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Host-Parasite Relationships in the Yeastlike Form of *Paracoccidioides brasiliensis* Strain IVIC Pb9

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The yeastlike form of *Paracoccidioides brasiliensis* strain IVIC Pb9 reduced the amount of α-1,3-glucan in its cell wall from 45 to 3% when subcultured in vitro for several years. This strain regained its α-1,3-glucan up to 25% of the total cell wall when grown in vivo. A mutant strain of *P. brasiliensis* Pb9, named IVIC Pb140, reported to have 1,3-mannan instead of α-glucan in the cell wall, could not be recovered from experimentally infected animals. The existence of some relationship between the presence of α-1,3-glucan in the cell wall of the yeastlike form and the pathogenicity of this fungus is suggested in this report.

Paracoccidioides brasiliensis, an imperfect fungus pathogenic for humans, undergoes thermal dimorphism (12). It develops a yeastlike (Y) form when grown at 37°C and a mycelial (M) form at room temperature.

Quantitative analyses of the cell walls of both Y and M forms have shown that their chemical compositions are similar (11), the most striking difference being with regard to the structural composition of the glucans found in both forms. In fact, the main glucose polymer in the Y form is α-1,3-glucan, whereas in the M form this polysaccharide is β-1,3-glucan (9). It has been proposed that the spherical shape of the Y form is due to the short rodlike fibers characteristic of the α-1,3-glucan, whereas the long narrow fibers of the β-1,3-glucan contribute to the M form (9).

In this report, the Y form of *P. brasiliensis* strain IVIC Pb9, formerly studied by Kanetsuna et al. (11), is shown to have halted the synthesis of α-1,3-glucan over the years of subculturing in vitro but to have retained the capacity to produce it after inoculation in hamsters. At the same time, a mutant of this strain, *P. brasiliensis* IVIC Pb140, reported to have 1,3-mannan instead of α-1,3-glucan in its cell wall (15), was unable to produce disease and could not be recovered from experimentally infected animals. It is suggested that the presence of α-1,3-glucan in the cell wall of the Y form of this fungus may play an important role in the pathogenicity of this microorganism.

MATERIALS AND METHODS

Organism and growth conditions. A human isolate of *P. brasiliensis* strain IVIC Pb9 (formerly strain 7193 of the Instituto Nacional de Tuberculosis, Caracas, Venezuela), maintained for several years in our laboratory on Sabouraud liquid broth modified agar (BBL, Cockeysville, Md.), was used in these studies. Exponentially growing populations of the Y form of this strain were obtained by inoculating two loopfuls of a 3-day-old culture, from a Sabouraud agar slant kept at 37°C, into 500-ml Erlenmeyer flasks containing 100 ml of brain heart infusion broth (BBL). Flasks were incubated for 4 to 5 days at 37°C with shaking. Slants were previously subcultured every 3 days at least 10 times (14).

Strain IVIC Pb140 is a morphological mutant isolated after treatment of the Y form of strain IVIC Pb9 with 50 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml (15).

Animal experimentation. Exponentially growing populations of the Y form of strains of *P. brasiliensis* were washed with distilled water, centrifuged at 2,000 × *g*, resuspended in 0.85% NaCl, and gently homogenized in a tissue grinder. The concentration of Y-like cells was adjusted to 2×10^6 colony-forming units per ml. Adult outbred male hamsters, weighing an average of 90 g, and male white mice (strains NMRY/IVIC and BALB/c), weighing 20 to 25 g, were inoculated intraperitoneally with 1 ml of the Y-like suspension of the fungal strains. A total of 12 animals of each species were used in testing each culture. Necropsy was not performed on animals dying in the first 2 weeks of the experiment. The survivors were sacrificed every month up to 6 months and examined for visible lesions on the viscera and for the presence of paracoccidioidomycotic nodules on the mesentery and peritoneal surface. Portions or whole viscera were minced, inoculated on Mycosel agar (BBL) slants, and incubated at 37°C. Care was taken to avoid dehydration of slants. Y colonies that developed on Mycosel agar were transferred and kept on Sabouraud agar.

Preparation and fractionation of cell walls. Cell wall preparation was performed as previously described (10) but using higher pressures in the Ribi cell fractionator (40,000 to 50,000 lb/in²).

The cell walls were separated into fractions essentially as described before (10). Three fractions were obtained: an alkali-insoluble residue (fraction 1), an alkali-soluble fraction precipitable by neutralization with acetic acid (fraction 2), and an alkali-soluble fraction not precipitable by neutralization with acetic acid (fraction 3).

Analytical procedures. Lipid, sugar, amino acid, and amino sugar determinations and periodate oxidation of polysaccharides were carried out as before (15). Infrared spectra were determined as previously described (13).

Source of enzyme and enzymatic procedure. α-1,3-Glucanase (EC 3.2.1. group) was prepared from *Cladosporium resinae* and tested against α-1,3-glucan from *Aspergillus niger* and β-1,3-glucan from *P. brasiliensis* (13). The enzymatic procedure was followed as before (15).

RESULTS

Experimental infection. The Y-like form of *P. brasiliensis* strain IVIC Pb9 at a concentration of 2×10^6 colony-forming units per ml was unable to produce disease or to be recovered from experimentally infected mice when inoculated intraperitoneally. However, using the same conditions, it was possible to recover the microorganism from hamsters 1 month after infection. This isolate was named strain IVIC Pb9H.

Table 1 shows a summary of the virulence of the three strains tested, taking as criteria for virulence both the spread of lesions and the recovery of viable Y-like cells from minced organs.

Autopsy performed on all animals infected with any strain, sacrificed in the first and second months, revealed paracoccidioidomycotic nodules of various sizes scattered through the mesentery. With strain IVIC Pb140 there was no evidence of infection of the viscera in any of the animals tested. Mesenteric nodules extracted from IVIC Pb140-inoculated animals, when treated with vital stain (2), contained only deteriorated single Y cells, most of them without protoplasm. No viable Y-like cells of this strain were recovered from any of the animals. Hamsters infected with strain IVIC Pb9 contained gross evidence of infection in the viscera and diaphragm. Evidence of lesions was not seen in the lungs. Viable cells were recovered from mesenteric nodules and spleen. NMRY and BALB/c mice contained only scattered small mesenteric nodules with no viable Y-like cells.

Strain IVIC Pb9H turned out to be the most virulent of the strains tested. Visible lesions were observed in all three species of animals containing viable cells from most of the cultured organs.

TABLE 1. *Virulence of strains of P. brasiliensis in laboratory animals after intraperitoneal inoculation*[a]

Strain	Hamster	Mice	
		NMRI/IVIC	BALB/c
IVIC Pb9	+	−	−
IVIC Pb9H	+	+	+
IVIC Pb140	−	−	−

[a] Animals were sacrificed every month up to 6 months after inoculation. Symbols: +, Recovery of viable Y-like cells and appearance of lesions; −, no recovery.

Chemical composition of cell walls. Table 2 summarizes the differences in chemical composition of the cell wall of the Y form of *P. brasiliensis* IVIC Pb9 as reported by Kanetsuna et al. (9) and as found in this work, including the strain injected in animals and subsequently recovered in vitro (IVIC Pb9H). Thin-layer chromatography of total acid hydrolysates of cell wall preparations showed the presence of glucose, galactose, and mannose in all of them. Lipids appeared to be present in lower amounts in the organism injected in hamsters. Some slight differences are recorded in the amounts of amino acids and amino sugars present in each of the preparations.

Fraction 1. *P. brasiliensis* IVIC Pb9H showed some increase in the amount of this fraction compared with either of the listed figures for Pb9. In any case, hydrolysates in this fraction contained a large amount of amino sugars (45 to 60% of the total fraction) and produced a single spot as glucosamine when analyzed by thin-layer chromatography. The infrared spectrum of this fraction was compatible with that of chitin, showing an absorption band at 890 cm^{-1} characteristic of β-linkages in glucans. This infrared spectrum was identical to that reported before for fraction 1 (15). Thin-layer chromatography showed the presence of glucose as the main neutral sugar in both Pb9 and Pb9H, although traces of galactose were present. Digestion of fraction 1 from Pb9 with β-1,3-glucanase released the whole of sugars as glucose, suggesting that this compound was polymerized under the form of β-1,3-glucan. However, fraction 1 of Pb9H released only 33% of its glucose content when in contact with β-1,3-glucanase, suggesting that some other kinds of linkages were present in the β-polymer. The presence of any α-linkage was discarded on the basis of the infrared spectrum. Amino acids were present in different amounts. It was not determined whether they existed isolated or bound in the form of glycoproteins.

TABLE 2. *Cell wall composition of Paracoccidioides brasiliensis strain IVIC Pb9 before and after passage through hamsters*

Strain	Composition (%)																
	Cell wall				Fraction 1 (alkali soluble)				Fraction 2 (alkali soluble, acid precipitable)				Fraction 3 (alkali soluble, nonprecipitable)				
	Hexoses	Amino sugars	Amino acids	Lipids	Yield[a]	Hexoses	Amino sugars	Amino acids	Yield[a]	Hexoses	Amino sugars	Amino acids	Yield[a]	Hexoses	Amino sugars	Amino acids	Lipids
IVIC Pb9[b]	38.4	43.4	10.1	11.0	40.0	19.0	44.0	20.6	45.0	100.0	0.0	0.0	5.0	25.0	0.0	0.0	
IVIC Pb9[c]	41.5	30.0	17.0	10.0	41.5	16.9	60.5	16.2	3.2	100.0	0.0	0.0	55.3	20.4	0.0	19.2	60.0
IVIC Pb9H[d]	37.7	41.0	18.4	5.0	53.2	9.8	51.5	43.2	19.9	91.0	0.0	0.8	26.9	38.7	0.0	23.0	38.0

[a] Percentage of each fraction in cell wall.
[b] As reported before (7, 9).
[c] As found during the course of this work, after several years of subculturing in vitro.
[d] After passage through hamsters.

Fraction 2. There were striking differences among the percentages of this fraction in the various strains listed in Table 2. It can be seen that the original 45% reported by Kanetsuna et al. (11) was reduced to 3% for the same strain, Pb9, after the strain had been subcultured in vitro for many years. When this strain, devoid of fraction 2, was isolated from hamsters after 60 days of infection, it was observed that the amount of fraction 2 increased to 19.9% when analyzed.

Chemically, fractions 2 from the different strains were composed of the same polysaccharide. Thin-layer chromatography of their hydrolysates showed a single spot of glucose. Enzymatic analysis indicated a 100% hydrolysis by α-1,3-glucanase. Periodate oxidation of this material produced no reaction, suggesting again the presence of only 1,3 linkages. The infrared spectrum of this material was identical to others reported for α-1,3-glucan (13, 15), showing a band at 840 cm^{-1} characteristic of α-glucans. Some proteins were also present in fraction 2 of Pb9H.

Fraction 3. As with fraction 2, there were remarkable differences among the contents of this fraction in the various strains, the percentages being inversely proportional to the amounts of fraction 2. Thus, in contrast to the 5% reported by Kanetsuna et al. (11), we obtained 55.3% for the same strain after it had been subcultured in vitro for many years.

In fractions 3 from all of the strains, thin-layer chromatography showed the presence of galactose, mannose, and traces of glucose, although their polymeric association was not studied. It is possible that they were galactomannans as reported before (1).

DISCUSSION

Previous reports (7, 9) indicated that glucans in the Y form of *P. brasiliensis* IVIC Pb9 were structured mostly in the α-1,3 configuration (45% of the cell wall) and that β-1,3-glucans were present in a lower proportion (5%). It has been observed in our laboratory that during continuous in vitro subculturing of this strain, its pathogenicity for laboratory animals decreases (L. M. Carbonell, personal communication). In this study it was found that the cell wall alkali-soluble fraction 2 was reduced in α-1,3-glucan content (Table 2). In fact, the amount diminished from 40% (8) to 3% of the total cell wall.

At the same time, a galactomannan present only in the M form of *P. brasiliensis* IVIC Pb9 (11) was detected in the Y form after subculturing in vitro in such a way that the whole quantity of sugars in the cell wall was now similar in both forms: 39% (11) and 41.5% (Table 2). Amino sugar and amino acid contents remained almost unchanged in both cases. It can also be seen that the α-1,3-glucan reappeared when strain IVIC Pb9 was injected into animals (IVIC Pb9H) or when it was cultured in fetal calf serum-containing medium (San-Blas and Vernet, Infect. Immun., in press).

The substitution of α-1,3-glucan by galactomannan in these two strains of IVIC Pb9 may have some implications in the decreased pathogenicity observed in the strain repeatedly subcultured in vitro. Recently, Azuma et al. (1) have demonstrated the serological activity of galactomannans from various pathogenic fungi, including *P. brasiliensis*. In this report, the authors pointed out that the Y form of *P.*

brasiliensis was devoid of galactomannan and that the galactomannans obtained from the mycelial cells of *Histoplasma*, *Paracoccidioides*, and *Blastomyces* species were common antigens to these fungi. It remains to be studied whether the galactomannan found in *P. brasiliensis* (Y form) after successive subculturing in vitro belongs to the same group of antigens.

With regard to the decrease in virulence of the mutant strain IVIC Pb140 and of the parental strain IVIC Pb9 devoid of α-1,3-glucan, it is suggested that the disappearance of this polysaccharide in the latter and its substitution by an amorphous 1,3-mannan in the former (15) may have some role in the process of phagocytosis, as has been indicated for a nonencapsulated mutant of *Cryptococcus neoformans* (3, 6). Thus, the peripheral α-1,3-glucan of *P. brasiliensis* could act as a protective layer, inasmuch as it could be resistant to digestion by enzymes present in the leukocytes even when the cells are phagocytized. This hypothesis is supported by various facts: (i) the existence of α-1,3-glucan in the pathogenic Y form of *P. brasiliensis* and not in its saprophytic mycelial form; (ii) its disappearance from the Y form when continuously subcultured in vitro and its reappearance when injected in animals, as shown in this paper; and (iii) induction of α-1,3-glucan synthesis in *P. brasiliensis* grown in culture medium to which fetal calf serum was added, to produce a strain as virulent as strain IVIC Pb9H (San-Blas and Vernet, Infect. Immun., in press).

It would be of interest to discover whether *P. brasiliensis* isolated from soil would have α-1,3-glucan as a component of the Y-form cell wall. In this respect, it is worth mentioning that previous studies on *Blastomyces dermatitidis* cell wall (4, 8) suggest an increased amount of α-glucan in human isolates compared with strains extracted from soil. Preliminary results in our laboratory suggest that growing *P. brasiliensis* IVIC Pb9 in synthetic culture media other than brain heart infusion does not induce the synthesis of α-1,3-glucan in the fungal cell wall, as was found to be the case with *A. niger* (16).

Kanetsuna et al. (9) suggested that the α-1,3-glucan may play a role in the maintenance of the spherical shape of the Y form of *P. brasiliensis*. However, our studies suggest that this may not be so, since strain Pb9 subcultured in vitro (i.e., the strain reduced in its α-1,3-glucan content) remained indistinguishable in its shape from the original strain. It seems, rather, that the α-1,3-glucan may play some important role in the protection of the fungus against the defensive mechanisms of the host.

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