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Cellular Immune Activity of a Galactomannan-Protein Complex from Mycelia of *Histoplasma capsulatum*

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A galactomannan-protein complex was extracted from defatted mycelium of *Histoplasma capsulatum* with 0.25 N alkali at 25 C. It accounted for 7.5% of the cell dry weight in nondialyzable form and had a galactose-mannose ratio of 2:5. The complex containing 68% protein was separated into polysaccharide and glycoprotein components by ion-exchange chromatography. The galactomannan formed a single precipitate in immunodiffusion, sharing a common antigen with the glycoprotein which contained two precipitating antigens. The glycoprotein elicited delayed-type hypersensitivity in guinea pigs as measured by skin-test and macrophage migration inhibitory factor assay, with 5 μg resulting in 86% specific inhibition. The galactomannan was skin-test negative, but 10 μg resulted in 80% specific inhibition of migration.

Previous antigenic analyses of the pathogenic fungus *Histoplasma capsulatum* have shown that histoplasmin purified derivative, the skin-test antigen obtained from culture filtrates, was composed of protein associated with carbohydrate (8, 22, 23). The nature of the carbohydrate component was not determined, but earlier, mannose and galactose were detected in a yeast-phase filtrate (20). Compositional analysis of the cell wall has revealed glucose and mannose as the only non-nitrogenous sugar constituents (5, 14). In a more recent study (7), an antigenic fraction extracted from the cell walls with ethylenediamine was found to contain 0.6% glucosamine, but neutral sugars were not determined. These findings raised the question of the existence in whole cells of a glycoprotein precursor of histoplasmin. The approach taken in the present work was to employ dilute alkali at ambient temperature for releasing peptidopolysaccharides from whole cells, a method which proved effective for the isolation of glucomannan complexes from yeast (18) and capsular polysaccharides from pneumococci (6). Material was then analyzed serologically and in an in vitro assay for cell-mediated immunity.

MATERIALS AND METHODS

Preparation of antigens. *Histoplasma capsulatum* NIH 6633, (−) mating type, isolated from a human patient with histoplasmosis, was the test strain. A batch of 5 liters of Salvin medium (20) modified by substituting yeast extract for the dialyzate was dispensed in 1-liter flasks, inoculated with a suspension obtained from week-old cultures on brain-heart infusion agar slants, and incubated for 10 days at 25 C on a gyrotory shaker at 150 rpm. The mycelium was sedimented at 1,300 × *g* for 30 min and washed with demineralized water. Culture supernates were pooled, concentrated in vacuo, dialyzed, and lyophilized. Subsequent steps were carried out at 25 C, except for centrifugation which was performed at 4 C. The wet, packed mycelium was extracted with 600 ml of chloroform-methanol (2:1, vol/vol) for 6 h under N_2 with one change of solvent. The defatted mycelium then was recovered by filtration at reduced pressure over coarse sintered glass and dried in vacuo. Dry defatted cells were extracted with 150 ml of 0.25 N NaOH for 16 h in a stoppered flask, the alkali-resistant residue was sedimented (1,300 × *g*, 20 min), and the supernate received 3 volumes of cold ethanol. The resulting precipitate was recovered (1,300 × *g*, 20 min), dissolved in water, and neutralized with concentrated acetic acid. The neutralized extract was dialyzed versus flowing demineralized water (5 liters/h for 18 h), and the nondialyzable portion was lyophilized. This material, termed histoplasmal extract I, was subjected to ion-exchange chromatography on a column (0.9 by 20 cm) of diethylaminoethyl-cellulose.

Biochemical characterization of antigens. Gel permeation chromatography employed Sephadex G100 superfine (Pharmacia Fine Chemicals) and Biogel A5M (Bio-Rad Laboratories). Column fractions were analyzed for protein with Folin-phenol (16) and for carbohydrate with phenol and sulfuric acid (10). Total N (micro-Kjeldahl) and total P were also determined (11). For carbohydrate analysis, 1-mg samples were hydrolyzed for 3 h in 2 N trifluoroacetic acid under N_2 at 100 C (2), and the acid was removed in vacuo. An alternative hydrolysis consisted of 1 N HCl for 1 h at 100 C under N_2. Alditol acetate

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derivatives were made (21), and samples were separated in a Perkin-Elmer 9000 gas chromatograph with ECNSS-M column packing (ethylene succinate-cyano-ethyl silicone polymer) and digital integration. Combined gas chromatography and mass spectrometry were carried out in an LKB Company instrument. For amino acid determination the sample was hydrolyzed (6 N HCl for 18 h at 105 C) under N_2 prior to separation in a Beckman Autolab amino acid analyzer.

Immunological procedures. Antiserum was obtained from male New Zealand albino rabbits infected intravenously with 10^7 yeast-phase cells of the test strain. After 4 weeks, sera were tested for antihistoplasmal antibodies by immunodiffusion. Animals were exsanguinated by cardiac puncture. Inbred guinea pigs, NIH strain 13/N, were sensitized with 2.5 mg of histoplasmal extract I in 0.5 ml of a water-in-oil incomplete or complete Freund adjuvant emulsion injected intradermally into several sites scattered in the nuchal area. *Mycobacterium tuberculosis* var. *hominis* (2.5 mg) was present in the complete adjuvant. Animals were skin-tested with the homologous antigen at 5 days and later at 21 days with fractions obtained by ion-exchange chromatography. Results were expressed as mean squared radii of induration from three animals in each group. The direct macrophage migration inhibitory factor (MIF) assay of David (4) with the following modifications was performed on guinea pigs sensitized with histoplasmal extract I in complete Freund adjuvant. The medium for tissue culture was RPMI 1640 containing 25 mM HEPES buffer (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; Grand Island Biological Co) and was supplemented with 0.03% L-glutamine prior to use. Sterile mineral oil (Drakeol, 20 ml) was instilled into the peritoneal cavity 28 days after the animals were sensitized, and after 3 more days the animals were anaesthetized with ether and exsanguinated by cardiac puncture. Peritoneal exudates pooled from three animals were exposed to isotonic NH_4Cl at 3 C to lyse red blood cells (19) followed by three washes in Hanks balanced salts solution. The system was constituted in Sykes-Moore chambers (Bellco), and the area of each tuft was measured at 24 h by photography and planimetry of the enlarged image. The mean migration value obtained in chambers without antigen (four chambers each containing two capillaries) was accepted as 100% migration. The inhibition of migration was measured for six replicate tufts at each antigen concentration, and the mean inhibition was expressed as a percentage of the migration in the absence of antigen.

As control antigens the purified protein derivative of tuberculin (Connaught, Toronto) and streptokinase-streptodornase (Lederle Laboratories) were used.

RESULTS

Composition of the alkali extract. From 12 g of defatted mycelium, 930 mg (7.75%) was extracted with dilute alkali and precipitated with ethanol. This parent material, histoplasmal extract I, was nondialyzable and contained carbohydrate (30.2%) and protein (68.8%). Gas chromatography revealed the presence of galactose and mannose (Table 1). A facile method of fractionating the extract was anion-exchange chromatography (Fig. 1). Of 100 mg applied to the column, 17.3 mg was eluted by 0.05 M NaCl, and 30.6 mg was eluted by a 0.15 to 0.25 M NaCl gradient, for a yield of 48%. The galactose-mannose ratio (2:5) in all fractions was constant. The gel permeation behavior of extract I on G100 superfine (Fig. 2) displayed two carbohydrate-containing components, one coeluting with protein, and a second peak retained by the gel along with a linearly decreasing amount of protein. On a 6% agarose gel (Biogel A5M) having a fractionation range of 1×10^4 to 5×10^6 molecular weight, all except a minor component was retained (Fig. 3). Carbohydrate and protein were present in both peaks. The monosaccharide and amino acid content of components eluted from the diethylaminoethyl-cellulose column was quantitated (Tables 1 and 2). The material eluting in fractions 1 to 10 was a galactomannan (GM) and fractions 13 to 33 contained a galactomannan protein (GP). A

TABLE 1. *Composition of galactomannan-containing fractions of Histoplasma capsulatum obtained by dilute alkali extraction and diethylaminoethyl-cellulose chromatography*

Sample	Component	Dry wt (%)	Galactose/ mannose
Extract I	Galactose[a]	3.83	2:5
	Mannose	9.72	
	Glucose	0.543	
	Total hexose	14.1	
	Total carbohydrate[b]	30.2	
	N	11.0	
	P	1.28	
Galactomannan	Galactose	11.7	2:5
	Mannose	28.5	
	Glucose	0.65	
	Total hexose	40.9	
	Total carbohydrate	65.7	
	N	2.63	
Galactomannan protein	Galactose	2.83	2:5
	Mannose	7.4	
	Total hexose	10.2	
	Total carbohydrate	19.9	
	N	10.3	

[a] Monosaccharide analysis by gas chromatography.
[b] Total carbohydrate by phenol-sulfuric acid.

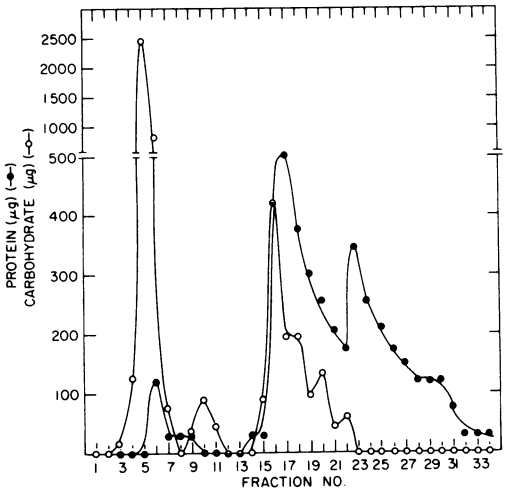

FIG. 1. *Column chromatography of extract I on diethylaminoethyl-cellulose. Column dimensions, 0.9 by 22 cm; fraction volume, 3 ml. Fractions 1 through 12 were eluted with 0.05 M NaCl; fractions 13 through 33 were eluted with a 0.15 to 0.25 M NaCl gradient.*

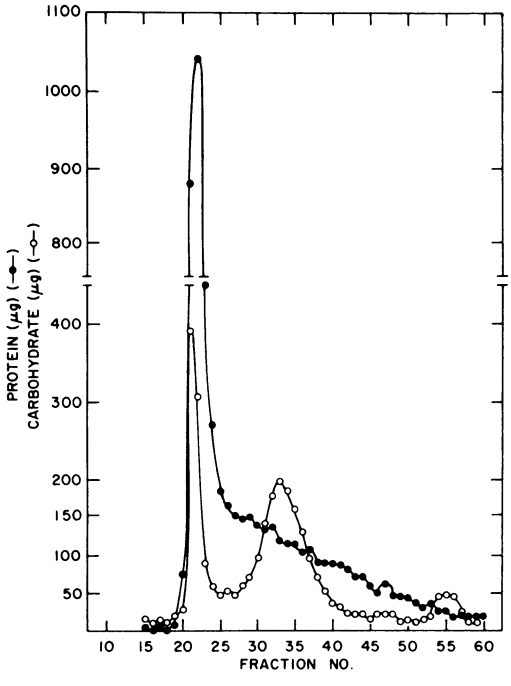

FIG. 2. *Gel-permeation chromatography of extract I on Sephadex G100 superfine. Column dimensions 1.2 by 100 cm; fractions of 1.8 ml eluted with phosphate buffer (5×10^{-3} M, pH 7) containing 0.05 M NaCl. The void volume marker blue dextran eluted in fraction 21.*

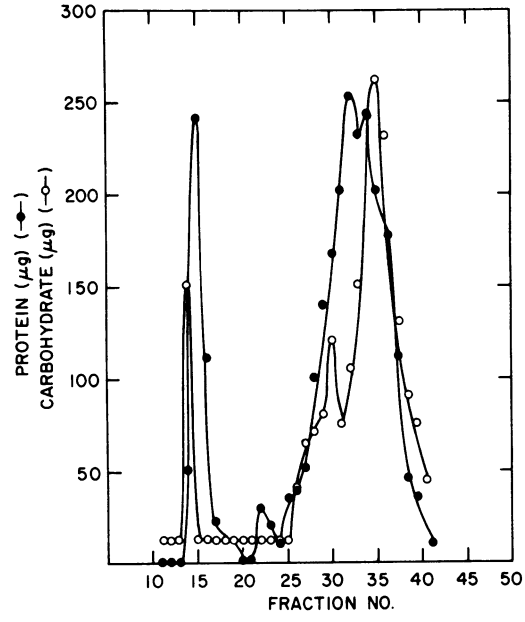

FIG. 3. *Gel-permeation chromatography of extract I on Biogel A5M. Column dimensions 1.2 by 75 cm; fractions of 2.1 ml eluted with phosphate buffer (3.5×10^{-4} M, pH 7) containing 0.05 M NaCl.*

TABLE 2. *Amino acid composition of the galactomannan protein complex of Histoplasma capsulatum*[a]

Amino acid	μmol/100 mg
Try	8.17
Lys	17.4
His	5.78
Arg	15.5
Asp	33.4
Thr	17.5
Ser	18.5
Glu	39.5
Pro	22.1
Gly	28.1
Ala	27.1
Val	26.1
Met	4.68
Ile	13.9
Leu	20.0
Tyr	7.56
Phe	8.85

[a] Total protein recovered as amino acid, 37.8% of GP dry weight. Diethylaminoethyl-cellulose fractions 13 through 33.

disparity was found between the total hexose recovered by gas chromatography and the total carbohydrate content estimated with phenol-sulfuric acid. Milder conditions for hydrol-

ysis were needed to detect a peak in the alditol acetate spectrum not previously seen. The unknown had a $R_{glucose}$ of 0.351. This retention time was not consistent with a hexose and some other sugars were ruled out: rhamnose, xylose, L-fucose, 2-deoxyribose, and 2-deoxyglucose. Preliminary mass spectroscopy indicated the presence of an alditol acetate but not a deoxyhexose derivative.

Serological and cutaneous reactivity. Histoplasmal extract I reacted in immunodiffusion with antiserum from an infected rabbit to form two immune precipitates (Fig. 4). The GM fraction obtained by ion-exchange chromatography formed a single precipitate line which fused with a line derived from GP. In addition, GP contained another component which formed a line only after 24 h of incubation. Two groups of guinea pigs immunized with histoplasmal extract I in either complete or incomplete Freund adjuvant were skin-tested with the homologous antigen after 6 days and with the GM and GP at 21 days (Table 3). Positive skin-tests in animals from both groups were observed at 6 days at both 10- and 100-μg doses, suggesting an early manifestation of cellular immunity. After 21 days, animals from both groups responded to extract I but induration was elicited by GP only at the 100-μg dose. Therefore, no apparent increase in specific skin activity was manifest using materials purified by ion-exchange chromatography. The GM gave negative skin tests even at the 100-μg dose level, in contrast to its potent serological activity.

TABLE 3. *Cutaneous hypersensitivity elicited by the galactomannan-protein complex in guinea pigs sensitized with histoplasmal extract I in adjuvant*

Antigen	Dose (μg)	Days	Induration, R^2, MM^2		
			4 h	24 h	48 h
CFA[a]					
Extract I	10	6	0	28	10
	100		15	68	44
Extract I	10	21	0	28	16
	100		21	65	46
GP	10		0	25	0
	100		33	64	33
GM	10		0	0	0
	100		0	0	0
IFA[b]					
Extract I	10	6	10	12	0
	100		38	40	10
Extract I	10	21	0	21	0
	100		34	39	21
GP	10		0	24	0
	100		36	19	0
GM	10		0	0	0
	100		0	0	0

[a] Complete Freund adjuvant.
[b] Incomplete Freund adjuvant.

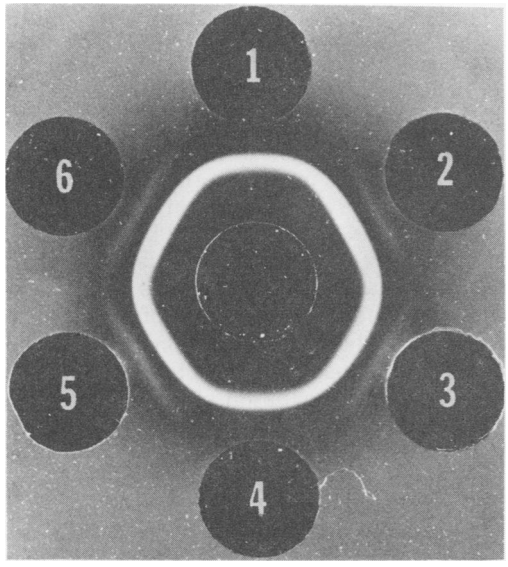

FIG. 4. *Immunodiffusion. Center well contained antiserum of an infected rabbit; outer wells contained antigens at 2 mg/ml. Wells 1 and 4, GM; 2 and 6, extract I; 3 and 5, GP.*

MIF assay. Extract I, as well as the GM and GP fractions derived from it, were capable of eliciting inhibition of macrophage migration in the peritoneal exudate cells of guinea pigs immunized with extract I in complete Freund adjuvant (Table 4, Fig. 5 and 6).

The inhibition was specific because the extracts were not toxic but stimulated migration in normal nonimmune animals and also because no significant reactions were observed with the cells from guinea pigs sensitized only with the mycobacterial component of Freund complete adjuvant. In the immunized group virtually complete inhibition was observed in the presence of 10 μg of extract I, and a dose-response relation was measured with GP to the extent that 1 μg resulted in 63% inhibition. The result obtained with GM, 80.2% inhibition at a dose of 10 μg, was surprising since this antigen had no skin reactivity. A culture filtrate-derived material was a less potent elicitor of MIF; 100 μg was necessary to obtain 60% inhibition. The filtrate material was obtained from the same cultures

TABLE 4. *Macrophage migration inhibitory factor assay of galactomannan-protein antigens of H. capsulatum*[a]

Antigen	Dose (μg)	Inhibition of migration (%)[b]		
		Sensitized		Normal
		Histoplasma	Mycobacterium	
Extract I	10	88.2	21.7	0
	100	92.7	14.1	(21)[a]
GM	10	80.2	19.3	(73)
	100	92.9	15.8	(25)
GP	1	63.0	—	—
	5	86.2	—	—
	10	—[b]	31.6	(23)
	25	89.0	—	
	100	93.1	21.1	(76)
Culture filtrate	10	9	—	—
	100	60.3	—	—
PPD	25	90.3	89.2	14.5
SKSD	50 U	(11.2)	18.2	(29)

[a] Abbreviations: PPD, purified protein derivative; SKSD, streptokinase-streptodornase.

[b] Parentheses denote percentage of stimulation. —, Not determined.

which were the source of the alkali extracts. Of the two antigens employed as controls, purified protein derivative resulted in MIF production as expected, and streptokinase-streptodornase gave negative results, suggesting that the guinea pigs had no streptococcal disease.

DISCUSSION

The nature of the antigens of *Histoplasma* capable of stimulating cellular immunity is of considerable importance because human susceptibility to this infection appears to be related to impaired lymphocyte responses (13). In the normal population the occurrence of positive skin-tests to histoplasmal filtrate antigens is widespread in 31 states, and in the states of highest prevalence positive reactors exceeded 50% of the inhabitants tested (1). Thus far emphasis has been placed on the characterization of culture filtrate antigens, the most purified form being histoplasmin purified derivative (22, 23). The present work sought to extract antigens from whole defatted cells by relatively mild means with dilute alkali. It was not surprising that the carbohydrate moiety of histoplasmal extract I was determined to be a galactomannan, since the principal antigen of other fungi shares this composition. Lloyd (15) characterized a peptidogalactomannan from *Cladosporium werneckii*, having a galactose-mannose ratio of 1:5 and 11% peptide. It was extracted by heating whole cells in phosphate buffer for 2 h and precipitating the peptidopolysaccharide with hexadecyl trimethyl ammonium bromide. The glycopeptide present in trichophytin, (3) had a galactose-mannose ratio of 1:3 to 1:8 and 10 to 12% protein. Trichophytin produced both immediate and delayed skin reactions in man and guinea pigs. In the two studies concerning the cell wall composition of *H. capsulatum*, galactose was not identified as one of the nonnitrogenous sugars (5, 14). Perhaps in the process of mechanical disruption employed for preparing cell walls, the galactomannan is partially sloughed off. It is of interest to speculate about the structural relationship between the GM and GP. If an analogy with the molecular architecture of yeast is valid (12), then the GM may represent an outer wall layer linked to the inner alkali-insoluble glucan by glycopeptide bonds of the GP.

Presumptive evidence was found for the existence of a sugar component of GM that was labile to 2 N trifluoracetic acid but stable after 1 N HCl hydrolysis for 1 h. Identification of this sugar proved elusive, but it appears not to be an artefact since mass spectrometry indicated it to be an alditol acetate but not a deoxyhexose derivative. Several common pentoses were ruled out, and the identity is being sought because it may represent an antigenic determinant.

The GM formed a strong immune precipitate in immunodiffusion, whereas GP revealed the presence of two components, one showing a line of identity with GM and a second component which required at least 24 h to precipitate. Since the serodiagnosis of human histoplasmal infection depends in part on the demonstration of two immune precipitates in immunodiffusion, the H and M lines, it would be of interest to study the relation of those components to the galactomannan fractions obtained from whole cells (9). Further separation of the GP is needed, possibly by affinity chromatography on Concanavalin A-sepharose which has recently been applied to separation of histoplasmin (S. C. Cheung, G. S. Kobayashi, and G. Medoff, Prog. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 156, 1973).

The GM had no skin reactivity in guinea pigs sensitized with histoplasmal extract I in complete Freund adjuvant at the doses supplied (10 μg, 100 μg), but 10 μg/ml was sufficient to elicit virtually complete and specific inhibition of macrophage migration. The basis for this dis-

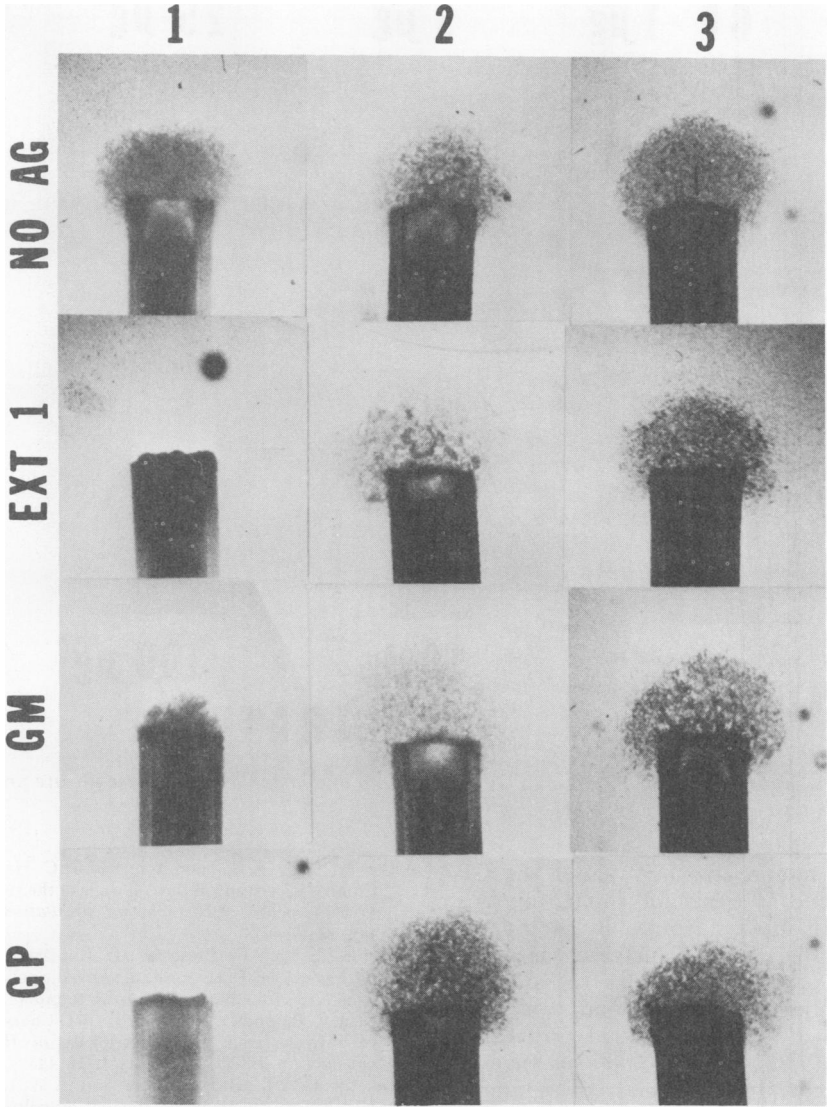

FIG. 5. *Response in an MIF assay to the galactomannan protein complex of H. capsulatum. Column 1, peritoneal exudate cells of animals immunized with extract I in complete Freund adjuvant. Column 2, cells of a normal nonimmune group. Column 3, cells from a group which received complete Freund adjuvant and no histoplasmal antigen. Antigen dose was 10 μg/ml.*

parity is not clear; it is possible that a small amount of peptide persists in the GM (2.63% N) sufficient to trigger MIF production, or that GM may have poor skin adherance properties and rapidly diffuse away. The observation that GP was a potent skin reactive and serologically active antigen gives reason to believe that it may be a precursor of the active component of histoplasmin. Goodman et al. (8) studied the kinetics of secretion of skin-reactive material as a function of culture age and found that at 10 days, which was the age of cultures in the present study, measurable polysaccharide was secreted but there was a low amount of protein released and the filtrate had low skin reactivity. At 8 weeks and beyond, the increased leakage of protein correlated with peak skin reactivity. Studies of the nature of histoplasmin purified derivative have implied that the active species' molecular weight is 1,000 to 5,000 (22) and that it is a glycopeptide. The gel permeation behavior of GP suggests a molecular weight of about

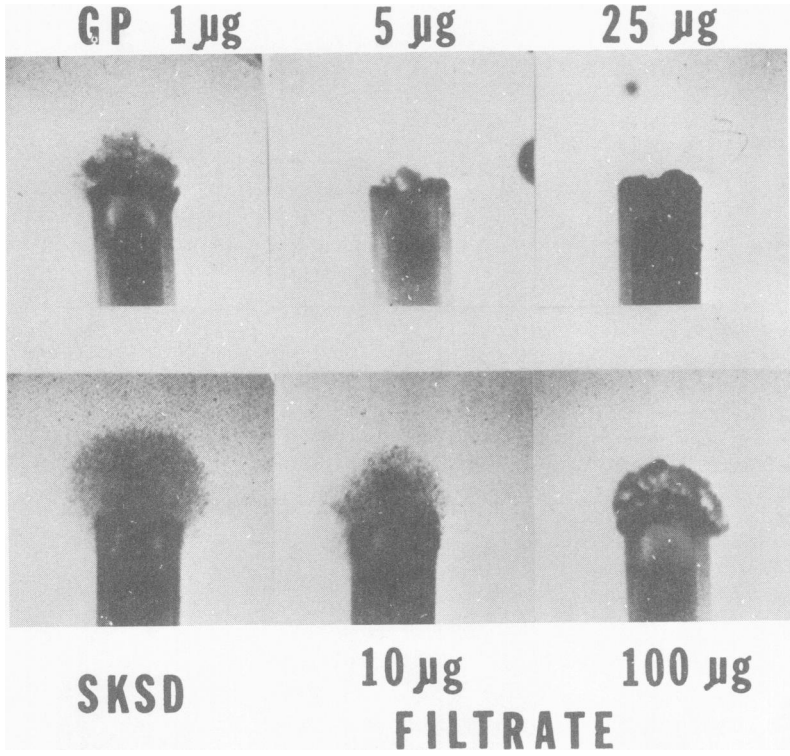

FIG. 6. *MIF assay showing dose response to GP and activity of a histoplasmal culture filtrate preparation, SKSD, Streptokinase-streptodornase, 50 U.*

100,000. The amino acid analysis of the GP fraction provides a basis for comparing this material with histoplasmin. It should be stressed that the material analyzed contained two protein peaks eluted from the diethylaminoethyl-cellulose column and was not further purified. The amino acid content of GMP revealed glutamic acid and aspartic acid to be the predominant species; these amino acids have been implicated in alkali-stable glycopeptide bond formation (17).

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