Mycopathologia **109**: 13–17, 1990.

Virulence of *Paracoccidioides brasiliensis*: The influence of *in vitro* passage and storage

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Accepted 24 April 1989

Key words: *Paracoccidioides brasiliensis*, paracoccidioidomycosis, passage *in vitro*, virulence

Abstract

Stability of virulence in *P. brasiliensis* isolates was studied with respect to the *in vitro* culture history and methods used for storage. Virulence in yeast-form *P. brasiliensis* isolates was tested in a chronic pulmonary murine model of paracoccidioidomycosis where progression of disease was quantitated in terms of colony forming units recoverable from lungs. Four isolates of *P. brasiliensis*, including recently isolated from patients or experimental animals, caused chronic progressive disease. Two isolates with a history of subculturing showed attenuation by causing resolving but chronic disease. An attenuated isolate became avirulent subsequent to 15 more years of subculturing. These findings suggest that virulence of *P. brasiliensis* can be attenuated or lost subsequent to cycles of subculturing over long periods. Our data suggest that the use of fresh *P. brasiliensis* isolates may be needed to provide reproducible virulence for experimental systems.

Abbreviations: ATCC = American Type Culture Collection, CFU = colony-forming units, LD = Lethal dose, MMv-M = modified McVeigh Morton, PMN = polymorphonuclear neutrophil

Introduction

Differences in virulence of *P. brasiliensis* isolates have been reported [6, 11, 12]. One basis for differences in virulence of *P. brasiliensis* has been associated with alpha-1,3 glucan cell wall content [10, 11]. With *P. brasiliensis* it was observed that the isolate IVIC Pb 9 halted synthesis of alpha-1,3 glucan over years of subculturing *in vitro* and lost capacity to infect mice but not hamsters [11].

The purpose of the present study was to investigate systematically the stability of virulence in several *P. brasiliensis* isolates maintained and/or stored under a variety of conditions. Newer and more quantitative methods for assessing virulence of isolates were used, for example, quantitation of inocula in terms of colony forming units (CFU), infection by the natural pulmonary route, and measurement of disease severity in terms of recoverable CFU of the pathogen.

Methods and materials

Animals

BALB/cByJ male mice, 8 to 12 weeks of age, from the specific pathogen-free breeding colony at the Institute for Medical Research, San Jose, CA., were used for the pulmonary infection model of paracoccidioidomycosis.

P. brasiliensis

Virulent isolates Mnt, Vel, Gra and Gar were cultured from patients with paracoccidioidomycosis in Medellin, Colombia (Table 1). Mnt and Vel were isolated in the mycelial form and tested *in vivo* the same year. All isolates in this study in the mycelial form were converted to yeast prior to *in vivo* challenge. Gra was subcultured in the mycelial form monthly for 4 years at room temperature prior to *in vivo* testing. Patient isolate LA was subcultured 20 years monthly in the mycelial form at room temperature. After the first 5 years of subculturing, this isolate was deposited at the American Type Culture Collection (ATCC), and the isolate designated as ATCC 32074. Isolate Ru, tested after 2 years of storage underwater and 3 years of subculturing monthly at 35 °C in the yeast form on agar slants [1, 8], was originally obtained from a patient in 1982.

Table 1. Isolates of *P. brasiliensis.*

Virulent		Attenuated and avirulent		
Name	Isolated	Name	Isolated	Deposited at ATTC
GarAP	1979 (Human) 1986 (Mouse)	LA #	1967 (Human)	1972 (ATCC 32074)*
Gra	1983 (Human)	Ru*	1982 (Human)	–
Mnt	1987 (Human)			
Vel	1987 (Human)			

* Attenuated.
\# Avirulent.

Inoculum preparation

Yeast-form *P. brasiliensis* from agar slants was seeded into 3 ml of modified McVeigh-Morton (MMv-M) broth and incubated at 35 °C on a rotary shaker (200 rpm) for 7 days. Multiple 3 ml broth cultures were then prepared from this to provide the challenge inocula. One isolate, obtained in 1979 from a patient (Gar), was used in 1986 to infect mice and then reisolated from them (GarAP) [2].

Inoculation of mice

Washed *P. brasiliensis* yeast cells were counted with a hemacytometer and percent viable units determined using the fluorescein diacetate-ethidium bromide viability stain [8]. Inocula were characterized and used to infect mice by the intranasal route as described [2]. The actual challenge was quantitated in terms of CFU by culturing lungs of infected mice less than one hour after infection.

Virulence assessment

Virulence of *P. brasiliensis* isolates was measured in terms of chronic progressive disease, quantitated by the number of CFU per lung. Lungs were removed aseptically, homogenized in 5 ml of RPMI-1640 medium containing 100 U penicillin and 100 mcg streptomycin per ml (GIBCO, Grand Island, NY) with a tissue grinder (Tissumizer, Tekmar TR-100, Cincinnati, OH). Ten-fold serial dilutions were plated (1 ml per plate) on an agar medium with good (greater than 50%) plating efficiency (Brain-Heart-Infusion agar +4% (vol./vol.) horse serum +5% (vol./vol.) MMcV-M 2 week broth culture filtrate)

as previously described [2, 3]. Plates were incubated at 35 °C for 1 day, then for an additional 7–9 days in plastic bags to prevent drying. After this incubation period CFU per plate were counted and CFU per lung calculated.

Statistics

Comparisons between groups were analyzed by the Student's t-test, with significance assumed to be $P < 0.05$.

Results

Virulence of P. brasiliensis isolates

Three recent patient isolates Gra, Mnt and Vel as well as one passaged in mice, GarAP, were virulent when tested in the chronic pulmonary model of paracoccidioidomycosis (Fig. 1). The mean infectious dose (CFU/lung at time 0) ranged between 1×10^6 to 4×10^6 for Gra, Mnt, and Vel. The CFU recoverable from lungs increased 10-fold over a 4 week period and then persisted, accompanied by chronic progressive disease and failure to gain weight. The weight of uninfected mice increased from 23.2 ± 1.4 to 34.0 ± 2.7 in 12 wks whereas mice infected with Vel, Gra, Mnt, or GarAP failed to gain weight and after 12 wks weighted 22.3 ± 1.1, 23.0 ± 4.2, 22.6 ± 1.5 and 23.0 ± 3.5 gm respectively. In contrast, the LA isolate (7×10^6 CFU/lung at time 0) was rapidly cleared (decline from time 0, $P < 0.001$) over a 4 week period (Fig. 1). The 4 week value was significantly different ($P < 0.001$) from the virulent isolates GarAP, Vel, Mnt and Gra. Another isolate, Ru, exhibited attenuated virulence in this system. For example, the infection started to resolve (10-fold reduction in CFU in 4 weeks, $P < 0.05$ compared to time 0) then persisted for 8 weeks. At both 4 and 8 weeks there were significantly fewer CFU/lung compared to virulent isolates GarAP, Vel, Mnt, Gra ($P < 0.001$) (Fig. 1). These findings show that recent patient isolates tested are generally virulent in this model; whereas, isolates with a history of extended subculturing can be attenuated or avirulent.

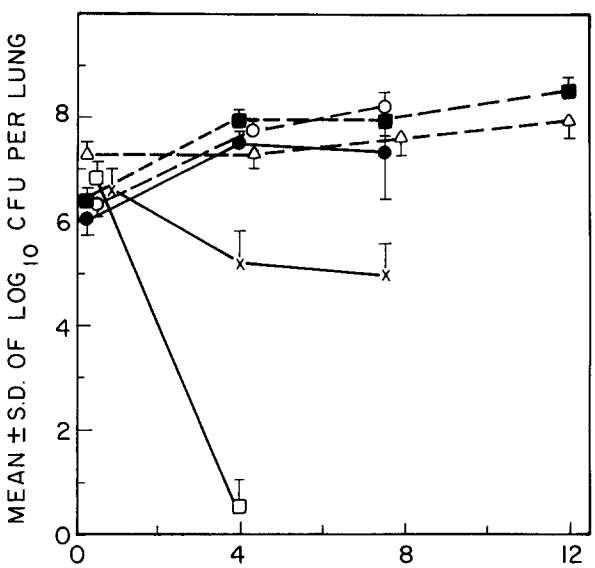

Fig. 1. Virulence of *P. brasiliensis* isolates. Groups of 15–20 mice were infected i.n. with yeast-form *P. brasiliensis* isolates GarAP (triangles), Vel (solid squares), Mnt (solid circles), Gra (open circles), Ru (x's), or LA (open squares) and $\log_{10}$ CFU ± S.D. of 5 mice determined at 0, 4, 8 or 12 weeks.

Loss of Virulence in P. brasiliensis

When the deposited isolate ATCC 32074 was obtained from ATCC in 1987 and tested for virulence, attenuated virulence was demonstrated compared to fresh patient isolates (Fig. 2). There was a gradual and significant clearance of this infection over 8 weeks (decline from time 0, $P < 0.001$) with persistence through 12 weeks (Fig. 2). The CFU/lung of ATCC 32074 at these times were significantly fewer than those of mice infected with virulent isolates ($P < 0.001$). In this experiment LA, subcultured in the laboratory for 20 years, showed lack of virulence and was significantly cleared in 4 to 8 weeks (Fig. 1, 2). At 4, 8, and 12 weeks the values for LA were significantly ($P < 0.001$) different from time 0 and from the values for ATCC 32074 at 4, 8 and 12 weeks, respectively. Mice (23.2 ± 1.3 gm) infected with ATCC 32074 did not gain weight as fast as mice (23.8 ± 1.4 gm) infected with LA, i.e., 24.2 ± 1.6

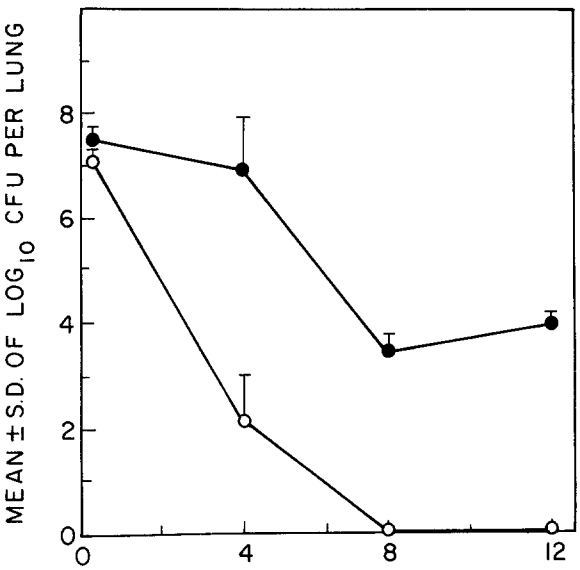

Fig. 2. Virulence of *P. brasiliensis* ATCC 32074 (a sub-line of LA isolate) vs. LA. Groups of 20 mice were infected i.n. with yeast-form *P. brasiliensis* ATCC 32074 (solid circles) or LA (open circles). Log_{10} CFU ± S.D. of lungs from 5 mice were cultured at 0, 4, 8 or 12 weeks post-infection.

vs. 28.0 ± 1.4 gm respectively at 4 weeks ($P < 0.01$). However, after 12 weeks of infection there was no significant difference in weight between the 2 groups (31.4 vs. 34.0 gm). These data suggest that subculturing of LA for 20 years rendered it avirulent.

Discussion

Virulent isolates of *P. brasiliensis* (Mnt, Vel, and Gra) were generally recently isolated; whereas two subcultured isolates (Ru and ATCC 32074) of *P. brasiliensis* had attenuated virulence. Subculturing of LA over 20 years resulted in avirulence. This latter finding confirms earlier observations that subculturing of *P. brasiliensis* leads to loss of virulence [2, 12]. It is possibly more relevant to this that Ru was subcultured at 35 °C for 3 years, as compared to none for Gra, prior to *in vivo* assay, than the elapsed time from initial isolation until assay. Discordance in the literature regarding pathogenesis of *P. brasiliensis* in murine models of pulmonary paracoccidioidomycosis, e.g. chronic progressive [2] vs. resolving disease [5], may be explained by our observation and the culture history of the isolates used.

Others, for example Zacharias *et al.* [12], have also described virulent (Pb 18), attenuated (Pb 192), and avirulent (Pb 265) isolates of *P. brasiliensis* assessed by development of lesions in mice after i.v. infection. Cell wall analysis, e.g. alpha-1,3 glucan, did not suggest striking differences among these strains. Specific culture history of these strains was not documented other than that they were isolated years ago from patients with paracoccidioidomycosis and maintained *in vitro*. Similarly, Kashino *et al.* [6] have reported virulent (Pb 18, Pb 2052) and avirulent (nonlethal) strains (Pb 265, IVIC Pb 267) of *P. brasiliensis* in B10. A mice infected i.p.. Virulent Pb 2052 was a recent patient isolate, whereas virulent Pb 18 was from a culture collection with undocumented culture history. *In vitro* growth, e.g. rapid growth with low generation time, did not correlate with *in vivo* virulence in these studies [6].

Although our studies did not try to identify 'virulence factors' that were lost when virulent isolates became avirulent, other have examined this in *P. brasiliensis* [11]. However, in our related studies on the interaction of *P. brasiliensis* and phagocytic cells we found that avirulence of LA correlated with susceptibility to killing by murine polymorphonuclear neutrophis (PMNs) *in vitro* (Abstract, Tenth Congress of Int. Soc. Human & Animal Mycology, 0–60, p. 25, 1988). The attenuated isolate ATCC 32074, a sub-line stored in liquid nitrogen, was resistant to killing by murine PMNs (unpublished results).

Animal passage of attenuated *P. brasiliensis* has been reported to restore virulence [2, 11]. Future experiments with Ru, ATCC 32074 and LA to confirm these observations are warranted. The cited reports on restoration of virulence in *P. brasiliensis* are consistent with observations made by Levine *et al.* [7] on virulence in another thermally dimorphic fungal pathogen *Coccidioides immitis*. It was reported that *C. immitis* virulence was markly attenuated after 84 serial transfers *in vitro*, e.g. LD_{20} increased from 20 to 3.7×10^5

arthroconidia, but regained virulence after passage in mice or on complex medium.

Catrenich and Johnson [4] have recently reevaluated the recovery of virulence in *Legionella pneumophila* by passage in guinea pigs. They point out that attenuated cultures may contain a mixture of fast-growing avirulent variant and viable slow-growing virulent cells on supplemented Muller-Hinton (SMH) agar. Individual colonies picked from SMH agar plates after 5 transfers failed to regain virulence when serially passaged (6 times) in guinea pigs. However, if pooled colonies were used as an inoculum, virulent *L. pneumophila* could be obtained by serial passage in guinea pigs. Moreover, if *L. pneumophila* was passaged 25 times on SMH agar, growth (pooled colonies) from such plates did not yield virulent *L. pneumophila* by serial passage in guinea pigs [4]. Attention should be paid to these findings when designing experiments to demonstrate recovery of virulence by dimorphic fungal pathogens by animal passage.

The basis for virulence in *P. brasiliensis* remains to be defined; however, isolates that have lost virulence, may, by serendipity, provide us with tools for identifying virulence factors. On the other hand, storage of isolates by the liquid nitrogen method used by the ATCC may preserve virulence of isolates.

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