

Yeast- and Mycelial-Phase Antigens of *Blastomyces dermatitidis*: Comparison Using Disc Gel Electrophoresis

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The disc gel electrophoretic patterns obtained with skin test-active (mycelial and yeast) antigens of *Blastomyces dermatitidis* were compared. As would be expected, the blastomycins (mycelial-phase) and the cytoplasmic ultrafiltrates (yeast-phase) were heterogeneous mixtures containing proteins, glycoproteins, lipoproteins, and carbohydrates. The skin test-active cytoplasmic ultrafiltrates and the blastomycins contained glycoproteins that had similar R_f values which allows the possibility that one or more of these components is responsible for the skin-test reactivity of these antigens. The electrophoretic migration of the alkali-soluble, water-soluble cell wall antigen differed from those of the cytoplasmic antigens and the two blastomycins. Electrophoresis, Sephadex chromatography, and ultrafiltration studies showed that the alkali-soluble, water-soluble cell wall antigen is comprised of lipid, polysaccharide, and protein and has a molecular weight range of 30,000 to 50,000. The increased number and mobility of both the protein and carbohydrate bands after denaturation and electrophoresis of this antigen in sodium dodecyl sulfate indicate that there are several cross-linkages between the polysaccharide and/or protein moieties, possibly via lipid or disulfide bridges.

In the preceding paper (3) data was presented showing that a yeast-phase cell wall antigen and, to a lesser extent, yeast-phase cytoplasmic ultrafiltrates exhibited greater specificities in eliciting delayed-type cutaneous reactions than did two blastomycins (mycelial-phase filtrates). Since the biological activities of these antigens differed considerably, we initiated a study to compare them on a chemical basis. Assuming that the skin test-active component(s) is a protein, or at least contains a protein moiety, and migrates as an anion at pH 8.3, then the antigens could be compared on the basis of their electrophoretic migrations in acrylamide gels.

MATERIALS AND METHODS

Blastomyces antigens. The skin test-active fractions of *Blastomyces dermatitidis* strain SCB-2 (ATCC 26199) were obtained from yeast-phase cells as previously described (3). These fractions include the cytoplasmic ultrafiltrates (XM-50, PM-30, and PM-10) and the alkali-soluble, water-soluble (ASWS) cell wall antigen.

Commercial blastomycin (Parke-Davis and Co., Detroit, Mich.) and blastomycin KCB-25 were concentrated by lyophilization prior to disc gel electrophoresis. Five milliliters of the undiluted antigens

was lyophilized and then resuspended in 0.1 to 0.5 ml of distilled water. A considerable amount of insoluble material was present in the commercial antigen after lyophilization and was removed by filtration (Millipore Corp., Bedford, Mass.; 0.45- μ m pore size) prior to electrophoresis.

Disc gel electrophoresis. The method used for acrylamide disc gel electrophoresis was essentially that described by Davis (4). Glass columns measuring 5 by 75 mm were filled with a 7.5% lower separator gel (55 mm in length) and an upper 3.5% spacer gel (7 mm). Samples containing 50 to 200 μ g of total protein were suspended in 10% sucrose and layered on the spacer gels. Electrophoresis was performed at room temperature in a tris(hydroxymethyl)aminomethane-glycine buffer (pH 8.3) at a constant current of 5 mA per column. When the tracking dye (bromophenol blue) had migrated to a point approximately 1 cm above the bottom of the gel column, electrophoresis was terminated, and the gels were stained with 1% Amido Schwartz. Afterwards, the excess dye was removed electrophoretically in 7.5% acetic acid, and the gels were scanned at 560 nm with a Gilford model 540 spectrophotometer.

Duplicate gels were stained with the periodate acid-Schiff reagent for carbohydrates. After electrophoresis, the gels were fixed in 7.5% acetic acid and then placed in a 20% periodate solution for 1 h. The periodate was removed electrophoretically, and the oxidized components were stained with Schiff reagent. For lipid-containing constituents, one part

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Sudan Black B (saturated in ethylene glycol) was added to two parts antigen sample, and the mixture was placed at 4 C for 24 h. Electrophoresis of the prestained samples was performed as described above. Gels were scanned at 545 and 590 nm to determine the mobilities of the periodic acid-Schiff stain-positive and Sudan Black-positive bands, respectively.

Electrophoresis of the ASWS antigen in the presence of sodium dodecyl sulfate (SDS) was performed as described by Shapiro et al. (9), except that 7.5% acrylamide gels were used. Samples were first denatured in 1% 2-mercaptoethanol and 1% SDS at 37 C for 3 h. Electrophoresis was then performed at a constant current of 8 mA per gel in a 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1% SDS. After electrophoresis, the gels were fixed in 20% sulfosalicylic acid for 16 h, stained with 0.25% Coomassie Blue for 5 h, and destained with 7.5% acetic acid. The gels were stained with the periodic acid-Schiff stain reagent (for carbohydrates) as described above.

Chemical analyses. Total protein content was determined by the procedure of Lowry et al. (7) with bovine serum albumin as the standard. The amino acid composition of the cell walls was determined by using 1-fluoro-2,4-dinitrobenzene, with DL-glutamic acid as the standard, by the method of Ghuyzen and Strominger (5). Samples were first acid-hydrolyzed in vacuo with 6 N HCl at 110 C for 18 h. For carbohydrates, samples were acid-hydrolyzed with 1 N HCl at 110 C for 7 h. The hydrolysates were neutralized with NaOH and filtered (Millipore Corp.; 0.45- μ m pore size). Total reducing groups were measured according to the procedure of Park and Johnson (8) using D-glucose as the standard. Glucose was assayed enzymatically with glucose oxidase (Glucostat, Worthington, Inc., Freehold, N.J.). Phosphorus was determined by the method of Chen et al. (1) using samples hydrolyzed with 6 N HCl at 110 C for 18 h.

Gel filtration. Approximately 25 mg of the ASWS cell wall antigen was applied to a Sephadex G-50 column (Pharmacia Fine Chemicals, Uppsala, Sweden) measuring 45 by 2.5 cm. Fractions (5 ml) were eluted with distilled water and assayed for protein and, after acid hydrolysis, for reducing groups.

RESULTS

Figures 1A and 1B depict the electrophoretic migrations of commercial blastomycin and blastomycin KCB-25. Four protein bands were detected in the commercial antigen, of which two were present in extremely small amounts. It should be pointed out, however, that the electrophoretic pattern of this antigen may be misleading because of the large amount of insoluble material that was removed after lyophilization. The other mycelial-phase filtrate, blastomycin KCB-25, contains at least six proteins (Fig. 1B). One of these, band B, has a retardation factor (R_f) value of .08 and may be similar or identical to protein band A in the commercial preparation. Otherwise, the two

antigens do not appear to share common protein-staining bands.

The gel electrophoretic patterns of the three yeast-phase cytoplasmic ultrafiltrates are shown in Fig. 2A, B, and C. For convenience, we designated the protein bands in the XM-50 ultrafiltrate A through K. In the subsequent fractions (PM-30 and PM-10 ultrafiltrates), the bands approximating the R_f values of those in the XM-50 ultrafiltrate were designated accordingly. The XM-50 ultrafiltrate (Fig. 2A) is comprised of at least 11 proteins. Filtration of this fraction through a PM-30 membrane resulted in the removal of protein bands D and J that had R_f values of 0.26 and 0.80, respectively. A protein (band b, R_f value 0.11) was detected in the PM-30 ultrafiltrate but not in the XM-50 fraction. Since the electrophoretic pat-

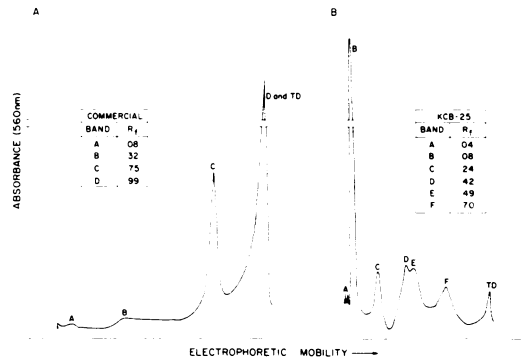


FIG. 1. Electrophoretic mobilities of proteins in commercial blastomycin (A) and in blastomycin KCB-25 (B). The tracking dye (TD) was used as a reference (R_f value, 1.0) for determination of R_f values. Arrows indicate the gel front (origin).

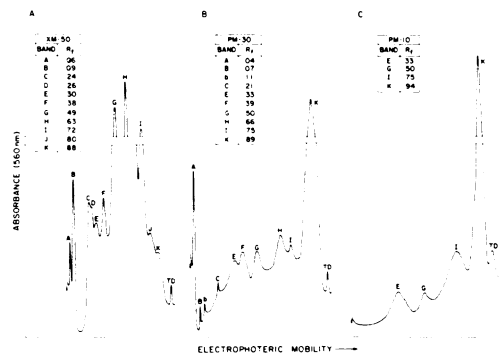


FIG. 2. Electrophoretic mobilities of proteins in the yeast-phase cytoplasmic ultrafiltrates: XM-50 (A), PM-30 (B), and PM-10 (C). The tracking dye (TD) was used as a reference (R_f value, 1.0) for determination of R_f values. Arrows indicate the gel front (origin).

terns depicted in Fig. 2A, B, and C are those obtained with the same cytoplasmic preparation, it seems likely that the presence of this protein was obscured in the XM-50 ultrafiltrate by the highly concentrated protein bands B and C. Equal amounts of the XM-50 and PM-30 filtrates were applied to the gel columns so that the relative concentrations of the proteins prior to and after ultrafiltration could be compared. Thus, protein bands A and K were concentrated by ultrafiltration, whereas bands B, C, G, H, and I were clearly reduced in concentration after passage through the PM-30 membrane.

It was reported in the preceding study (3) that filtration of the PM-30 fraction through a PM-10 membrane resulted in an almost complete loss in the skin-test reactivity of the cytoplasmic antigen. We were, therefore, particularly interested in comparing the electrophoretic patterns of the skin test-active PM-30 fraction with that of the inactive PM-10 ultrafiltrate. As shown in Fig. 2C, the PM-10 ultrafiltrate contains protein bands with R_f values that correspond to those of bands E, G, and I in the PM-30 fraction and a band with a slightly higher R_f value (0.94) than that of band K (0.89) in this same fraction. The results of the skin tests (3) and those of disc gel electrophoresis indicate that neither bands E, G, I, or K are responsible for the skin-test reactivity of the cytoplasmic antigens. Likewise, since bands D and J in the XM-50 ultrafiltrate were not detected in the skin test-active PM-30 fraction, neither of these bands would have attributed to the skin-test reactivity of this antigen.

In examining the reproducibility of the method used to fractionate the yeast-phase cytoplasm, we found that both the number and the relative concentration of the proteins varied from one preparation to another. However, protein bands A, B, C, and F were consistently detected in the skin test-active PM-30 ultrafiltrate. To determine which, if any, of these might be medium constituents, brain heart infusion broth (Difco, Inc., Detroit, Mich.) was treated in the same manner as the cytoplasmic preparation, i.e., it was extracted for lipids, dialyzed, and then passed through ultrafilter membranes. A protein band with an R_f value of 0.40 was retained on the PM-10 membrane and may be similar to that of protein band F in the PM-30 cytoplasmic ultrafiltrate. Otherwise, the R_f values of the medium components did not correspond to those of the cytoplasmic antigens.

The R_f values of the carbohydrate-containing bands in the two blastomycins and the XM-50 cytoplasmic ultrafiltrate are presented in Table 1. Three periodic acid-Schiff stain-positive

bands were detected in commercial blastomycin. The band designated II has an R_f value of 0.07 which corresponds to that of protein band A (Fig. 1A). Of the five periodic acid-Schiff stain-positive components in the KCB-25 antigen, the first three have R_f values approximating those of protein bands A, B, and C (Fig. 1B). Seven periodic acid-Schiff stain-positive bands were detected in the XM-50 ultrafiltrate. Of these, five (bands I, II, IV, VI, and VII) had mobilities corresponding to those of protein bands (Fig. 2A, bands A, B, C, G, and H).

Only two lipid-containing bands were detected after electrophoresis of samples pre-stained with Sudan Black. One of these (R_f value, 0.99) corresponded to the protein band in the commercial blastomycin, and the other lipid-containing band had an R_f value (0.92) which approximated that of the fastest-moving protein band in the XM-50 ultrafiltrate.

By comparing the R_f values of the proteins, glycoproteins, and carbohydrates in the yeast- and mycelial-phase antigens, it can be seen that each of the skin test-active antigens contains a glycoprotein with an R_f value of 0.07 to 0.09. Also, two glycoprotein bands in the XM-50 and PM-30 cytoplasmic antigens have R_f values (0.04 to 0.06, and 0.24) corresponding to two glycoproteins (R_f values, 0.04 and 0.24) in blastomycin KCB-25. Neither of these components were detected in the commercial blastomycin. Other bands in the yeast- and mycelial-phase antigens have similar mobilities but differ in their combined staining reactions to Amido Schwartz, the periodic acid-Schiff stain reagent, and Sudan Black.

In the preceding paper (3), it was shown that of the yeast-phase antigens isolated, only the ASWS cell wall fraction exhibited both sensitivity and specificity. For this reason, we directed much of our work toward elucidating the nature of this antigen. The electrophoretic pattern, depicted in Fig. 3 (solid line), revealed three, maybe four, protein bands. The number and migration of these bands were not consistent, i.e., bands A and/or B were not always detected, even when electrophoresis was performed on the same sample; nor were we able to achieve better separation of the bands in separator gels measuring 90 mm in length. The R_f value of the periodic acid-Schiff stain-positive band also varied, ranging from 0.11 to 0.17. The only lipid-containing band had an R_f value of 0.99.

The inconsistency in the electrophoretic patterns of the ASWS antigen (in duplicate gels of the same sample) suggested that the antigen was partially dissociated (or degraded) during gel electrophoresis. We then determined the

TABLE 1. R_f values of periodic acid-Schiff stain-positive components in blastomycins and yeast-phase cytoplasmic antigen (XM-50) of *B. dermatitidis* strain SCB-2^a

Band designation	R_f values		
	Blastomycins		Yeast-phase XM-50
	Commercial	KCB-25	Ultrafiltrate
I	0.02	0.03	0.05
II	0.07	0.07	0.09
III	0.86	0.25	0.13
IV		0.62	0.25
V		0.82	0.42
VI			0.50
VII			0.63

^a R_f values were obtained by comparing the mobility of periodic acid-Schiff stain-positive bands with that of the reference dye (bromophenol blue).

electrophoretic pattern of the antigen after denaturation with SDS. This anionic detergent minimizes the native charges of proteins, and, as a result, the electrophoretic mobility of the bands is strictly dependent upon molecular weight, i.e., lower molecular weight proteins have increased mobility (9). The results are depicted in Fig. 3 (dotted line). The increased R_f values of the bands clearly indicate that the protein(s) was dissociated into smaller molecular weight subunits. Similar results were obtained with the polysaccharide(s). The one periodic acid-Schiff stain-positive band (R_f value, 0.11 to 0.17) detected prior to electrophoresis in SDS was dissociated into three bands (R_f values, 0.04, 0.20, and 0.52).

Ultrafiltration of the ASWS cell wall antigen on PM-30 and UM-20 E membranes, which retain substances with molecular weights of 30,000 and 20,000, respectively, did not effectively fractionate the antigen. In other words, both the residues and the filtrates were equally reactive as skin-test antigens (3). These results are consistent with our finding that the electrophoretic migration patterns did not differ appreciably from those of the PM-10 residue (Fig. 4, solid line). The only skin test-inactive fraction obtained from the ASWS antigen was that of the PM-10 ultrafiltrate. Electrophoretically, this fraction was comprised of two fast-moving protein bands having R_f values of 0.78 to 0.86 and 0.99.

Figure 4 shows the elution pattern of the cell wall antigen from Sephadex G-50. A single polysaccharide-protein peak was obtained, and, although the polysaccharide-protein ratio varied from 0.51 at the column's void volume to 0.28

in the subsequent fractions, there was no significant difference in the skin-test reactivity of these fractions.

We next sought to quantitate the total amino acid, carbohydrate, and phosphorus content of the cell wall antigen after acid hydrolysis. As

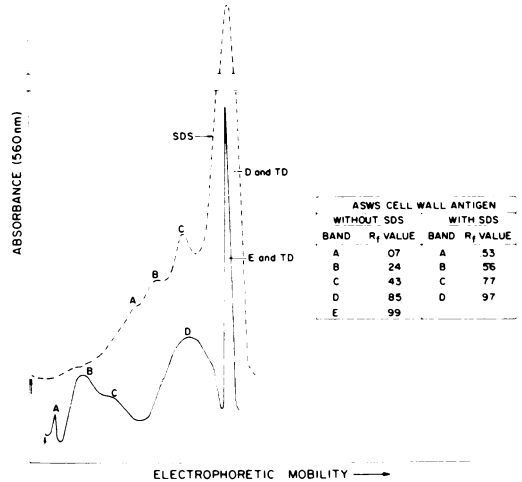


FIG. 3. Electrophoretic migration of the yeast-phase ASWS cell wall antigen without SDS (solid line) and in the presence of SDS (dotted line). The gels were stained with Amido Schwartz qr, for SDS gels, Coomassie Blue as described in Materials and Methods. The tracking dye (TD) was used as a reference (R_f value 1.0) for determination of R_f values. Arrows indicate the gel front (origin).

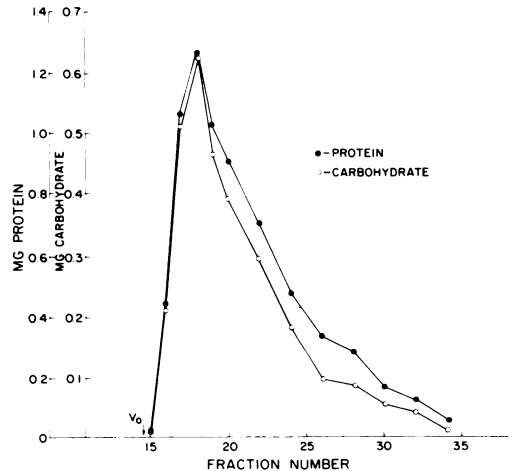


FIG. 4. Elution of the ASWS cell wall antigen (25 mg) on a Sephadex G-50 column (45 by 2.5 cm). Fractions (5 ml) were collected and analyzed for protein and carbohydrate content as described in Materials and Methods.

shown in Table 2, approximately 40 and 31% of the antigen is comprised of amino acids and carbohydrates, respectively. Descending paper chromatography with *n*-propanol-ethyl acetate-water (70:1:2, vol/vol/vol) as the solvent system and aniline hydrogen phthalate as the detecting spray revealed that the polysaccharide is comprised of mannose, galactose, and glucose. This latter carbohydrate constitutes only a minor portion (0.28 $\mu\text{mol}/\text{mg}$) of the total polysaccharide content (1.74 $\mu\text{mol}/\text{mg}$). The small amount of phosphorus present in the cell wall antigen is liberated only after strong acid hydrolysis (6 N HCl, 110 C, 18 h) and may represent a diester linkage of the polysaccharide moieties, phospholipid phosphorus, or both.

DISCUSSION

Disc gel electrophoresis of blastomycin antigens and the yeast-phase cytoplasmic ultrafiltrates of *B. dermatitidis* established the extent of the heterogeneity of these antigens and revealed certain similarities in their electrophoretic patterns. The results obtained in this study and those reported in the preceding study (3) suggest that the skin-test reactivity of the yeast-phase cytoplasmic antigen resides in one (or more) of four protein-containing bands. This conclusion is based upon the following: of the ten protein-containing bands in the PM-30 ultrafiltrate, four were detected in the skin test-inactive PM-10 ultrafiltrate, and two of the remaining six bands were not consistently detected in PM-30 ultrafiltrate preparations.

Three of the four bands that are thought to be responsible for the skin-test reactivity of the cytoplasmic antigen are glycoproteins. The slowest-moving glycoprotein (R_f value, 0.04) and a glycoprotein with an R_f value of 0.24 may be similar or identical to two glycoproteins that were detected in blastomycin KCB-25 (R_f values, 0.04 and 0.24). Neither of these were detected in the commercial blastomycin. A glycoprotein with an R_f value of 0.07 to 0.08 was detected in the yeast-phase cytoplasmic antigen

(R_f value, 0.07), blastomycin KCB-25 (R_f value, 0.08), and commercial blastomycin (R_f value 0.08). In comparing the relative concentration of this component with others within each antigen, we found that the glycoprotein was partially retained on the PM-30 ultrafilter membrane and, therefore, was less concentrated in the most active of the cytoplasmic antigens (PM-30 ultrafiltrate). On the other hand, this was the most concentrated component (relative to others) in the KCB-25 antigen. If the skin-test reactivity of this antigen is attributed to this glycoprotein, then it would account for the greater reactivity of blastomycin KCB-25 in comparison with the commercial antigen (in which the glycoprotein was barely detected). Unfortunately, we have not yet been able to separate the glycoproteins in the PM-30 ultrafiltrate from each other, and, until such purifications are achieved, we cannot determine which of these is actually responsible for the skin-test reactivity of the cytoplasmic antigen. The availability of the blastomycins was limited, so no attempts were made to fractionate these antigens.

The electrophoretic pattern of the ASWS cell wall antigen differed from those of the cytoplasmic ultrafiltrates and the blastomycins. The protein-staining bands with R_f values of 0.07 and 0.24 did not stain with the periodic acid-Schiff stain reagent. Rather, the only carbohydrate-containing band had an R_f value of 0.11 to 0.17 and did not correspond to the mobilities of the protein bands or the lipid band (R_f value, 0.99). Whereas the results obtained in electrophoresis, ultrafiltration, and Sephadex chromatography clearly indicate that the ASWS cell wall antigen has a molecular weight range of 30,000 to 50,000 and is comprised of protein, polysaccharide, and lipid, they do not establish whether these are chemically bonded to form one or more complexes. However, since SDS should not affect glycosidic linkages, the electrophoretic pattern of the antigen after denaturation would indicate that there are several cross-linkages between the polysaccharide and/or protein moieties, possibly via lipid(s) or disulfide bridges.

Previous studies (2, 6) have shown that the ASWS fractions of three additional strains of *B. dermatitidis* are chemically similar to that of strain SCB-2. The method used to isolate the cell wall antigen, namely alkaline hydrolysis followed by ultrafiltration (PM-10 membrane), is a relatively simple process and could be adapted to a large scale production of the antigen. Of interest is the report by Kanetsuna

TABLE 2. Chemical analyses of the ASWS cell wall antigen of *B. dermatitidis* strain SCB-2^a

Amino acids ($\mu\text{mol}/\text{mg}$)	Carbohydrates		
	Total ($\mu\text{mol}/\text{mg}$)	As glucose ($\mu\text{mol}/\text{mg}$)	Phosphorus ($\mu\text{mol}/\text{mg}$)
4.04	1.74	0.28	0.27

^a All values depicted represent the average obtained in analyses of at least three separate ASWS preparations.

and Carbonell (6) that the mycelial-phase walls of this fungus contain considerably more (4%) of an ASWS fraction than do the yeast-phase cell walls (0.5%). Since little is known about the antigenic complexity of this dimorphic fungus, studies should be directed towards comparing the ASWS fractions of the mycelial- and yeast-phase cell walls, both on a chemical and an antigenic basis.

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