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Cryptococcal Skin Test Antigen: Preparation Variables and Characterization

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Antigen capable of eliciting delayed hypersensitivity reactions in the skin of sensitized guinea pigs could be extracted from *Cryptococcus neoformans* cells by stirring the cells from 3 to 5 days in concentrated urea or guanidine. Hydrolysis of urea to ammonia by cryptococcal urease accompanied urea extraction, but alkalinity appeared neither necessary nor sufficient for extraction. Antigen from live cells gave larger delayed skin reactions than did antigen from Formalin-killed cells. Peak skin test reactivity appeared to reside in a protein-rich fraction having an elution volume on Sephadex G50 corresponding to a molecular weight of 10^4. Activity precipitated with half-saturated ammonium sulfate and could be detected in a single, narrow, rapidly migrating band on disc electrophoresis. Dialyzable proteinaceous antigen and high-molecular-weight, serologically active polysaccharide were present in the antigen, but not active in the delayed hypersensitivity reactions.

Conditions which impair delayed hypersensitivity seem to predispose to cryptococcosis, as evidenced by the fact that more than one-third of patients with cryptococcosis have Hodgkin's disease, non-Hodgkin's lymphoma, sarcoidosis, or adrenal corticosteroid therapy (5, 10). Yet, knowledge remains meager about cutaneous delayed hypersensitivity responses of cryptococcosis patients. Among the problems which beset such studies are the infrequency of infection and interference introduced by underlying conditions or their therapy. There is no lack of candidate cryptococcal antigens with which to pursue such studies, although none has been compared with another. Successful demonstrations of delayed hypersensitivity reactions have been reported in mice (7, 8, 13), guinea pigs (1, 3, 13, 16), human volunteers (3, 4, 6, 12, 15), and cryptococcosis patients (1, 3, 6, 17). In the last category were groups of 25 (3), 27 (17), and 38 (1) patients from this institution and 8 patients from another (6). The current paper adds to our prior reports by characterizing the antigen in use here, evaluating the steps used in preparation, and describing the histological response in the skin test site of a sensitized guinea pig.

MATERIALS AND METHODS

Skin test antigen. The method employed for preparation of skin test antigen was a small modification of that reported previously (1). A single, poorly encapsulated, serotype A isolate of *Cryptococcus neoformans* was used for the inoculum. The yeastlike cells were grown at 30°C for 72 h on 1% neopeptone (Difco Laboratories, Detroit, Mich.)-2% glucose agar or on yeast extract (Difco) dialysate agar. For the latter, yeast extract was dialyzed against distilled water until the dialysate had an optical density at 280 nm equivalent to 0.3 to 0.4% (wt/vol) yeast extract in water. Purified 1% agar (Difco) and 0.5% glucose were added to the dialysate. The mixture was autoclaved for sterilization. The medium was devised to contain proteins that should be different from those in neopeptone and of such low molecular weight that they would be removed by dialysis. In this way, it was hoped that animals sensitized to neopeptone contaminating the fungal inoculum would not react to culture medium antigens contaminating the skin test antigen. Cryptococci were harvested, washed three times in saline by centrifugation, cultured for bacterial and fungal contaminants, and centrifuged at 16,300 × *g* for 30 min. Cultures for fungal contamination were performed by streak dilution on Sabouraud agar. Bacterial contamination was sought by culturing on blood agar containing amphotericin B. Cultures were incubated aerobically at 30°C and observed periodically for 2 weeks. The packed cells were mixed with sterile 11.6 M urea in distilled water (1 ml of urea per g of wet cells). The slurry was stirred for 72 h at 4°C and centrifuged, and the supernatant was dialyzed in ethylene oxide sterilized tubing against three changes of sterile normal saline over 72 h. Dialyzed cryptococcal extract was checked for contamination as described above, centrifuged to remove insoluble precipitate, and filter sterilized. The usual yield was 3 mg of protein per g of wet cells. The final product contained protein at 2 to 4 mg/ml, hexose at 0.2 to 0.5 mg/ml, and deoxyribonucleic acid at 0.015 to 0.088 mg/ml.

Sensitization. Cryptococci were grown on neopep-

tone-glucose, washed three times in saline, and killed by stirring for 4 h at room temperature in 1% formaldehyde buffered to pH 7 with 7.5 mM phosphate. Alternatively, cryptococci were killed by heating at 100°C for 30 min. In either case, killed cells were washed three times in phosphate-buffered saline (0.15 M NaCl–6.7 mM PO_4, pH 7.4) and counted in a hemocytometer. An equal volume (2×10^8/ml) of killed cryptococci in phosphate-buffered saline and Freund incomplete adjuvant was homogenized and mixed with *Mycobacterium tuberculosis* H37Ra (Difco) to give 1 mg of mycobacteria per ml of suspension. Hartley guinea pigs weighing 300 to 500 g were injected with 1.0 ml of suspension per animal, divided between footpads and nuchal area. Control animals received phosphate-buffered saline in complete Freund adjuvant.

Skin testing. Animals were shaved and skin tested on their flanks with 0.1 ml of undiluted antigen 2 weeks after sensitization. Reactions were read at 4 and 24 h as the mean of two perpendicular diameters of induration. Reactions at 4 h were observed only with antigens that reacted equally in sensitized and control animals. These reactions, due to nonspecific inflammation, are not reported here, but no skin test result is reported unless the reaction in the control animal was less than 5 mm at 4 and 24 h.

Chemical analysis. Protein was measured by the method of Lowry et al. (11) with a bovine serum albumin standard. Hexose was assayed by anthrone (9) with a glucose standard. Deoxyribonucleic acid was determined by diphenylamine (9) with salmon sperm deoxyribonucleic acid as a standard. Ammonia was measured by distillation and acidometric titration. Urea was measured by the technique of Wibenga (18).

Separatory techniques. Preparative ultracentrifugation was performed in 0.15 M NaCl–0.2 M sodium phosphate (pH 7.0) with a linear 5 to 20% sucrose gradient by spinning 4.8-ml samples at 100,000 × *g* for 64 h at 3°C in a Beckman model L ultracentrifuge. Analytical disc electrophoresis was performed in 7% polyacrylamide-tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9.5) with 3-μl samples mixed in 150 μl of sample gel, polymerized, and electrophoresed in a Canalco model 12 at 5 mA per tube for 25 min. Preparative disc electrophoresis was performed with a Buechner Fractophorator in Tris-glycine buffer (pH 8.6). Sample volumes of 250 μl were mixed with an equal volume of 10% sucrose in water and layered on the gradient. Electrophoresis was at 15 mA.

Molecular weight standards for Sephadex column chromatography included cytochrome *c* (Nutritional Biochemical Corp., Cleveland, Ohio), human serum albumin (Merck Sharpe & Dohme, West Point, Pa.), α-trypsin inhibitor 5× crystalized (Nutritional Biochemical Corp.), and pancreatic trypsin inhibitor (Mann Research, New York, N.Y.).

Serology. A complement fixation test for cryptococcal polysaccharide was performed as described previously (2). The lowest concentration of polysaccharide detectable was 0.25 μg/ml.

RESULTS

Variables of the extraction process. Results obtained when a saturated solution of urea in water (11.6 M) was used were compared with those obtained with 10 and 8 M urea. No difference was noted in dialyzed unconcentrated extracts obtained after 24 and 48 h of extraction in regard to the protein yield, which ranged from 0.26 to 0.85 mg/g of wet cryptococci. At 72 h, protein yield had increased substantially (Table 1, experiment 1). The highest protein yield was obtained with 11.6 M urea. In a subsequent experiment, prolonging extraction from 3 to 5 days increased the protein yield (Table 1, experiment 2). However, the magnitude of the differences in yield within experiments 1 and 2 was not great compared with the differences between

TABLE 1. *Effect of variables on the extraction of skin test antigen*

Experiment no.	Fungus		Extraction process			pH		Yield	
	Culture medium[a]	Viability	Days	Dissociating agent (M)	Buffer (M)	Initial	Final	Protein (mg/g of wet cells)	Skin test[b]
1	NG	Live	3	Urea (8.0)	None	7.0	9.2	1.6	12 (1)
	NG	Live	3	Urea (10.0)	None	7.0	9.1	2.7	
	NG	Live	3	Urea (11.6)	None	7.0	9.2	4.1	12 (1)
2	NG	Live	3	Urea (11.6)	None	7.0	9.7	1.1	9 (1)
	NG	Live	4	Urea (11.6)	None	7.0	9.6	2.1	10 (1)
	NG	Live	5	Urea (11.6)	None	7.0	8.1	2.7	9 (1)
3	NG	Live	3	None	Borate (0.1)	9.0	5.2	0.052	7.0 (3)
	NG	Formalin killed	3	None	Borate (0.1)	9.0	6.4	0.056	6.0 (3)
	NG	Formalin killed	3	Urea (11.6)	Borate (0.1)	9.0	8.9	4.7	9.8 (3)
	NG	Live	3	Urea (11.6)	Phosphate (0.1)	7.0	9.6	5.1	13.0 (3)
	NG	Live	3	Urea (11.6)	Borate (0.1)	9.0	9.3	3.5	11.2 (3)
4	DYE	Formalin killed	3	Urea (11.6)	Borate (0.1)	9.0	9.0	7.5	10.5 (4)
	DYE	Formalin killed	3	Guanidine (7.0)	Borate (0.1)	9.0	7.9	2.9	13.0 (4)
	DYE	Formalin killed	3	Guanidine (7.0)	Phosphate (0.1)	7.0	6.8	2.0	9.0 (4)

[a] NG, Neopeptone-glucose; DYE, dialysate of yeast extract.

[b] Results expressed as diameter in millimeters of induration in sensitized guinea pigs with number of animals within parentheses. All antigens in each experiment were tested in the same animal(s).

experiments. Skin testing is a poor means of quantitation, but did not reflect these relatively small differences in protein within experiments 1 and 2.

Table 1 also shows the marked rise in pH accompanying urea extraction. Because urease is a known constitutive enzyme of *C. neoformans*, ammonia in the extract was measured before dialysis and found to average 0.345 M in three separate experiments (range, 0.298 to 0.435). As seen in Table 1, experiment 3, extraction of live or Formalin-killed cells with 0.1 M borate buffer in the absence of urea failed to maintain the pH at 9.0 and also failed to extract useful amounts of protein. Skin test reactivity with those extracts was poor. In the presence of urea, 0.1 M phosphate buffer failed to maintain a neutral pH. These results failed to clarify whether an alkaline pH was necessary or desirable in the urea extraction. Guanidine often can be substituted for urea as a dissociating agent and is not known to be hydrolyzed by the fungus, as is urea. A 7 M solution was found to extract cryptococcal antigen, with lower protein but with potency similar to that obtained with urea (Table 1, experiment 4). Ammonia concentration in the undialyzed guanidine extract was only 0.054 to 0.065 M, and pH was controlled easily. The results showed that an alkaline pH was not decidedly harmful or beneficial.

Formalinization of cryptococci before extraction had no consistent effect upon protein yield or pH rise during urea extraction. However, the mean diameter of skin test reactions was consistently reduced in all five comparisons of formalinized cells with viable cells (results of one comparison are shown in Table 1, experiment 3). The mean reduction in diameter due to Formalin from five experiments was 25%.

Based upon these experiments, subsequent extractions were performed in 11.6 M urea with 0.1 M borate buffer (pH 9) for 3 days with viable cryptococci. Urea was preferred to guanidine because the former was available in a sterile, pyrogen-free form (Ureaphil; Abbot Laboratories, North Chicago, Ill.). Borate was selected because of its buffering capacity at pH 9 and its antimicrobial activity. The remainder of the studies to be described used antigen prepared in this way.

Characterization of antigen. Use of dialysis to remove urea resulted in passage across the dialysis membrane of 72% of the material reacting in the Lowry test for protein. Therefore, Sephadex G25 column chromatography was employed to remove urea. Monitoring column effluent at 280 nm showed a large single peak which began at the void volume and trailed off into the urea-containing fractions. Table 2 shows the fractions obtained when 10 ml of urea extract was placed on a Sephadex 25 column (15 by 900 mm) and eluted with phosphate-buffered saline. Only 19% of the eluted protein was contaminated with urea, compared with 72% with dialysis, but the protein-rich fraction II had no significant skin test reactivity. This suggested that the low-molecular-weight antigen lost during dialysis may not be useful as a skin test antigen anyway. The other type of antigen loss during dialysis, precipitation of insoluble debris, also occurred during Sephadex chromatography and made the column unable to be reused. To investigate directly the biological activity of antigen passing through dialysis tubing, urea extract was dialyzed against 0.2 M $(NH_4)HCO_3$ instead of saline, and the dialysate was lyophilized and hydrated in saline. Urea was removed by Sephadex G25 column chromatography, and the protein concentration was adjusted to that of the dialyzed antigen (3.45 mg/ml). The lyophilized reconstituted antigen did not elicit a skin test response in sensitized guinea pigs. A reaction of less than 5 mm was obtained with two other low-molecular-weight fractions. One of the these was obtained by ultrafiltration of the dialyzed antigen through a Diaflow UMO5 membrane (Amicon Co., Lexington, Mass.) having a nominal cutoff of 5000 daltons. The other antigen was obtained by vacuum filtration through dialysis tubing. Failure of this latter antigen to elicit delayed hypersensitivity reactions led to vacuum dialysis being used to concentrate antigen for some subsequent experiments.

Preparative ultracentrifugation (Fig. 1) found all the protein to be of relatively low molecular weight, whereas the serologically reactive polysaccharide was of higher molecular weight. For studying an amount of antigen adequate for skin testing, skin test antigen was concentrated threefold on vacuum dialysis, and 1.7 ml was placed on a Sephadex G200 column (2.5 by 100 cm). Borate-buffered saline (0.15 M NaCl-0.035 M borate, pH 8.0) was used to elute 6.5-ml frac-

TABLE 2. *Sephadex G25 chromatography of urea extract*

Fraction	Tubes	Volume (ml)	Protein (mg)	Complement-fixing antigen titer	Urea	Skin test (mm)[a]
Void volume	0–15					
I	16–24	21.0	50	1:4,096	0	13.3
II	25–30	14.5	20	1:4	0	2.0
III	31–34	7.0	7	1:4	+	0
IV	35–40	12.5	9	1:4	++++	Irritation

[a] Mean 24-h diameter of induration in four sensitized guinea pigs skin tested with undiluted fractions.

tions. The protein eluted in the lower-molecular-weight fractions (Fig. 2). The same column was equilibrated with phosphate-buffered saline, and 16 ml of unconcentrated antigen was chromatographed. The starting material had a polysaccharide titer of 1:1,600 (determined by complement fixation) and 128 mg of protein. The void volume was discarded, and the subsequent eluate was divided into two fractions (Table 3). The second of these contained virtually all of the protein. The dividing line between the two fractions corresponded to the elution volume of human immunoglobulin G (molecular weight, 160,000) on this column. Both fractions were concentrated on vacuum dialysis. This step resulted in the loss of 41% of the protein in G200 fraction II, but none in G200 fraction I. Skin test reactivity could be detected in the concentrate of G200 fraction II, but not fraction I (Table 3). Of the 6.5 ml in the concentrate of G200 fraction II, 4.7 ml was placed on a Sephadex G100 column (15 by 900 mm) and eluted with phosphate-buffered saline, and three fractions were obtained (Fig. 3). These fractions were concentrated by vacuum dialysis. After concentration, they contained 44.2 of the 54 mg placed on the column (Table 3). Only G100 fractions I and II had skin test reactivity. The boundary between

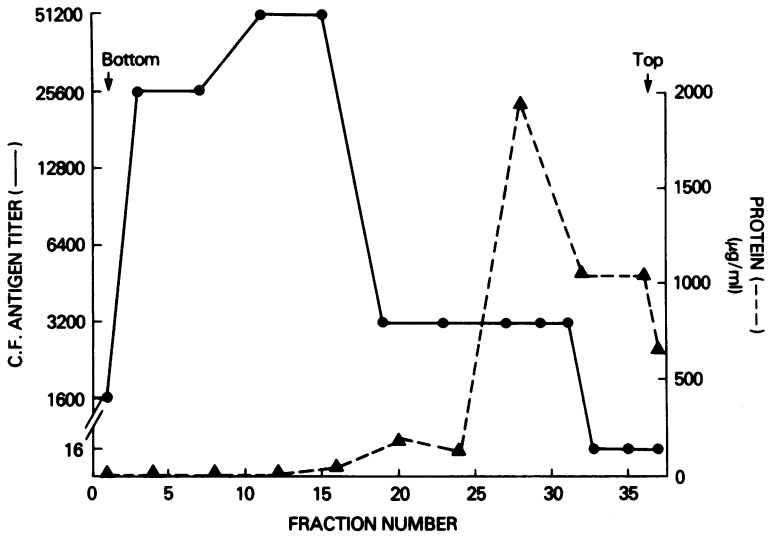

FIG. 1. *Preparative ultracentrifugation of 0.2 ml of skin test antigen on a 5 to 20% sucrose gradient.*

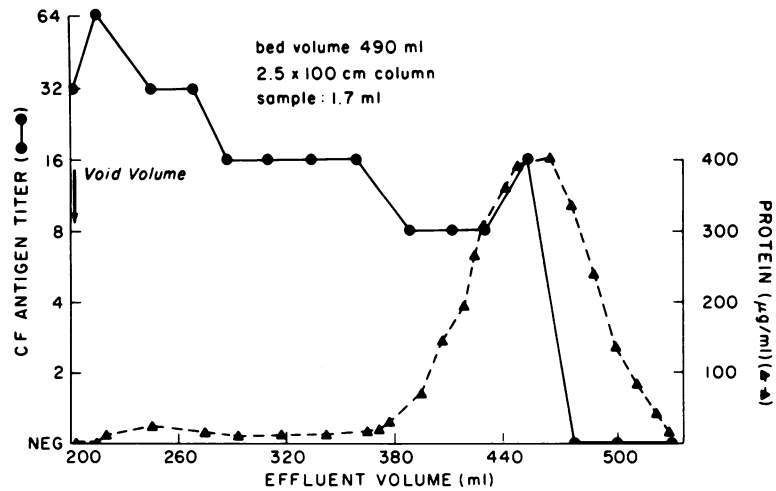

FIG. 2. *Column chromatography of skin test antigen on Sephadex G200.*

TABLE 3. *Chromatographic separation of skin test antigen*

Fraction	Tubes	Eluted volume (ml)	Concentrated fractions			
			Volume (ml)	Protein (mg)	Complement-fixing antigen test titer	Skin test (mm)[a]
G200[b]						
Void volume	1-16					
I	17-31	108	7.0	<2	1:1,024	0
II	32-62	246	6.5	75	1:256	11.6
G100[c]						
Void volume	1-9					
I	10-15	22	4.5	1.2	1:1,024	8.3
II	16-29	50	12.0	43	1:512	9.6
III	30-39	37	4.2	<1	1:2	1.3

[a] All five skin tests were placed on the same three sensitized guinea pigs, and mean induration diameters were recorded.

[b] Chromatography on a Sephadex G200 column.

[c] Rechromatography of G200 fraction II on a Sephadex G100 column.

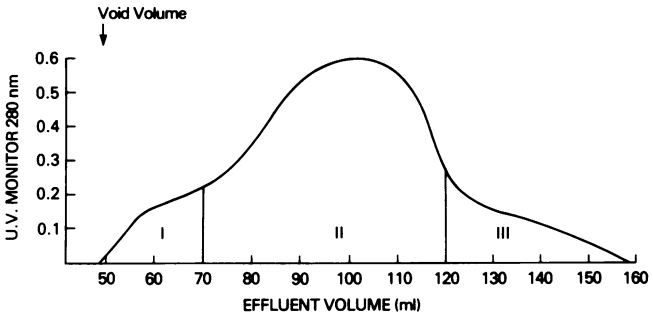

FIG. 3. *Rechromatography of fraction II from a Sephadex G200 column on Sephadex G100.*

G100 fraction II and G100 fraction III, the unreactive fraction, corresponded to a molecular weight of approximately 6,300.

To confirm the molecular weight of the major protein peak more accurately, 5 ml of antigen was placed on a standardized Sephadex G50 column (25 by 1,000 mm) and eluted with borate-buffered saline. The protein peak corresponded to a molecular weight of 10,000 (Fig. 4).

Starch block electrophoresis of skin test antigen at pH 8.2 found the proteinaceous antigen to migrate more rapidly than complement fixation-reactive material, but separation was incomplete. Disc electrophoresis of both skin test antigen and ultrafiltrate of that antigen showed a single, rapidly migrating anodal band as stained by amidoschwarz (Fig. 5). The same band was found in the previously described G100 fraction II. Periodic acid Schiff staining of duplicate gels was negative with the ultrafiltrate, but showed faint staining of the migrating band as well as staining in the sample gel and interface between separator and stacking gel. Preparative disc electrophoresis of the same antigen shown in the left of Fig. 5 yielded 30 tubes with 3.0 ml/tube (Fig. 6). The protein peak, tubes 5 to 9, and the complement fixation reactive antigen, tubes 10 to 30, were concentrated 9.3- and 7.6-fold, respectively, by vacuum dialysis. The concentrate of tubes 5 to 9, which contained 42% of the protein applied to the column, gave a definite delayed hypersensitivity response in three sensitized guinea pigs, albeit of only 5.7-mm mean diameter of induration. The concentrate of tubes 10 to 30 elicited no reaction in the same sensitized animals.

Ability of the skin test-reactive components to precipitate in half-saturated $(NH_4)_2SO_4$ was assessed. Dialyzed antigen (5 ml) was mixed with an equal volume of saturated $(NH_4)_2SO_4$ at 4°C. The precipitate was dissolved in normal saline and dialyzed free of sulfate ion. The precipitated dialyzed antigen was concentrated to 3.0 ml by vacuum dialysis. This material contained 40% of the protein in the original 5.0 ml, but the complement fixation titer was reduced from 1:512 to 1:32. The original and final materials, containing 3.9 and 2.6 mg of protein per ml, respectively, gave mean skin test diameters in three sensitized guinea pigs of 17 and 18 mm, respectively. Both antigens gave the same rapidly moving band in disc electrophoresis that is

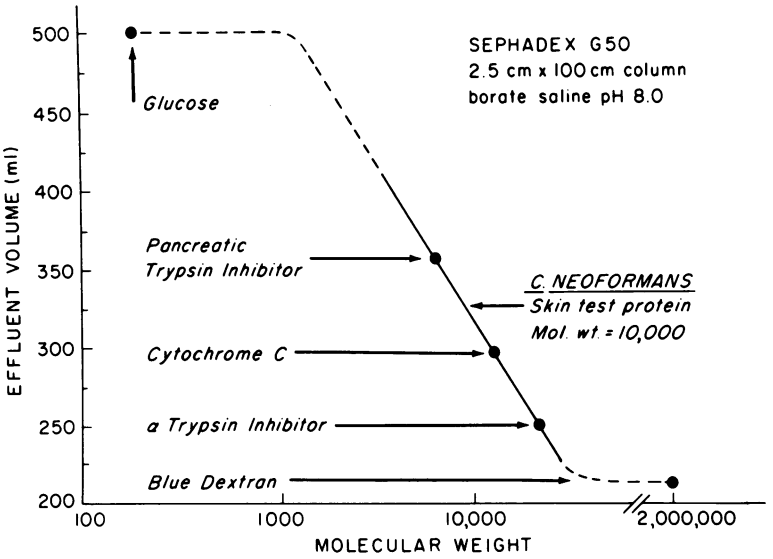

FIG. 4. *Elution volumes of peaks given by skin test protein and standards on Sephadex G50.*

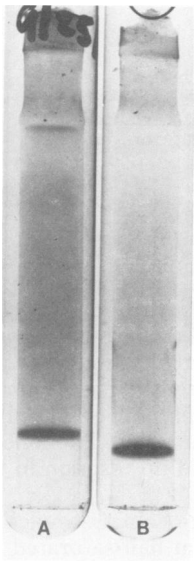

FIG. 5. *Disc electrophoresis of skin test antigen with amidoschwarz stain. A, Skin test concentrated 10-fold by lyophilization. B, Vacuum dialysis ultrafiltrate concentrated by lyophilization.*

shown for other antigens in Fig. 6.

Histology. Biopsies of skin test sites at 24 and 48 h in sensitized guinea pigs showed a perivascular, deep dermal inflammatory response consisting largely of lymphocytes and neutrophils (Fig. 7). A less prominent infiltrate of similar composition appeared in the more superficial layers of the dermis. Control animals had no inflammatory response.

DISCUSSION

Successful extraction of skin test antigen from *C. neoformans* was found to depend upon the presence of a dissociating agent such as 8 to 11.6 M urea or 7 M guanidine. The extract became alkaline when fungal urease converted urea to ammonia, but alkalinity alone was not sufficient to extract useful antigen and did not facilitate extraction by guanidine. When the urea or guanidine was removed from the extract, some of the extracted antigen precipitated from solution. The extract contained so much low-molecular-weight antigen that 72% of the Lowry-reactive material was lost during dialysis. All efforts failed to detect skin test reactivity in this dialyzable antigen. Biological activity did reside in a low-molecular-weight proteinaceous antigen that precipitated in half-saturated $(NH_4)_2SO_4$ and migrated anodally in a single band on disc electrophoresis. Molecular weight was not determined under conditions that would dissociate aggregates, but in Sephadex G50 the peak centered at an elution volume corresponding to a protein of 10,000 molecular weight.

The antigen reported here has some of the characteristics found by Murphy and Pahlavan in dialyzed culture filtrate antigen (14). In their experience, skin test reactivity was lost when the antigen was passed through an Amicon XM50 membrane with a nominal retention limit of 50,000. Disc gel electrophoresis in 3% polyacrylamide showed a rapidly migrating band that stained strongly for protein and weakly for polysaccharide, similar to the results reported here for urea-extracted antigen.

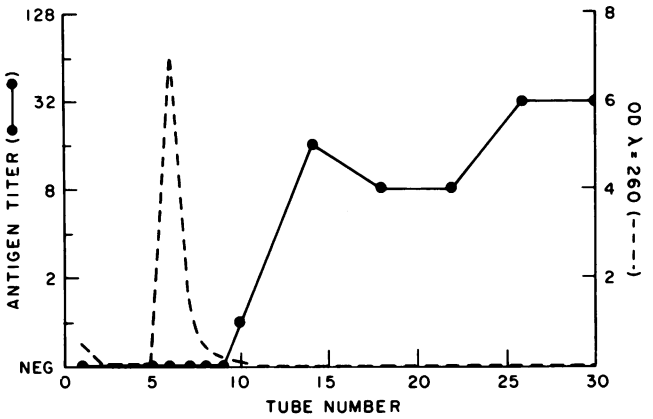

FIG. 6. *Preparative disc electrophoresis of skin test antigen concentrated 10-fold by lyophilization to give 6 mg of protein in 250 μl.*

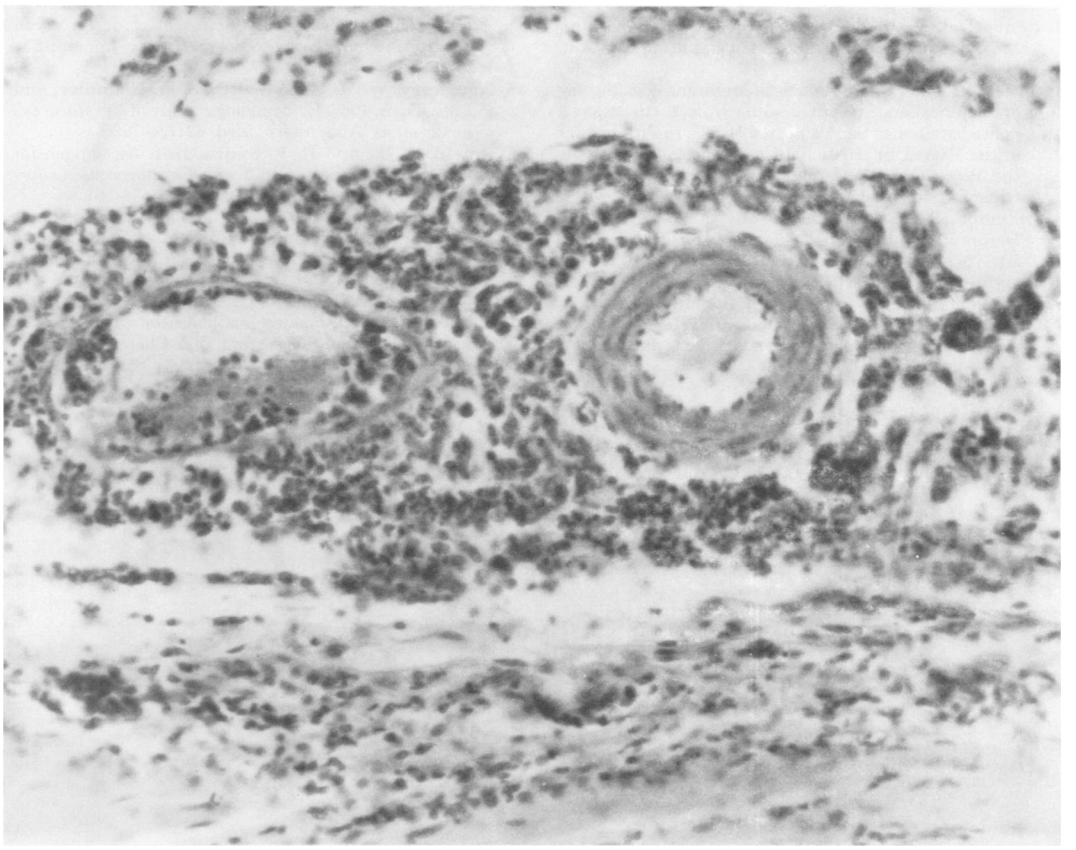

FIG. 7. *Deep dermal perivascular inflammatory response in sensitized guinea pig 24 h after skin testing. Hematoxylin and eosin. Original magnification, ×400.*

Hay and Reiss (8) evaluated subcellular fractions of homogenized *C. neoformans* for footpad swelling in sensitized mice. The most active antigen was particulate material that did not sediment at 20,000 × *g* in 30 min, but did sediment at 100,000 × *g* in 60 min. Soluble extract was only weakly reactive. Culture filtrate was unsatisfactory because of intense local reactions 4 h after skin testing of sensitized mice. As in the present report, there was no correlation between

delayed hypersensitivity reactivity and ability to react with antibody against cryptococcal polysaccharide.

Biopsies of guinea pig skin test sites and the time course of the skin reaction support the contention that the antigen described here elicits delayed hypersensitivity responses. Previously published skin test biopsies using different antigens were taken from guinea pigs (13) and from a renal transplant recipient (6). The histology was similar to that reported here.

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