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Immune Response to *Cryptococcus neoformans* Soluble Polysaccharide

I. Serological Assay for Antigen and Antibody

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Chromium chloride was used as a coupling agent for the conjugation of purified cryptococcal polysaccharide to sheep erythrocytes. Sensitized erythrocytes were used in a passive hemagglutination (PHA) assay for antibody to cryptococcal polysaccharide and a passive hemagglutination inhibition (PHI) assay for antigen. The PHA assay was more sensitive than complement fixation, agglutination, or precipitation tests for antibody. The PHI assay could detect submicrogram quantities of soluble polysaccharide. Antigen or antibody could be detected in serum or spinal fluid from seven of eight patients with cryptococcosis. Tests for antigen or antibody were negative with sera from patients with histoplasmosis, blastomycosis, coccidioidomycosis, aspergillosis, or allescheriosis. A low frequency (3%) of positive reactors for antibody was found among sera from normal persons and from persons with unrelated diseases; whereas, all tests for antigen were negative. The assay showed a high degree of sensitivity for immunoglobulins of the immunoglobulin M class; however, cryptococcal antibody of the immunoglobulin G class was also detected. The immunological specificity of the polysaccharide preparation was due to carbohydrate rather than to protein associated with the polysaccharide.

Several serological methods have been used with varying degrees of success for the assay of cryptococcal antigen and antibody in body fluids. Campbell (4) noted the absence of suitable serological procedures and antigens for the demonstration of circulating antibodies in cryptococcosis. Both the tube agglutination (8) and indirect fluorescent-antibody (22) procedures have become accepted methods for the detection of antibody to *Cryptococcus neoformans*; moreover, latex particle agglutination (3) and complement fixation (23) have been used for the detection of *C. neoformans* antigen.

Seeliger (17) suggested that passive hemagglutination (PHA) with its inherent high sensitivity could be more useful than present assays for antibody. Pollock and Ward (15) adsorbed a crude capsular polysaccharide to human type O erythrocytes and used these erythrocytes to detect cryptococcal antibody. They reported low-order cross-reactions with sera from several patients with disseminated histoplasmosis and with elevated cold agglutinins. Widra et al. (24) were unable to adsorb cryptococcal polysaccharide to human erythrocytes by using the experimental conditions employed by Pollock and Ward (15). They were able to adsorb polysaccharide to formalinized erythrocytes, but their PHA assay proved no more sensitive than the whole cell agglutination technique. In addition, Widra et al. (24) reported that preparations of the soluble polysaccharide were not immunologically stable and eventually became reactive to normal human, rabbit, and bovine serum components.

The purpose of our study was to investigate the use of chromiun chloride as a coupling agent for the conjugation of purified soluble polysaccharide to sheep erythrocytes. This method was used successfully by Gold and Fundenberg (7) for coupling proteins to erythrocytes and later by Baker et al. (1) for use with pneumococcal polysaccharides. After successfully conjugating cryptococcal polysaccharide to sheep red blood cells, a PHA assay for cryptococcal antibody and a passive hemagglutination inhibition (PHI) assay for cryptococcal polysaccharide were readily developed. Several of the problems reported by investigators with earlier PHA assays for cryptococcal antibody were not encountered with this method.

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MATERIALS AND METHODS

Organism. *C. neoformans* strain 613 was used throughout this study. This strain was isolated in 1961 from spleen and lung specimens obtained at autopsy from a patient with a clinical diagnosis of lobar pneumonia. The organism produces a capsule 1.2 μm in width on Littman's medium and it conforms to the standard description of *C. neoformans* outlined by Kreger-van Rij (13).

Rabbit and mouse antisera. The immunization schedule used to prepare *C. neoformans* antisera in rabbits was described in a previous report (12). Mice were immunized subcutaneously with 20 μg of purified cryptococcal polysaccharide emulsified in Freund's incomplete adjuvant (BBL, Bioquest). Seventeen days after immunization, the mice were bled from the ophthalmic venous plexus.

Human sera. Sera and cerebral spinal fluid submitted from patients with suspected or culturally proven mycotic infections were obtained at the University of Iowa Hospitals. Normal sera were obtained from freshman medical students at the University of Iowa.

Purification of soluble polysaccharide. The method used to purify cryptococcal polysaccharide was a slight modification of that used by Evans and Theriault (6) and has been described by a previous report (12).

Pronase treatment of polysaccharide. A 100-mg sample of polysaccharide was dissolved in 50 ml of 0.067 M sodium phosphate buffer (*p*H 7.4). A total of 1,000 PUK units of Pronase (Calbiochem) was added and incubated with the polysaccharide for 24 hr at 40 C. The polysaccharide was then deproteinized, precipitated with ethanol, dialyzed, and dried as described previously (12).

PHA. Polysaccharide was coupled to sheep erythrocytes by a modification of the method used by Baker et al. (1) for pneumococcal polysaccharide. Red cells stored in Alsever's solution were washed three times with saline. One milliliter of saline containing 1.0 mg of purified polysaccharide was added to 0.6 ml of packed sheep erythrocytes. One milliliter of a fresh solution of saline containing 0.5 mg of chromium chloride ($CrCl_3 \cdot 6H_2O$) was added dropwise with constant stirring. The cell suspension was incubated for 7 min at room temperature, washed four times with saline, and adjusted to a final concentration of 0.5%.

All serum specimens were heat inactivated at 56 C for 30 min before use. Antisera were titrated by the Microtiter method in "V" plates. Serial dilutions were done in 25-μliter volumes in saline containing 1% inactivated normal rabbit serum (NRS). After addition of 25 μliters of the 0.5% sensitized erythrocyte suspension to serum dilutions, the plates were incubated at room temperature for 2.5 hr. The highest dilution of serum that caused complete agglutination of sensitized erythrocytes was regarded as the end point. Two controls were included to determine the specificity of any agglutination observed in titration of sera. Serum diluted in saline containing 1% normal rabbit serum and 100 μg of polysaccharide per ml served as a control for specificity of the observed agglutination. Hemagglutination due to a specific antigen-antibody reaction was inhibited by polysaccharide in the diluent. In addition, presence of natural sheep red blood cell agglutinins was determined by incubating serum dilutions with unsensitized sheep erythrocytes. When human serum specimens contained nonspecific agglutinins that interfered with interpretation of PHA results, the agglutinins were removed by adsorption of sera for 10 min at room temperature with an equal volume of washed, packed sheep erythrocytes. A control antiserum of known titer was included in each assay.

PHI assay. The PHA test was modified to a PHI assay for cryptococcal polysaccharide. This test was performed using the Microtiter method by serially diluting the specimen suspected of containing antigen in saline containing 1% normal rabbit serum. To each well was added 25 μliters of immune rabbit serum diluted to contain 2 hemagglutination units (twice the concentration of antibody required to cause complete agglutination of sensitized erythrocytes as determined by prior titration). After incubation at room temperature for 30 min, a 25-μliter volume of 0.5% sensitized erythrocytes was added to each well, and the plates were incubated at room temperature for 2.5 hr. The PHI titer was considered to be the highest dilution of antigen that would inhibit blanket formation of agglutinated cells. Using known concentrations of cryptococcal polysaccharide in saline, the sensitivity of this assay system was determined to be 0.2 to 0.5 μg of polysaccharide.

Complement fixation, agglutination, and precipitation technique. All serological methods were done using a block dilution of antigen and antiserum. The complement fixation test was performed by the Microtiter method using 5 $C'H_{50}$ units of complement in the reaction mixture and the soluble cryptococcal polysaccharide as antigen. Agglutination of Formalin-killed cryptococci was accomplished by both the Microtiter and tube agglutination technique (9). Precipitation of the soluble polysaccharide by immune antiserum was attempted by the qualitative tube precipitation technique (9).

Sucrose density gradient centrifugation. Zonal centrifugation was done on a 3.9-ml linear gradient of 10 to 27% (w/w) sucrose in saline contained in a polyallomer tube (11.11 by 60.32 mm). The gradient was made with a Beckman density gradient former. A 0.2-ml serum sample was layered on the gradient, and the tubes were centrifuged in a Beckman SW-56 titanium rotor on a model L4 ultracentrifuge for 4.5 hr at 56,000 rev/min at 20 C. After centrifugation, a hole was punched in the bottom of the tube, 40% sucrose was pumped in from the bottom, and the gradient was removed from the top of the tube. The gradient was assayed for absorbance at 278 nm, and 3-drop fractions were collected and assayed for PHA activity. When human serum was fractionated, the relative concentrations of immunoglobulin (IgM and IgG in the fractions were determined by immunodiffusion against rabbit anti-human IgM or IgG (Immunology Inc., Lombard, Ill.).

RESULTS

Optimal conditions for coupling cryptococcal polysaccharide to sheep erythrocytes were assessed by the reaction of sensitized erythrocytes

with immune cryptococcal rabbit serum. Sheep erythrocytes sensitized with 250 to 2,000 μg of soluble polysaccharide displayed similar serological activity. The concentration of chromium chloride, however, influenced the serological activity of sensitized erythrocytes. The optimal amount of chromium chloride present in the coupling reaction was found to be 500 μg in a total reaction volume of 2.6 ml. Varying degrees of nonspecific agglutination occurred when 1,000 to 2,000 μg of chromiun chloride were used. Erythrocytes were not sensitized in the absence of coupling agent and were only weakly reactive when 100 μg of chromium chloride was used. Cells treated with Formalin by the method of Csizmas (5) and subsequently exposed to soluble polysaccharide and chromium chloride showed no serological activity with immune rabbit serum.

The serological activity of sensitized erythrocytes remained unchanged after storage in saline at 4 C for as long as 28 days. The PHA assay could be repeated with different preparations of polysaccharide and sensitized cells on different days with no more than a twofold change in titer of rabbit, human, or murine antisera.

Disposable styrene Microtiter plates (Microtiter V, rigid, disposable) and permanent Lucite plates were employed in this study. Occasional nonspecific agglutination of sensitized erythrocytes was observed when styrene plates were used. This nonspecific agglutination could be minimized by immersing the plates in 1% NRS for 12 hr before use. Nonspecific agglutination was never observed with Lucite plates, and they could be used without treatment with NRS.

TABLE 1. *Comparative sensitivity of serological methods*[a]

Serological method	Antigen	Maximum titer[b]
Passive hemagglutination (Microtiter)	Soluble polysaccharide	1/512
Complement fixation (Microtiter)	Soluble polysaccharide	1/128
Agglutination (Microtiter)	Whole cell	1/16
Agglutination (tube)	Whole cell	1/8
Precipitation (tube)	Soluble polysaccharide	No reaction

[a] All titrations were done on single serum specimen from rabbit immunized with Formalin-killed cryptococcal cells.

[b] Highest dilution of antisera showing presence of antibody.

TABLE 2. *Antigen and antibody assay of specimens from cryptococcal patients*

Patient	Date collected	Specimen	Reciprocal of titer	
			Antibody (PHA)[a]	Antigen (PHI)[b]
C. F.	9/13/60	Serum	<2	8
L. F.	2/20/63	Serum	<2	<2
C. W.	2/3/70	Serum	8	<2
	2/17/70	Serum	64	<2
L. M.	2/19/70	Serum	<2	8
	3/9/70	Serum	<2	2
	3/26/70	Serum	<2	<2
M. T.	3/24/70	Serum	16	<2
	3/26/70	Serum	16	<2
	4/6/70	Serum	<2	<2
V. H.	6/16/70	Serum	8	<2
R. W.	8/31/70	CSF[c]	<2	32
A. H.	10/27/70	Serum	2	<2

[a] Passive hemagglutination.

[b] Passive hemagglutination inhibition.

[c] Cerebrospinal fluid.

The PHA assay was found to be more sensitive than a number of other methods used in the serodiagnosis of cryptococcosis. Table 1 shows the maximum titer obtained with each of several serological techniques by using a specimen of immune rabbit serum. All titrations were done by block titration of antigen and antiserum. The PHA assay was found to be most sensitive, followed by complement fixation and whole cell agglutination. Precipitation of polysaccharide by immune rabbit serum could not be detected by the tube precipitation method.

The PHA assay was able to detect anti-cryptococcal antibody in serum from patients with culturally proven cryptococcosis (Table 2). Positive results for antibody were obtained with specimens from four of eight patients. Cryptococcal antigen was detected by PHI in specimens from three of the remaining four patients. All specimens have been assayed several times using different preparations of sensitized erythrocytes without a change in PHA titer of more than one twofold dilution.

The PHA assay was negative with six sera from patients with histoplasmosis, one serum specimen from a patient with blastomycosis, three sera from coccidioidomycosis patients, and three serum specimens from allescheriosis patients. Sera from two aspergillosis patients produced questionable results on styrene Microtiter plates, but were negative when assayed using Lucite plates. Positive titers of 1/8 and 1/32 were obtained with 2 of 50 presumably normal sera. One of 40 sera from patients at the University of Iowa Hospitals

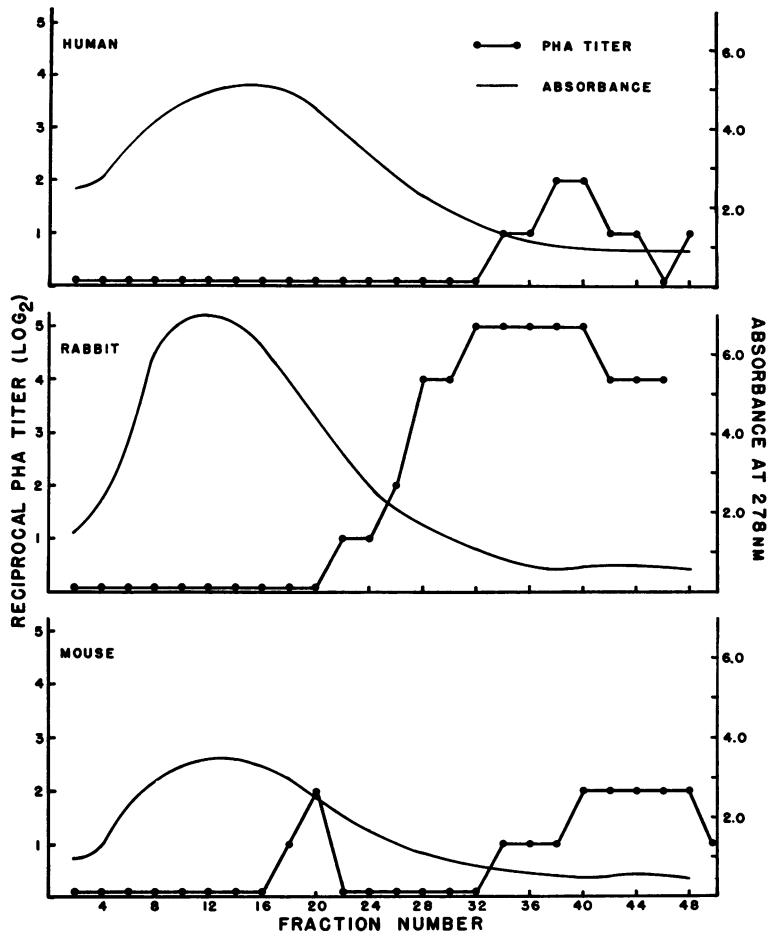

FIG. 1. *Immune human, rabbit, and mouse sera fractionated by sucrose density gradient centrifugation. Distribution of serum proteins and cryptococcal polysaccharide-specific antibodies detected by PHA. Fraction 1 is the first fraction at the top of the gradient.*

reacted with cryptococcal polysaccharide to a titer of 1/4. Antibody found in all three of these normal control sera could be neutralized with cryptococcal polysaccharide.

PHA assays for antibody have usually shown a greater sensitivity to globulins of the IgM class than globulins of the IgG class. The immunoglobulin specificity of the PHA assay for cryptococcal antibody was determined by sedimentation through a sucrose gradient. Two separate specimens of human cryptococcal serum, rabbit cryptococcal serum, and two pooled sera from individual groups of three immunized mice were sedimented through a sucrose gradient. Fractions collected from the gradient were assayed by PHA, and the antibody titer was plotted against fraction number along with ultraviolet absorption at 278 nm (Fig. 1). Serum components migrating at a rate of 4*S* and 7*S* were not resolved, and they are identified as the leading protein peak. Although only a single specimen from each source is shown, all human and rabbit sera showed peak serological activity in the 19*S* globulin region located between 20 and 30 fractions after the initial protein peak. The murine serum pool shown in the figure demonstrated activity in both an early and a late peak. A second pool of murine serum showed only a late peak.

A 5 mg/ml sample of purified soluble polysaccharide isolated from *C. neoformans* strain 613 was assayed by various chemical means to determine the presence of non-polysaccharide material (Table 3). The total nitrogen content of the product could be accounted for mainly as protein nitrogen, assuming a 6.25 conversion factor (18). Further treatment of the polysaccharide by the

TABLE 3. *Noncarbohydrate constituents of Cryptococcus neoformans strain 613 soluble polysaccharide*[a]

Constituent	Content (μg/ml)	Total weight (%)
Protein[b]	65	1.3
Nitrogen[c]	10	0.2
DNA[d]		
RNA[e]		

[a] Analysis based on sample containing 5,000 μg of soluble polysaccharide per ml.
[b] Lowry assay (sensitivity ≈ 25 μg/ml).
[c] Micro-Kjeldahl with nesslerization (sensitivity ≈1 to 2 μg/ml).
[d] Diphenylamine assay (sensitivity ≈ 50 μg/ml).
[e] Absorption at 260 nm (sensitivity ≈ 5 μg/ml).

modification of Sevag's method of deproteinization would not reduce or alter the percentage of of protein or nitrogen. Deoxyribonucleic acid (DNA) as determined by the diphenylamine reaction (19) or ribonucleic acid (RNA) as determined by adsorption at 260 nm was absent.

Since the polysaccharide preparations used in this study contained a small amount of protein, it was necessary to demonstrate that the PHA assay was specific for antibody to the polysaccharide rather than the protein portion of the preparation. A solution of 100 mg of cryptococcal polysaccharide was incubated with 1,000 PUK units of Pronase as described above. Assay of a 5 mg/ml sample of polysaccharide by the Lowry and micro-Kjeldahl assays indicated that no protein could be detected, and nitrogen content was reduced from 10 μg/ml before Pronase treatment to 1.8 μg/ml after Pronase treatment. The nitrogen content was near the lower limit of assay sensitivity.

The Pronase-treated polysaccharide was coupled to sheep erythrocytes with chromium chloride and assayed for serological activity. Removal of protein did not affect the ability of the polysaccharide to react with immune serum (Table 4). In addition, protein-free polysaccharide and normal cryptococcal polysaccharide did not differ in their ability to inhibit PHA.

DISCUSSION

Within the limits of assay sensitivity, the *C. neoformans* soluble polysaccharide was free of contaminating DNA and RNA. The soluble polysaccharide preparation did, however, contain a small amount of protein. Despite the presence of protein, the preparation used throughout this study was referred to as soluble polysaccharide. It is not known whether the protein component may have been simply trapped within the highly viscous polysaccharide or whether it may have been chemically bound to the polysaccharide. The latter possibility appears most likely since deproteinization of the preparation resulted in removal of protein only until the protein content was reduced to about 1%. Further deproteinization removed both polysaccharide and protein but did not alter the relative concentration of either component in the supernatant fluid. Since chloroform acts as a liquid adsorbent (20) during the deproteinization procedure, adsorption of both polysaccharide and protein would be expected if the protein were bound to the polysaccharide. Other investigators (2, 23) have also noted the presence of a small amount of protein in preparations of cryptococcal polysaccharide.

The PHA assay for cryptococcal antibody was found to be satisfactory, and the procedure has several attributes that recommend its further use. The assay was easy to perform, it was more sensitive than several methods in current use, and titers were reliably reproducible. The loss of immunological activity of soluble polysaccharide reported by Widra et al. (24) was not encountered. Polysaccharide preparations used by Widra et al. were not deproteinized, nor was the polysaccharide dried by lyophilization.

As a result of utilizing a highly purified antigen, cross-reactions in the PHA assay were found to be minimal. A survey of normal sera and sera from other mycotic infections indicated that there was, in fact, a 3% frequency of positive reactions, all of which could be neutralized by the specific antigen. Vogel et al. (22), using fluorescent-antibody techniques, reported an 8% frequency of positive reactors among 339 sera from normal individuals and persons with unrelated diseases. Using the indirect fluorescent-antibody test, Kaufman and Blumer (10) found an incidence of 21% positive reactors among normal subjects and

TABLE 4. *Effect of Pronase on serological activity of cryptococcal polysaccharide*

Specimen	Reciprocal PHA titer[a]	
	Treated	Untreated
Rabbit serum 1	2,048	2,048
Rabbit serum 2	64,000	32,000
Mouse serum 1	128	128
Mouse serum 2	256	256
Human serum 1	16	16
Human serum 2	4	8

[a] Erythrocytes sensitized with Pronase-treated or untreated soluble polysaccharide. PHA, passive hemagglutination.

patients with systemic fungus disease other than cryptococcosis. Unlike the PHA assay reported by Pollock and Ward (15), a high incidence of positive reactors among histoplasmosis patients was not found. This may be due to differences in the type of red blood cell used in the test, the degree of purity of the antigen, or the method of coupling polysaccharide to the erythrocyte.

The PHI assay for soluble polysaccharide was able to detect submicrogram quantities of polysaccharide. When the test was used on specimens from cryptococcal patients, low titers of polysaccharide were detected in sera or spinal fluids from several patients. The PHI technique was less sensitive than the latex particle agglutination assay (3), since that method has been shown to detect as little as 0.025 μg/ml (M. Gordon, *personal communication*). Like the latex agglutination assay (3, 10), studies of normal sera and serum from patients with several mycotic infections showed no positive reactors.

The ease with which the PHI assay technique may be quantitatively standardized may make it a useful tool for resolving differences in the relative merits of other serological tests for cryptococcosis reported in the literature. Walter and Jones (23) reported that the complement fixation assay was able to detect antigen in 16 of 40 sera from cyrptococcosis patients, whereas the latex fixation test detected antigen in only 5 of the same 40 sera. On the other hand, Kaufman and Blumer (10) subsequently reported that the latex particle agglutination assay detected antigen in 46 of 49 serum and spinal fluid specimens from cryptococcal patients; whereas, the complement fixation test detected antigen in only 27 of the 49 specimens.

Data obtained in this study indicate that PHA specificity of the polysaccharide preparation resides with the carbohydrate portion since removal of protein did not alter the ability of the polysaccharide to react with immune serum. The inhibition of agglutination between sensitized erythrocytes and immune serum was also unaffected by Pronase treatment of the polysaccharide.

The PHA assay readily detected globulins of the IgM class. Strong activity was noted in the IgM region of six sera fractionated on a sucrose gradient. Activity in the IgG region was noted in a single pool of immune mouse serum. The indirect fluorescent-antibody test (21) and bentonite flocculation technique (11), presently used for assay of cryptococcal serum, both have shown specificity only for immunoglobulins of the IgG class. The immunoglobulin specificity of the tube agglutination assay (8) has not been determined but is presumed to be of the IgM class.

The significance of various immunoglobulin classes of antibody in cryptococcosis is not known. Knowledge of immunoglobulin types produced in coccidioidomycosis and candidosis has been quite useful. Sawaki et al. (16) showed that tests with a high sensitivity for IgM were positive only in the early stage and after exacerbation of preexisting coccidioidomycosis. Results from antibody assays more sensitive to IgM reflected the clinical condition of the patient more accurately than antibody assays with a high sensitivity for IgG. Lehrer (14) found significant titers of IgG antibody in 78% of 65 patients with candidosis, IgM antibody was present in 51%, and IgA in 30%. Some differences were found in incidence, titer, and immunoglobulin class between the four clinical types of candidosis. Patterns of antibody production of various classes of immunoglobulins may prove to be significant in the serodiagnosis of cryptococcosis. A full evaluation would, however, require the survey of considerably more sera than were available for this study.

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LITERATURE CITED

1. Baker, P. J., P. W. Stashak, and B. Prescott. 1969. Use of erythrocytes sensitized with purified pneumococcal polysaccharides for the assay of antibody and antibody-producing cells. Appl. Microbiol. 17:422–426.
2. Bennett, J. E., and H. F. Hasenclever. 1965. *Cryptococcus neoformans* polysaccharide: studies of serologic properties and role in infection. J. Immunol. 94:916–920.
3. Bloomfield, N., M. A. Gordon, and D. F. Elmendorf, Jr. 1963. Detection of *Cryptococcus neoformans* antigen in body fluids by latex particle agglutination. Proc. Soc. Exp. Biol. Med. 114:64–67.
4. Campbell, C. C. 1967. Serology in the respiratory mycoses. Sabouraudia 5:240–259.
5. Csizmas, L. 1960. Preparation of formalinized erythrocytes. Proc. Soc. Exp. Biol. Med. 103:157–160.
6. Evans, E. E., and R. J. Theriault. 1953. The antigenic composition of *Cryptococcus neoformans*. IV. The use of paper chromatography for following purification of the capsular polysaccharide. J. Bacteriol. 65:571–577.
7. Gold, E. R., and H. H. Fundenberg. 1967. Chromic chloride: a coupling reagent for passive hemagglutination reactions. J. Immunol. 99:859–866.
8. Gordon, M. A., and D. K. Vedder. 1966. Serologic tests in diagnosis and prognosis of cryptococcosis. J. Amer. Med. Ass. 197:961–967.
9. Kabat, E. A., and M. M. Mayer. 1967. Experimental immunochemistry, 2nd ed. Charles C Thomas, Springfield, Ill.
10. Kaufman, L., and S. Blumer. 1968. Value and interpretation of serological tests for the diagnosis of cryptococcosis. Appl. Microbiol. 16:1907–1912.
11. Kimball, H. R., H. F. Hasenclever, and S. M. Wolff. 1967. Detection of circulating antibody in human cryptococcosis by means of a bentonite flocculation technique. Amer. Rev. Resp. Dis. 95:631–637.

12. Kozel, T. R., and J. Cazin, Jr. 1971. Nonencapsulated variant of *Cryptococcus neoformans*. I. Virulence studies and characterization of soluble polysaccharide. Infec. Immun. 3:287–294.
13. Kreger-van Rij, N. J. W. 1961. Taxonomy of *Cryptococcus neoformans* and its variety *uniguttulatus*. Antonie van Leuwenhoek J. Microbiol. Serol. 27:59–64.
14. Lehrer, T. 1970. Serum fluorescent antibody and immunoglobulin estimations in candidosis. J. Med. Microbiol. 3:475–481.
15. Pollock, A. Q., and L. M. Ward. 1962. A hemagglutination test for cryptococcosis. Amer. J. Med. 32:6–16.
16. Sawaki, Y., M. Huppert, J. W. Bailey, and Y. Yagi. 1966. Patterns of human antibody reactions in coccidioidomycosis. J. Bacteriol. 91:422–427.
17. Seeliger, H. P. R. 1964. Use of serological methods for the diagnosis of cryptococcosis. Ann. Soc. Belge Med. Trop. 44:657–672.
18. Seibert, F. B., and L. F. Affronti. 1963. Kjeldahl-Nessler method for determination of nitrogen, sec. II., p. 5–8. *In* Methodology manual for investigation of mycobacteria and fungal antigens. American Thoracic Society.
19. Seibert, F. B., and L. F. Affronti. 1963. Diphenylamine reaction for DNA, sec. II., p. 25–27. *In* Methodology manual for investigation of mycobacterial and fungal antigens. American Thoracic Society.
20. Sevag, M. G., D. B. Lackman, and J. J. Smolens. 1968. The isolation of the components of streptococcal nucleoproteins in serologically active form. J. Biol. Chem. 124: 425–436.
21. Vogel, R. A. 1966. The indirect fluorescent antibody test for the detection of antibody in human cryptococcal disease. J. Infec. Dis. 116:573–580.
22. Vogel, R. A., T. F. Sellers, and P. Woodward. 1961. Fluorescent antibody techniques applied to the study of human cryptococcosis. J. Amer. Med. Ass. 178:921–923.
23. Walter, J. E., and R. D. Jones. 1968. Serodiagnosis of clinical cryptococcosis. Amer. Rev. Resp. Dis. 97:275–282.
24. Widra, A., S. McMillen, and H. J. Rhodes. 1968. Problems in serodiagnosis of cryptococcosis. Mycopathol. Mycol. Appl. 36:353–358.