Lipid Composition of *Paracoccidioides brasiliensis*: Possible Correlation with Virulence of Different Strains

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The lipid content and composition of four strains of *Paracoccidioides brasiliensis* were analysed to determine any possible correlation with their virulence for hamsters and mice. Two strains, Pb168 and Pb141, were equal in virulence, Pb9 was slightly virulent and Pb140 was avirulent under the experimental conditions. No correlation was observed between virulence and the total lipid or phospholipid content of the strains. The lipid yield was highest in Pb9 and lowest in Pb168. Polar lipids were highest in Pb9 and least in Pb140. Phosphatidylcholine was the dominant phospholipid in all strains but its percentage was lower in the avirulent strain Pb140. Diphosphatidylglycerol, the least saturated lipid in all strains, was less abundant in Pb140 than in the virulent strains Pb168 and Pb141. In all four strains, neutral lipids constituted the major fraction of total lipids and triglycerides were the predominant individual lipid class, being more abundant in the avirulent and slightly virulent strains than in the virulent strains. The fatty acid profiles of total lipids and individual lipid classes of neutral and polar lipids obtained from the four strains were similar; however, the individual lipid classes showed patterns of preferential distribution of these fatty acids.

INTRODUCTION

Qualitative and quantitative analysis of the lipids in pathogenic fungi and their relationship to virulence has been the subject of several studies. Peck (1947) suggested an association of lipid content with the virulence of pathogenic fungi. Di Salvo & Denton (1963) studied the lipid contents of four strains of *Blastomyces dermatitidis* and suggested a positive correlation between the amount of total lipid in each strain and its virulence for mice. Nielsen (1966) investigated the possibility that a similar relationship between lipid content and virulence might also exist in another fungus, *Histoplasma capsulatum*, causing systemic infection in man. His data suggested that in the six isolates studied neither total extractable lipid nor phospholipid showed a quantitative correlation with virulence. However, the possibility that the quantity of phosphatidylcholine correlates with the virulence of a given isolate of *H. capsulatum* was not ruled out. A possible relationship between phospholipids and virulence of *B. dermatitidis* strains was suggested by Cox & Best (1972). In an attempt to explain the cause of attenuation of virulence, Anderes *et al.* (1973) studied the lipids of an auxotrophic avirulent mutant of *Coccidioides immitis* and compared its lipid composition with that of the wild-type.

Virtually nothing is known about the lipid composition of *Paracoccidioides brasiliensis*, the causal organism of South American blastomycosis. It was therefore considered profitable

to undertake an extensive study of the lipid composition of some selected strains of *P. brasiliensis* and to investigate the correlation, if any, which exists between this and their virulence for hamsters and mice. This paper reports the results of such a study.

METHODS

Fungal strains and cultures. Strains of *Paracoccidioides brasiliensis* Pb168, Pb141 and Pb140 were obtained by treating the yeast form of *P. brasiliensis* Pb9 of the Instituto Venezolano de Investigaciones Científicas (IVIC) with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The method of their isolation and purification has been described elsewhere (San-Blas & Centeno, 1977). All strains were grown routinely on a medium consisting of (g l⁻¹ in distilled water): glucose, 20; glycine, 10; yeast extract, 5; agar, 15. The pH of the medium was adjusted to 7.2. The cultures were grown at 37 °C for 4 to 5 d, i.e. until the mid-exponential phase of growth. Harvested cultures were washed with distilled water and immediately lyophilized.

Virulence tests. Exponentially growing cultures of *P. brasiliensis* strains were washed with distilled water, centrifuged at 2000 g, resuspended in 0.85% (w/v) NaCl and gently homogenized in a tissue grinder. The concentration of yeast-like cells was adjusted to 2×10^8 colony-forming units ml⁻¹. Adult outbred male hamsters, weighing an average of 90 g, and male white mice (strains NMR1/IVIC and BALB/c), weighing 20 to 25 g, were inoculated intraperitoneally with 1.0 ml cell suspension. Twelve animals of each species were used to test the virulence of each strain of *P. brasiliensis*. Necropsy was not performed on animals dying in the first 2 weeks of the experiment. From the survivors, one animal of each species was sacrificed each month up to 6 months and examined for visible lesions on the viscera and for the presence of paracoccidioidomycotic nodules on the mesenteric and peritoneal surfaces. Portions of whole viscera, removed at autopsy, were macerated and cultured on Mycosel agar (BBL) slants. Yeast colonies that developed on Mycosel agar were transferred and kept on Sabouraud dextrose agar (Difco).

Lipid extraction. The lyophilized cultures were extracted with 20 vol. each of chloroform/methanol as 2:1, 1:1 and 1:2 (v/v) mixtures. The cultures were suspended in chloroform/methanol (2:1, v/v) and homogenized in a Sorvall Omni-Mixer (Ivan Sorvall, U.S.A.) for 1 h. The extract was separated by centrifuging at 5000 g for 5 min and the pellet, suspended in chloroform/methanol (1:1, v/v), was macerated in a Potter Elvehjem tissue grinder (Fisher Scientific Co., Fairlawn, N.J., U.S.A.). The extract was separated by centrifuging and the pellet was resuspended in chloroform/methanol (1:2, v/v) with constant stirring. The combined extracts were reduced to near dryness on a rotary evaporator, taken up in known volume of Folch's lower phase solvent and washed with the upper phase solvent (Folch *et al.*, 1957). The lipid extract in the lower phase was evaporated to a small volume, dried with Na₂SO₄ and centrifuged. The supernatant was dried to constant weight, first by a stream of N₂ and then under vacuum in a desiccator.

Separation of lipids. Total lipids (100 to 120 mg, in 3 ml chloroform) were loaded on to a silicic acid column. Neutral lipids were eluted with chloroform and polar lipids with chloroform/methanol (1:1, v/v) followed by methanol. The eluted fractions were reduced to a known volume and samples containing 2 to 3 mg lipid were spotted on heat-activated 20 × 20 cm plates coated with 250 µm thick Kieselgel G (E. Merck, Darmstadt, Germany). Neutral lipids were separated in the single solvent system consisting of hexane/diethyl ether/acetic acid (85:15:1, by vol.). For two dimensional separation of polar lipids a double solvent system was used. The successive solvent systems were: (1) chloroform/methanol/aqueous NH₄OH (75:25:3, by vol.); (2) chloroform/methanol/acetic acid/water (170:25:25:4, by vol.). All solvents contained 0.01% (w/v) butylated hydroxytoluene to prevent oxidation of the lipids.

Identification and quantitative estimation of lipids. Lipids were visualized by first spraying the plate with 50% H₂SO₄ followed by heating at 90 °C for 15 to 20 min. They were identified by (i) co-chromatography with standard lipids (Applied Science Laboratories, College Park, Penn., U.S.A.), (ii) comparison of the patterns obtained with published diagrams for these solvent systems (Deven & Manocha, 1975) and (iii) reactions of specific classes of lipids with various reagents.

For quantitative estimation and fatty acid analysis of individual lipid classes, the plates were sprayed with 0.02% (w/v) Rhodamine 6G and examined under fluorescent light. Visualized spots of lipid were scraped off from 12 to 15 plates and the lipids were eluted from the gel with chloroform/methanol (2:1, v/v). The eluted fractions were dried to constant weight and the amount of each individual lipid class was determined gravimetrically. The recovery of lipids was 84 to 86% in all cases. Calculations are based on 100% recovery for sake of convenience and comparison between the strains.

Gas-liquid chromatography of fatty acids. Fatty acid methyl esters from various lipid classes were prepared as described by Deven & Manocha (1975). A gas chromatograph (Varian model 3700; Varian Instrument Division, Palo Alto, Calif., U.S.A.) equipped with a flame ionization detector was used for analysis. A glass column (180 cm × 2 mm i.d.), packed with 3% (w/v) SP-2330 on 100 to 120 Supelcoport, was temperature

programmed from 120 to 220 °C at 3 °C min⁻¹. The injector and detector temperatures were 200 °C and 300 °C, respectively. Identification of fatty acids was achieved by (i) comparison of retention times with authentic standards and (ii) co-chromatography with known fatty acid samples. Quantitative estimation of the peak areas was made according to the method of Carrol (1961).

RESULTS

Virulence of P. brasiliensis strains

The four strains of *P. brasiliensis* used in this study demonstrated a marked difference in their virulence for hamsters and mice. Autopsy performed on all animals infected with any strain, sacrificed in the first and second month, revealed paracoccidioidomycotic nodules of various sizes scattered throughout the mesentery. There was no evidence of infection of the viscera in any of the animals challenged with strain Pb140. However, mesenteric nodules removed from these animals and stained with a vital stain (Berliner & Reca, 1966) revealed non-viable, deteriorated yeast-like cells mostly without their cytoplasm. Thus, strain Pb140 proved to be avirulent whereas strains Pb141 and Pb168 were virulent under our laboratory conditions. Lesions containing viable cells of the pathogen were observed in both the hamsters and two strains of mice inoculated with Pb141 and Pb168. Inoculation with Pb9, a strain of relatively low virulence, led to the development of lesions in the hamster but it did not produce disease and could not be recovered from experimentally infected mice.

Lipid yield and fatty acid composition

The total lipid yields of *P. brasiliensis* strains were as follows: Pb140, 7.4 to 8.2%; Pb9, 9.0 to 9.8%; Pb141, 6.3 to 6.8%; Pb 168, 4.6 to 5.3%. There was a marked similarity in the fatty acid profiles of total lipids obtained from these strains. The major fatty acids in all the strains were palmitic acid (C_{16:0}), palmitoleic acid (C_{16:1}), stearic acid (C_{18:0}), oleic acid (C_{18:1}) and linoleic acid (C_{18:2}). Oleic acid was present in the greatest amount in all the strains followed by linoleic and palmitic acid. These three fatty acids together accounted for about 94% of the fatty acids of the virulent strains Pb141 and Pb168, up to 84% of those of the slightly virulent strain Pb9 and up to 80% of those of the avirulent strain Pb140. Both strains Pb9 and Pb140 contained a relatively higher proportion of an unidentified fatty acid than the virulent strains Pb141 and Pb168. The peak of the methyl ester of this fatty acid appeared after C_{24:0} and its mass spectrum did not conform to any known fatty acid. Besides these major fatty acids, a number of minor ones were also detected, e.g. myristic acid (C_{14:0}), myristoleic acid (C_{14:1}), pentadecanoic acid (C_{15:0}), heptadecanoic acid (C_{17:0}), arachidic acid (C_{20:0}), eicosenoic acid (C_{20:1}), eicosadienoic acid (C_{20:2}), behenic acid (C_{22:0}), docosadienoic acid (C_{22:2}) and lignoceric acid (C_{24:0}).

Polar and neutral lipid composition

Analysis of the polar and neutral lipid fractions and the further separation of these fractions into individual lipid classes by thin-layer chromatography showed that, regardless of the fungal strain, the neutral lipids were always in greater amount than their corresponding polar fractions (Table 1). The ratio of relative proportions of neutral and polar lipids varied from 3:2 in Pb9 and Pb141 to 2:1 in Pb140 and Pb168.

The four strains had similar patterns of lipid composition. Phosphatidylcholine (PC) was the dominant phospholipid followed by phosphatidylethanolamine (PE), except in the avirulent strain Pb140 where cerebroside (CER) was the second highest. The avirulent strain Pb140 contained less PC in both the phospholipid and total lipid fractions than the virulent strains. The content of diphosphatidylglycerol (DPG), expressed as a percentage of the total lipid and, in particular, of the phospholipid fraction, was greatest in Pb168 followed, in descending order, by Pb141, Pb9 and Pb140. The other phospholipids identified were

Table 1. Polar and neutral lipid content of four strains of *Paracoccidioides brasiliensis*

Lipid class*	Lipids from strain:											
	Pb140			Pb9			Pb141			Pb168		
	mg (g dry wt) ⁻¹	% of total PL or NL	% of total lipids	mg (g dry wt) ⁻¹	% of total PL or NL	% of total lipids	mg (g dry wt) ⁻¹	% of total PL or NL	% of total lipids	mg (g dry wt) ⁻¹	% of total PL or NL	% of total lipids
Polar lipids (PL)												
PI	3.4	13.3	4.2	5.1	13.2	5.4	2.6	10.2	4.1	2.5	14.8	5.0
PS	2.6	10.4	3.3	3.5	9.0	3.7	2.1	8.0	3.2	1.9	11.2	3.8
PC	4.1	16.1	5.1	9.9	25.7	10.5	6.8	26.1	10.4	4.4	26.4	8.8
PE	3.7	14.5	4.6	7.6	19.6	8.0	5.5	21.4	8.6	2.6	15.5	5.2
CER	3.9	15.6	5.0	6.3	16.4	6.7	3.4	13.2	5.3	1.8	10.8	3.6
DPG	0.9	3.7	1.2	2.5	6.6	2.7	2.5	9.5	3.8	1.8	10.7	3.6
PA	3.1	12.4	3.9	2.4	6.3	2.6	1.5	5.8	2.3	1.7	10.3	3.4
Unidentified	3.5	13.9	4.4	1.2	2.9	1.2	1.4	5.4	2.1	ND	ND	ND
Total	25.2		31.7	38.5		40.8	25.8		39.8	16.7		33.4
Neutral lipids (NL)												
Sterol and fatty acid esters												
Triglycerides	5.3	9.9	6.7	10.1	17.9	10.6	2.9	7.5	4.5	5.3	16.0	10.6
Free fatty acids	35.6	66.1	44.9	36.7	65.1	38.6	16.4	42.0	25.3	15.1	45.3	30.1
Diglycerides	4.8	8.9	6.0	0.8	1.5	0.8	2.2	5.7	3.4	5.5	16.6	11.0
Sterols	1.1	2.0	1.4	3.7	6.6	3.9	11.2	28.6	17.2	1.3	4.0	2.6
Monoglycerides	4.8	8.9	6.0	3.8	6.9	4.0	5.1	12.9	7.8	4.9	14.6	9.7
Total	2.1	4.0	2.7	1.0	1.8	1.1	1.3	3.2	1.9	1.1	3.3	2.2
	53.7		67.7	56.1		59.0	39.1		60.1	33.2		66.2

ND, Not detected.

* PI, Phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CER, ceresbroside; DPG, diphosphatidylglycerol; PA, phosphatidic acid.

phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA). There was no marked difference in the pattern of their distribution within the four strains. The unidentified lipid was not detected in Pb168, probably because of its presence in extremely small amounts.

The major fatty acids in the various polar lipid classes of the four strains of *P. brasiliensis* were palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) and together they comprised 82 to 98% of the total fatty acids of the individual polar lipid classes (Fig. 1). Small amounts of the other fatty acids previously listed were also detected. The avirulent strain Pb140, and to a lesser extent the slightly virulent strain Pb9, contained relatively greater amounts of the unidentified fatty acid in their phospholipid classes than the two virulent strains Pb141 and Pb168. In all four strains PI and PS had a similar fatty acid distribution with palmitic acid being present in greatest amount whereas both PC and PE showed similar fatty acid profiles with oleic acid as the predominant fatty acid. In all four strains DPG was the least saturated lipid with linoleic acid as the predominant fatty acid. The CER of different strains differed slightly with respect to the relative levels of oleic acid and linoleic acid whereas in PA the distribution of fatty acids was random and revealed no pattern.

The separation of neutral lipids into various classes (Table 1) clearly showed that triglycerides represented the most predominant lipid class in all strains. They constituted 25 to 45% of the total lipid with the greater amount being present in the avirulent Pb140 and slightly virulent Pb9 strains [36 mg (g dry wt)⁻¹] as compared to the virulent strains Pb141 and Pb168 [15 to 16 mg (g dry wt)⁻¹]. Free fatty acids, sterols and sterol esters apparently did not show any preferential distribution in the four strains. Similarly, these classes of neutral lipid did not develop any pattern in the distribution of their fatty acids. However, the triglycerides, diglycerides and monoglycerides of the virulent strains Pb141 and Pb168 differed from the avirulent Pb140 and slightly virulent Pb9 strains with respect to the percentage of unidentified fatty acid. The latter two strains possessed greater amounts of this acid than of oleic acid, which was the predominant fatty acid of the virulent strains Pb141 and Pb168.

DISCUSSION

The total lipid content of the four strains of *P. brasiliensis*, cultured under identical conditions, varied considerably. However, the quantity of lipid obtained from a particular strain appeared characteristic and showed reasonable agreement between replicate studies. The lipid composition of *P. brasiliensis* was qualitatively similar to other human pathogenic fungi, e.g. *H. capsulatum* and *B. dermatitidis* (Domer & Hamilton, 1971) and *C. immitis* (Anderes *et al.*, 1973). In all these fungi neutral lipids are the major component of the total lipids and triglycerides are the greatest single lipid class in the extracts. Among phospholipids, phosphatidylcholine and phosphatidylethanolamine are the most prominent fractions.

Paracoccidioides brasiliensis differed from other pathogenic fungi in the lack of any apparent correlation between virulence and the total lipid or phospholipid content of different strains. In four strains of *B. dermatitidis*, Di Salvo & Denton (1963) observed a positive correlation between total lipid in yeast-phase cells and mouse virulence, though they did not observe a corresponding correlation for phospholipid fractions. Cox & Best (1972), on the other hand, suggested a possible relationship between phospholipids and virulence in *B. dermatitidis*. Similar results showing a greater amount of total lipid in the arthrospores of the virulent strain of *C. immitis* as compared to the non-virulent mutant were obtained by Anderes *et al.* (1973). Probably, a higher lipid content protects the micro-organism against the host defence mechanisms. This possibility was tested by Anderes *et al.* (1971) who suggested that exposure of a micro-organism to antimicrobial agents tended to

Fig. 1. Content of palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids in the polar lipid classes of the four strains of *Paracoccidioides brasiliensis*: ▲, Pb141; △, Pb168; ●, Pb140; ○, Pb9. Polar lipid classes: PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CER, cerebroside; DPG, diphosphatidylglycerol.

produce a much higher lipid content. Nielsen (1966) examined six isolates of *H. capsulatum* and found that neither the total lipid nor phospholipid showed any correlation with virulence: 50% or more of the phospholipids were phosphoinositides, and PE was present in much greater concentration than PC. The latter was absent in the less virulent strains. These findings were very different from those of Domer & Hamilton (1971), who observed higher concentrations of PC than of PE and PI in different strains of *H. capsulatum* and *B. derma-*

titidis. Such discrepancies could be due to differences in cultural conditions, extraction procedures, methods of separation and of identification of lipid classes, all of which could influence the results.

Our data showed that PC was the predominant phospholipid with the concentration in virulent and slightly virulent strains being considerably greater than in the avirulent strain. The four strains of *P. brasiliensis* also contained appreciable amounts of cerebrosides, not reported in other pathogenic fungi. A near relative, sphingolipid, has been reported in *H. capsulatum* (Nielsen, 1966). In *B. dermatitidis* and *C. albicans*, besides PC and PE, Peck (1947) reported the presence of an unidentified carbohydrate-containing lipid and stated that this lipid was found mostly in association with pathogenic as opposed to non-pathogenic fungi. It is possible that the unidentified substance was a cerebroside. The biological function of these lipids is not fully understood, but their role in immunological activity and as constituents of cellular membranes is well known.

Most of the literature dealing with lipid analyses in relation to virulence of pathogenic fungi pays little attention to the neutral lipids, even though the latter constitute 60% or more of the total yield. Analyses of neutral lipids from extracts of *P. brasiliensis* strains revealed six different classes of lipids with triglycerides being the most abundant. The amount of triglycerides was greatest in the avirulent strain and least in the virulent strains. The latter strains also possessed a smaller percentage of an unidentified fatty acid in their triglycerides than the avirulent and slightly virulent strains. Although triglycerides represent the most efficient form of energy storage, their relative amounts depend on the culture conditions and stage of growth. The contribution triglycerides make to the virulence of strains of *P. brasiliensis* remains to be established.

The only indication, if any, of the possible association of phospholipids with virulence was provided by the DPG content of different strains of *P. brasiliensis*. Diphosphatidylglycerol was the least saturated of the lipids present and contained greater amounts of linoleic acid than of either oleic or palmitic acid. The greatest amount was found in the virulent strains and the least in the avirulent strain. This probably reflects the higher metabolic status of the virulent strains since DPG is generally associated with mitochondria or cellular organelles of high metabolic activity.

There is strong evidence for the participation of lipid intermediates in the synthesis of β -glucan and cellulose in plants (Hopp *et al.*, 1978). The literature on the role of lipid-like intermediates in fungal cell wall biosynthesis is sparse, but the involvement of a lipid intermediate in β -glucan biosynthesis has been postulated (Brett & Northcote, 1975). In a recent review on the biochemistry of the cell wall of *P. brasiliensis*, San-Blas & San-Blas (1977) have shown a definite correlation between the cell wall composition, especially the α -glucan content, and the degree of virulence of a particular strain of *P. brasiliensis*. They have suggested that the constituent macromolecules of the cell wall play an important role in the active protection of the fungus against the defensive mechanisms of the host. It seems pertinent to undertake further investigations on the macromolecular biosynthesis and architecture of the cell wall of *P. brasiliensis* in order to identify those molecules that might be correlated with virulence.

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