etc., as well as different *P. brasiliensis* strains (2, 12–14, 16).

The strains of *P. brasiliensis* studied were isolated from different sources at geographically distinct

places and maintained under different conditions. It is a well-known fact that fungi are extremely variable organisms. Strains of the same fungus obtained from different sources may not necessarily behave in the same manner. Only strain IVIC Pb 9 had its growth curve determined by different authors (12, 13, 17) as well as in the present study. Even in this particular case, comparisons of data are rendered extremely limited, due to the differences in methodology already mentioned. In previous studies growth of the fungi was assessed by dry-weight measurements (12, 13), turbidimetric determinations (16) or on the basis of the viable cell population present in the cultures. This last information was obtained either by counting the colony-forming units (2, 14, 16) or by vital staining (16). In the present work we counted total cells in hemocytometric chambers and the percentage of viable cells after vital staining.

There is not yet available an adequate totally defined medium, universally adopted, for cultivation of *P. brasiliensis*. Most of the comparative studies, have been made with samples taken from yeasts grown in complex media: BHI infusion broth (16); GGY broth (12, 13); modified GGY medium (12); Kelly's hemoglobin agar medium (2); in the present work, PYG and Fava Netto's medium (6) were used.

It is obvious, therefore, that the comparisons made with data from different authors are of limited value. However, the growth patterns of *P. brasiliensis* described by some of the mentioned authors (2, 16) are somewhat similar to those obtained in our study: the growth periods were of approximately equal length; the sharp decrease in viability that was observed by these authors was also here confirmed for the strains grown in PYG medium (IVIC Pb 9 and IVIC Pb 267). The maintenance of higher percentage of viability of those strains cultivated in Fava Netto's medium for longer periods may be due to its richness in nutrients.

The pathogenicity determinations were carried out using *P. brasiliensis* on the 8th day of culture since around this time period maximum percentage of viable cells was observed for the six studied strains.

The mortality studies showed that Pb 18 and Pb 2052 are very virulent strains for B10.A mice, while Pb 265 and IVIC Pb 267 are non-lethal; strains Pb SN and IVIC Pb 9 elicited an intermediate pattern of mortality. These results confirmed previous findings concerning variation in the lethality of different *P. brasiliensis* isolates (5, 9, 15). The slight virulence of IVIC Pb 9 for hamster and mice (10) and the avirulence of IVIC Pb 267 for hamsters (G. & F. San-Blas, personal communication) were also confirmed in the present work.

The LD 50% estimations allow us now to state that the differences on lethality between strains can be very high since, for instance, the LD 50% of the strain Pb 18 is approximately 34 times lower than that of IVIC Pb 9 and much lower, although impossible to estimate, than that of IVIC Pb 267 or Pb 265.

The anatomopathological data here obtained varied among the studied strains and the cumulated total number of lesions found, after three months, showed correlation with the mortalities observed: the pathogenic strains (Pb 18 and Pb 2052) showed LD 50% values lower than 2.0×10^6 viable yeasts and cumulated number of granulomata higher than 130; the non-lethal strains (Pb 265 and IVIC Pb 267) presented a cumulated number of lesions lower than 13; both, LD 50% values and cumulative number of granulomata obtained with strains IVIC Pb 9 and Pb SN were intermediate.

The dissemination pattern of the infection and the target organs seem to vary among the different *P. brasiliensis* strains, but to well establish these characteristics further studies by means of histopathology as well as longer periods of observation are necessary. However, the anatomopathological survey of mice challenged with Pb 18 or Pb 2052 showed gross lesions that were more intense and disseminated with the evolution of the infection. These results disagree with those of Moscardi & Franco (11) who observed peritoneal inflammation with resolution of the infection from the 4th week on, when infecting outbred mice i.p. with Pb 18. This discrepancy between our results and those of the above mentioned authors may be due to the genetic heterogeneity of the outbred mice: since we have previously observed the decisive influence of the genetic pattern of the host on the development of experimental murine paracoccidioidomycosis; furthermore only three out of the nine inbred mouse strains studied were susceptible to *P. brasiliensis* i.p. infection (4). Then the probability to deal with genetically sensitive invididuals, when outbred mice are used, is low. Based on this fact, we must emphasize the importance of working with genetically homogenous animals, when the influence of a given factor, on the disease, is under in-

vestigation. Taking in account our previous work (4) and the present investigation, we can conclude that both, the host genetic background as well as the *P. brasiliensis* strain, have marked influence on the outcome and evolution of the disease.

In the present work absence of correlation was observed regarding the time period lapsed between the isolation of one particular strain of *P. brasiliensis* and its virulence: strain Pb 18, isolated 50 years ago has similar pathogenicity to that of Pb 2052, isolated three years ago. In order to verify if the strain Pb 18 diminished its pathogenicity by *in vitro* subculturing, virulence studies with this strain after successive passages *in vivo* or after addition of fetal calf serum to the growth medium (19) is under investigation.

The present work is original in comparing both curve patterns of *P. brasiliensis* strains with their pathogenicity to inbred mice. Our studies show that there is no correlation between these factors. Strains Pb 265 and Pb 18, which both grow slowly in culture medium, showing low total and viable numbers of yeasts have opposite behaviour *in vivo:* Pb 18 is lethal, originates high numbers of granulomata and Pb 265 is apathogenic. On the other hand, strains IVIC Pb 267 and Pb 2052, which both grow very well in culture are, respectively, apathogenic and lethal to inbred mice.

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