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Cell Wall Composition of Two Strains of *Blastomyces dermatitidis* Exhibiting Differences in Virulence for Mice

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Cell walls isolated from two strains of *Blastomyces dermatitidis* were examined. Whereas strain Ga-1 was practically avirulent for mice, strain KL-1 produced death by 21 days in 50% of the mice inoculated. Analyses of the trypsin-treated cell walls of the two strains revealed a higher chitin and protein content in strain KL-1, whereas a higher polysaccharide content was observed in the cell walls of strain Ga-1. Extraction of the walls with 1 N NaOH revealed a threefold difference in the amount of alkali-soluble cell wall material present. The alkali-soluble material could be further fractionated into a water-soluble and a water-insoluble fraction. Previous reports have indicated that the water-insoluble fraction of *B. dermatitidis* consists of an alpha-linked glucan; however, we report that in addition a phospholipid moiety is covalently bound to the polysaccharide. Furthermore, on the basis of organic phosphorus content, considerably more phospholipid is associated with the alpha-linked glucan of the more virulent KL-1 strain. These results suggest that this cell wall constituent might be one of the factors related to the virulence of this fungus.

Few attempts have been made to determine what cellular factor(s) might be responsible for the virulence of *Blastomyces dermatitidis*. Di Salvo and Denton (4) previously investigated the relative virulence of four *B. dermatitidis* strains. These authors observed a higher total lipid content in the yeast-phase cells of the more virulent strains. This is the only study which has attempted to relate the chemical composition of strains of *B. dermatitidis* to their virulence.

Kanetsuna and Carbonell (7) analyzed the cell wall of the yeast-phase of one strain of *B. dermatitidis*. These workers reported that the walls contain 37% amino sugar, 36.2% total hexoses, 7.8% amino acids, 4.3% nitrogen, and 0.12% phosphorus.

The present investigation was initiated to explore the chemical basis for the differences observed in the mouse virulence of two *B. dermatitidis* strains. The availability of a virulent and an avirulent strain suggested that a comparison of the chemical composition of cell walls might reveal significant differences in the two strains.

MATERIALS AND METHODS

Cultures. *B. dermatitidis* strain Ga-1 was isolated from a human case of chronic cutaneous blastomycosis. Mice inoculated intravenously with viable yeast-phase cells of this strain survived for 90 days (4). The other strain, KL-1, was isolated from a Lexington, Kentucky, soil sample and produced death within 21 days in 50% of the mice injected (3, 4).

Yeast-phase cells of the two strains were grown on Brain Heart Infusion agar slants (Difco, Detroit, Mich.) for 7 days at 37 C and inoculated into Kolle flasks containing 125 ml of Brain Heart Infusion agar. After 7 days of incubation at 37 C, the cells were harvested in 0.01 M potassium phosphate buffer (*p*H 7.2) and killed by five cycles of freezing and thawing.

Preparation of cell walls. Approximately 20 g of killed, whole yeast-phase cells were suspended with an equal amount of glass beads (0.45 to 0.55 mm in diameter) in 0.01 M potassium phosphate buffer (*p*H 7.2) and shaken in a Braun model MSK mechanical homogenizer for 90 seconds. This procedure resulted in complete rupture of more than 95% of the cells as indicated by microscopic examination. A CO_2-cooling device was employed to minimize heat effects during this procedure. Cell walls were recovered by differential centrifugation at 4 C and washed repeatedly with distilled water to remove cytoplasmic constituents.

The crude cell walls were partially purified by incubation with trypsin (100 μg/ml) in 0.01 M potassium phosphate buffer (*p*H 7.2) for 4 hr at 37 C. After trypsin treatment, the suspensions were centrifuged at 27,000 $\times$ *g* for 10 min, the supernatant solution was discarded, and the cell wall pellet was washed repeatedly with distilled water.

Extraction of cell walls with NaOH. Trypsin-treated

cell walls (100 mg) were suspended in 60 ml of 1 N NaOH and incubated for 3 hr at 37 C in a shaking water bath. After centrifugation at 27,000 × *g* for 15 min, the supernatant solutions were combined and passed through a membrane filter (0.45-μm pore size; Millipore Corp.) to remove any particulate material. The insoluble cell walls and the alkali-soluble supernatant material were dialyzed at 4 C for 72 hr against distilled water to remove the NaOH. The preparations were lyophilized and then weighed to determine the amount of nondialyzable material released by NaOH.

Lipid extraction of NaOH-soluble wall material. To remove any lipid not covalently bound, the alkali-soluble material was suspended in 10 ml of ethanol and incubated at 55 C for 15 min. Twenty ml of chloroform was then added, and the mixture transferred to 25 C for an additional 30 min. The insoluble residue was collected by filtration on glass fiber filters (Millipore Corp., Bedford, Mass., 0.45-μm pore size) and washed twice with 30 ml of chloroform-ethanol (2:1, v/v). The chloroform-ethanol filtrate, which contained readily extractable lipids, was not examined further. Covalently bound lipid, however, was then dissociated from the particulate wall material by hydrolysis with 0.05 N HCl at 95 C for 30 min and extracted with chloroform-methanol (2:1, v/v).

The chloroform-methanol-soluble lipid was chromatographed on thin layers of Silica Gel G with diisobutylketone-acetic acid-water (40:25:5, v/v/v) and petroleum ether-diethyl ether-acetic acid (90:10:1, v/v/v) as the solvent systems. Lipids were detected using rhodamine 6G, iodine vapor, ninhydrin, and the phospholipid spray of Dittmer and Lester (5). The method of Levine and Chargaff (9) was used to detect lipids containing choline.

Chemical analyses. The total hexosamine content present in the two cell-wall preparations was determined by the procedure of Strominger et al. (14) after acid hydrolysis. Preliminary experiments indicated that the maximal release of amino sugars was obtained with 6 N HCl, 110 C, for 16 hr. Descending paper chromatography with butanol-pyridine-water (9:5:4, v/v/v) indicated that only glucosamine was present in the hydrolysates. This amino sugar was located on chromatograms using aniline hydrogen phthalate or ninhydrin spray reagents, or both.

The Morgan-Elson procedure (11) was used to measure quantitatively the *N*-acetylglucosamine released from the cell walls by chitinase (Calbiochem, Los Angeles, Calif.). For these experiments, 100 mg of trypsin-treated cell walls was incubated in 12 ml of 0.1 M sodium-acetate buffer (*p*H 5.0) to which 18 mg of chitinase was added. The walls were incubated at 37 C for 7 days in a shaking water bath. After incubation, the suspensions were filtered (Millipore Corp., 0.45-μm pore size) to remove insoluble cell wall residue, and the supernatant fraction was analyzed for *N*-acetylglucosamine. This procedure was essentially that described by Kanetsuna et al. (8).

Maximal release of polysaccharide glucose was obtained after hydrolysis with 1 N HCl at 110 C for 7 hr. The hydrolysates were neutralized with NaOH and filtered (Millipore Corp., 0.45-μm pore size), and the glucose content was determined by glucose oxidase (Glucostat, Worthington Biochemicals, Inc., Freehold, N. J.) with D-glucose as the standard.

The amino acid composition of the cell walls was determined using 1-fluoro-2,4-dinitrobenzene with DL-glutamic acid as the standard by the method of Ghuysen and Strominger (6). The walls were first hydrolyzed with 6 N HCl at 110 C for 18 hr. All hydrolysates were neutralized with NaOH and filtered (Millipore Corp., 0.45-μm pore size) prior to analysis. These hydrolysates were also compared by automatic amino acid analysis using a Beckman-Spinco amino acid analyzer.

Phosphorus was determined by the method of Chen et al. (2) using samples hydrolyzed with 6 N HCl at 110 C for 18 hr. The extremely small amount of inorganic phosphorus present prior to acid hydrolysis was subtracted from the total to achieve an indication of the organic phosphorus content.

RESULTS

In preliminary experiments, isolated cell wall preparations from the yeast phase of each strain of *B. dermatitidis* were acid-hydrolyzed under various conditions and analyzed for likely constituents using paper chromatography. These results confirmed previous reports (7) that the major hexose is glucose, that the only amino sugar detectable after acid hydrolysis is glucosamine, and that a large number of amino acids are present after trypsin purification of the cell walls.

Since there were no readily apparent qualitative differences in the wall composition of the two strains, quantitative comparisons of known wall constituents were made. First, an indication of the relative amounts of chitin present in the walls of the two strains was obtained by measuring the release of hexosamine by acid hydrolysis or chitinase (Table 1). The *B. dermatitidis* strain analyzed by Kanetsuna and Carbonell (7) was reported to contain 37% chitin. Assuming all hexosamine liberated from the walls by acid hydrolysis originated from chitin and there was no destruction of amino sugar during hydrolysis, the chitin content in the cell walls of strains Ga-1 and KL-1 is 24 and 43%, respectively (Table 1). This indication of a higher chitin content in the more virulent strain was confirmed by the finding that more *N*-acetylglucosamine could be released from this strain by commercial chitinase. Since the purity of this enzyme preparation is not known and no chitin standard was available for comparison, the results with acid hydrolysis were considered more indicative of the chitin content.

Kanetsuna and Carbonell (7) found that 35% of the yeast-phase cell wall could be solubilized with 1 N NaOH at 20 C. As shown in Table 2, alkaline extraction of cell walls from Ga-1 and KL-1 revealed significant differences in the amount of cell wall which could be solubilized

TABLE 1. *Chitin content of trypsin-treated cell walls of Blastomyces dermatitidis strains Ga-1 and KL-1*

Strain	Hexosamine released by	
	Chitinase[a] (μmoles/mg)	6 N HCl[b] (μmoles/mg)
Ga-1	0.88	1.03
KL-1	0.97	1.59

[a] Trypsin-treated cell walls (100 mg) of each strain were incubated with chitinase as described in Materials and Methods. Hexosamine content (as *N*-acetylglucosamine) was obtained by the method of Morgan and Elson (11).

[b] Trypsin-treated cell walls (100 mg) were hydrolyzed at 110 C for 16 hr with 6 N HCl (in vacuo), neutralized with NaOH, and filtered to remove insoluble material, and the filtrate was analyzed for amino sugar (with standard glucosamine) by the method of Strominger et al. (14). All values represent the average of at least three separate analyses.

TABLE 2. *Alkaline extraction of cell walls of Blastomyces dermatitidis strains Ga-1 and KL-1*[a]

Strain	Trypsin-treated cell walls (mg dry wt)	NaOH-extracted cell walls (mg dry wt)	NaOH-soluble extract (mg dry wt)	Total recovery (%)
Ga-1	851.0	398.5	449.8 (52.8%)	98.5
KL-1	991.0	734.9	169.0 (17.1%)	91.2

[a] Cell walls of the two strains were extracted with 1 N NaOH as described in Materials and Methods. The insoluble wall residue remaining after alkali extraction and the nondialyzable, soluble extract were then compared on a dry weight basis. The percentage of the wall recovered as nondialyzable, soluble material is depicted in parentheses and represents the average of two separate analyses.

with NaOH. Whereas 53% of the dry weight of cell walls from Ga-1 could be solubilized by this treatment, only 17% of the wall from strain KL-1 could be recovered as nondialyzable, soluble material after alkaline extraction. Another difference apparent in this table is the lower recovery of material from KL-1 extracted walls. This could represent the loss of low-molecular-weight material during dialysis to remove the NaOH, however it would not account for the significant differences in the two strains and was not pursued.

Since a significant difference in the amount of cell wall material solubilized by alkali was observed, additional analyses were made to determine the nature of the alkali-soluble material and the residual insoluble fraction of the two strains. Kanetsuna et al. (7, 8) reported that two types of glucans, distinguished by their solubility in 1 N NaOH, were present in the cell walls of their strain. An alkali-soluble glucan, comprised of alpha-1,3-glycosidic linkages, represented about 95% of the total cell-wall glucan. The remaining alkali-insoluble glucan was reported to be beta-1,3-linked. The data presented in Table 3 compare the glucan content of cell walls before and after alkaline extraction. As indicated, no significant differences were detected in the total glucose content of the trypsin-treated cell walls. Fractionation of the glucans with 1 N NaOH, however, revealed that 84.3% of the cell wall glucose is alkali-soluble in strain Ga-1 as compared to only 72.5% in the KL-1 strain. Thus, these results would indicate that trypsin-treated walls of the less virulent Ga-1 strain contain more alpha-linked glucan, whereas those of the KL-1 strain contain more of the alkali-insoluble (β-linked) glucan.

Since the cell walls and the available fractions from the walls were insoluble, an indication of the protein content was made indirectly by measuring the release of amino acids after acid hydrolysis. As shown in Table 4, considerably more protein is present in the trypsin-treated cell walls of the more virulent KL-1 strain. Kanetsuna and Carbonell's report (7) of 7.8% protein in trypsin-treated walls is comparable to our finding of 5.8 and 10.5% in strains Ga-1 and KL-1, respectively. Automatic amino acid analyses of trypsin-treated cell walls revealed that both strains possess the same amino acids and in approximately the same molar ratio (*unpublished data*). After alkali extraction, the protein content of the residual wall material is 0.96 and 0.87 μmole (per mg dry weight) in strains Ga-1 and

TABLE 3. *Relative glucan composition of cell walls of Blastomyces dermatitidis strains Ga-1 and KL-1*[a]

Strain	Trypsin-treated cell walls (μmoles/mg)	NaOH-extracted cells walls (μmoles/mg)
Ga-1	1.7–1.8	0.25–0.30
KL-1	1.4–1.5	0.38–0.42

[a] Cell walls of the two strains which had been purified by trypsin treatment were compared for glucose content before and after mild alkaline extraction. In each case, the walls were hydrolyzed in vacuo with 1 N HCl at 110 C for 7 hr, neutralized, and filtered, and the clear filtrate was analyzed for glucose with the Glucostat reagent as described in Materials and Methods. The values indicated are the ranges observed in at least three separate analyses.

KL-1, respectively (Table 4). These values could reflect a difference in the NaOH solubility of the cell wall proteins, or alternatively, could reflect differences in the molar ratio of solubilized constituents.

An analysis of the NaOH-soluble cell wall material (Table 5) showed 4.4 and 3.73 μmoles of glucose per mg dry weight. Assuming no loss of glucose during hydrolysis, this fraction consists of 79 and 67% glucan in strains Ga-1 and KL-1, respectively. Infrared absorption spectra (KBr pellet) revealed a prominent band at 845 cm^{-1} which could be consistent with Kanetsuna's previous report (7) that the glucan in this fraction is composed of alpha-glycosidic linkages (Cox and Best, *unpublished data*).

As shown in Table 5, the protein content of the alkali-soluble material of strain KL-1 is 0.93 μmole per mg compared to only 0.48 μmole in strain Ga-1. A surprising finding, based on the analyses of Kanetsuna and Carbonell (7), was the presence of organic phosphorus in the alkali-soluble material. Since considerably more phosphorus was associated with the alkali-soluble fraction of the more virulent KL-1 strain, additional analyses were conducted to determine the nature of this component. A number of attempts were made to dissociate the phosphorus-containing moiety from the alpha-linked glucan. So far only mild hydrolysis liberates the organic phosphorus. The phosphorus-containing constituent has not yet been identified, but it could be a phospholipid since it can be detected on thin-layer chromatograms by rhodamine 6G, iodine, the phospholipid spray of Dittmer and Lester (5), and the periodate-Schiff reagent (10). Although the relative migration on thin-layer plates using diisobutylketone-acetic acid-water (40:25:5, v/v/v) and petroleum ether-diethyl ether-acetic acid (90:10:1, v/v/v) as solvents

TABLE 4. *Protein content of cell walls of Blastomyces dermatitidis strains Ga-1 and KL-1*[a]

Strain	Trypsin-treated cell walls (μmoles/mg dry wt)	NaOH-extracted cell walls (μmoles/mg dry wt)
Ga-1	0.58	0.96
KL-1	1.05	0.87

[a] Cell walls from each strain were analyzed for amino acid content before and after mild alkali extraction. In both cases, the walls were hydrolyzed with 6 N HCl at 110 C for 18 hr, neutralized, and filtered, and the filtrate was analyzed for *N*-terminal amino acids with 1-fluoro-2,4-dinitrobenzene as described in Materials and Methods. The values indicated are the average of three separate analyses.

TABLE 5. *Chemical composition of alkali-soluble material from Blastomyces dermatitidis cell walls*

Strain	Glucose[a] (μmoles/mg)	*N*-acetylglucosamine[b] (μmoles/mg)	Protein[c] (μmoles/mg)	Organic phosphorus[d] (nmoles/mg)
Ga-1	4.40	0.01	0.48	7.2
KL-1	3.73	0.04	0.93	105.3

[a] After hydrolysis with 1 N HCl, 110 C, 7 hr; analyzed for glucose with Glucostat reagent.

[b] After hydrolysis with 6 N HCl, 110 C, 16 hr; analyzed for glucosamine as described in Table 1.

[c] After hydrolysis with 6 N HCl, 110 C, 18 hr; analyzed for amino acids as described in Table 4 and Materials and Methods.

[d] After hydrolysis with 6 N HCl, 110 C, 18 hr; analyzed for phosphorus by the method of Chen et al. (2).

approximates those of a lecithin standard and the material is ninhydrin negative, no choline could be detected with the reagent described by Levine and Chargaff (9). The absence of fatty-acid side chains, as indicated by a positive periodate-Schiff reaction, could result from saponification during the alkaline extraction required to isolate this fraction. Whether further chemical destruction occurs during the subsequent mild acid hydrolysis required to dissociate the phospholipid from the glucan is not yet known. These results indicate that the cell walls of the KL-1 strain contain significantly greater amounts of a material which behaves like a phospholipid, but final identification will depend on the development of extraction techniques which will allow its removal from the walls without chemical alteration. Alternative methods such as extraction with ethylenediaminetetraacetic acid and hot phenol-water have not been successful.

Recently, the alkali-soluble material has been further fractionated into a water-soluble and a water-insoluble fraction (*unpublished data*). Preliminary studies indicate that, although considerably more of the water-soluble fraction is present in the alkali-soluble extract of the KL-1 strain, this material is chemically identical in the two strains and is comprised of protein, organic phosphorus, and a polysaccharide of glucose, galactose, and mannose. These results would be consistent with the analyses of the alkali-soluble extracts of strains KL-1 and Ga-1 (Table 5).

The water-insoluble fraction is composed primarily of the alpha-linked glucan, but trace amounts of the phosphorus constituent were detected in the water-insoluble fraction from Ga-1, and significantly greater levels of this component

were present in this fraction from the KL-1 strain. Since these studies suggest a possible correlation between organic phosphorus content and the virulence of these two strains, additional studies are now in progress to further characterize the alkali-soluble fractions of the two strains.

DISCUSSION

Trypsin-treated cell walls of a relative avirulent and a virulent strain of *B. dermatitidis* showed only slight differences in total polysaccharide, protein, and chitin content. In general, more chitin and protein were observed in the cell walls of the virulent strain (KL-1), whereas a slightly higher polysaccharide (as glucose) content was present in the walls of the less virulent strain (Ga-1).

Fractionation of the trypsin-treated walls by the method of Kanetsuna et al. (8) with 1 N NaOH revealed significant differences in the relative amounts of cell wall material solubilized by alkali. Whereas Kanetsuna and Carbonell (7) previously reported 35% of the cell wall to be alkali-soluble, we found 52.8 and 17.1% in strains Ga-1 and KL-1, respectively.

Our data are in basic agreement with those of Kanetsuna and Carbonell (7) in that the solubilized cell wall component is comprised mainly of an alpha-linked glucan polymer. Whereas these authors did not detect organic phosphorus in the alkali-soluble extract from their organism, material of this nature can be detected in trace amounts from strain Ga-1 and in significant amounts in the strain KL-1. Furthermore since this component, tentatively identified as a phospholipid, was dissociated only by mild acid hydrolysis, its covalent linkage to the alpha-glucan seems apparent. Since the virulence of Kanetsuna's strain was not reported, it would be of interest to determine if a phospholipid is also present in addition to the alpha-linked glucan of his strain and, if so, to examine the possibility that a direct relationship exists between this cell wall constituent and virulence.

A number of years ago Peck and Hauser (13) obtained small amounts of a phospholipid-containing carbohydrate fraction from yeast-phase cells of *B. dermatitidis*, and, although the chemical characterization was not pursued by these authors, a later study (12) indicated that the constituent is associated with pathogenic rather than nonpathogenic fungi. Baker (1) subsequently reported that a "phosphotide" fraction obtained from whole cells of *B. dermatitidis* (yeast phase) evoked a mononuclear response in mice. Interestingly, the phospholipid preparation, isolated by the method of Peck and Hauser (13), would have contained small amounts of the phospholipid-containing carbohydrate fraction. Since the mononuclear cell is the predominant cell type in the granulomatous response of blastomycosis, studies are presently in progress to determine if the phospholipid-polysaccharide cell wall constituent isolated in this study might be directly responsible for this pathological reaction.

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LITERATURE CITED

1. Baker, R. D. 1942. Experimental blastomycosis in mice. Amer. J. Pathol. 18:463–478.
2. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem. 28:1756–1758.
3. Denton, J. F., E. S. McDonough, L. Ajello, and R. J. Ausherman. 1961. Isolation of *Blastomyces dermatitidis* from soil. Science 133:1126–1127.
4. Di Salvo, A. F., and J. F. Denton. 1963. Lipid content of four strains of *Blastomyces dermatitidis* of different mouse virulence. J. Bacteriol. 85:927–931.
5. Dittmer, J. C., and R. J. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. 5:126–127.
6. Ghuysen, J., and J. L. Strominger. 1963. Structure of the cell wall of *Staphylococcus aureus* strain Copenhagen. I. Preparation of fragments by enzymatic hydrolysis. Biochemistry 2:1110–1119.
7. Kanetsuna, F., and L. M. Carbonell. 1971. Cell wall composition of the yeastlike and mycelial forms of *Blastomyces dermatitidis*. J. Bacteriol. 106:946–948.
8. Kanetsuna, F., L. M. Carbonell, R. E. Moreno, and J. Rodriquez. 1969. Cell wall composition of the yeast and mycelial forms of *Paracoccidioides brasiliensis*. J. Bacteriol. 97:1036–1041.
9. Levine, C., and E. Chargaff. 1951. Procedures for the microdetermination of nitrogenous phosphatide constituents. J. Biol. Chem. 192:465–479.
10. Marinetti, G. V. 1967. Paper chromatography of phosphatides and glycolipids, p. 8. *In* Lipid chromatographic analysis. Marcel Dekker, Inc., New York.
11. Morgan, W. T. J., and L. A. Elson. 1934. A colorimetric method for the determination of N-acetylglucosamine and N-acetylchondrosamine. Biochem. J. 28:988–995.
12. Peck, R. L. 1947. The lipids of fungi with special reference to pathogenic fungi, p. 167–1888. *In* W. J. Nickerson (ed.), Biology of pathogenic fungi. Chromica Botanica Co., Waltham, Mass.
13. Peck, R. L., and C. R. Hauser. 1938. Chemical studies of certain pathogenic fungi. I. The lipids of *Blastomyces dermatitidis*. J. Amer. Chem. Soc. 60:2599–2603.
14. Strominger, J. L., J. T. Park, and R. E. Thompson. 1959. Composition of the cell wall of *Staphylococcus aureus*: its relation to the mechanism of action of penicillin. J. Biol. Chem. 234:3263–3268.