Applied Microbiology, Dec. 1968, p. 1907–1912
Vol. 16, No. 12
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Value and Interpretation of Serological Tests for the Diagnosis of Cryptococcosis

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Received for publication 24 September 1968

Maximal serological diagnosis of cryptococcosis may be accomplished through the concurrent use of three tests: the latex agglutination (LA) test for cryptococcal antigen, and the indirect fluorescent antibody (IFA) and tube agglutination (TA) tests for *Cryptococcus neoformans* antibodies. These tests were applied to 141 serum and cerebral spinal fluid specimens from 66 culturally proven cases of cryptococcosis and to 42 sera from normal subjects and from patients with other systemic mycotic diseases. The LA test was sensitive and completely specific; of the sera from proven cases, 55% were positive. With the TA test, 37% of the specimens were positive and the test was highly specific. With the IFA test, 38% of the specimens were positive and the test appears to be the least specific of the three. Cross-reactions were most evident with blastomycosis and histoplasmosis case sera. When the three tests were used concurrently, 87% of the cryptococcosis case specimens were positive and permitted a presumptive diagnosis of *C. neoformans* infections in 61 (92%) of the 66 patients whose specimens were examined.

Cryptococcosis is a pulmonary disease that frequently spreads to other parts of the body, particularly the central nervous system. Clinically, it may be confused with other infectious and malignant diseases. The diagnosis of cryptococcosis may be difficult because *Cryptococcus neoformans* is an opportunist which readily coexists with malignant diseases of the reticuloendothelial and lymphatic systems, diabetes, histoplasmosis, and tuberculosis. Furthermore it is often implicated as a secondary invader when broad spectrum antibiotics, steroids, or cytotoxic agents are administered (3, 5, 6).

The actual incidence of cryptococcosis is not known; however, various authorities estimate that the disease is more common than suspected. J. P. Utz estimated that 200 to 300 cases of cryptococcal meningitis occur annually in the United States (*The Evening Star*, Washington, D.C., 16 Nov. 1964). Littman and Schneierson (11) state that that 5,000 to 15,000 subclinical and clinical pulmonary cases of cryptococcosis occur annually in New York City alone. Since 1952, the number of deaths reported due to *C. neoformans* within the United States has averaged 66 per year (1). The almost 100% mortality rate of cryptococcal meningitis before the era of amphotericin B therapy (5) stresses the paramount need for accurate and rapid diagnosis of this disease and its control.

Until recently, individuals suffering from cryptococcosis were considered essentially immunologically inert. Consequently, diagnosis was limited to time-consuming cultural and biochemical procedures. Within the last few years, in response to the increased awareness of cryptococcal infections, a number of serologic procedures have been developed and reported. This study was undertaken to develop and evaluate new procedures and to evaluate the specificity and sensitivity of established ones.

Materials and Methods

Serum and spinal fluid specimens. Specimens received in the Fungus Immunology Unit, NCDC, were from culturally proven cases of blastomycosis, candidiasis, coccidioidomycosis, cryptococcosis, and histoplasmosis and from apparently healthy humans. The clinical diagnosis in each case was obtained from the attending physician. All specimens were preserved with Merthiolate (1:10,000) and stored at −20 C.

Complement-fixation (CF) test. Sera and cerebral spinal fluids (CSF) were titrated for cryptococcal antibody and antigen by the Laboratory Branch Complement Fixation (LBCF) test (14). This test involves five 50% units of complement in 0.4 ml in a total volume of 1.0 ml. The antigen-antibody-complement mixture is incubated for 15 to 18 hr at 4 C. The antigen used for detecting antibodies was a 10% suspension of whole cells of *C. neoformans* culture B-551 (8). An optimal dilution of 1:8 for the whole cell antigen was determined by box titration

with rabbit anti-*C. neoformans* serum. Rabbit anti-*C. neoformans* serum lot DR, produced according to an earlier protocol (8) and demonstrating a tube agglutination titer of 1:320, was used to detect the presence of cryptococcal antigen in sera and spinal fluids. The optimal dilution for this serum was 1:128. This was determined by box titration with human serum containing cryptococcal antigen as demonstrated by latex agglutination. Yeast-form antigens of *Histoplasma capsulatum* NCDC lot 35, diluted 1:32, and *Blastomyces dermatitidis* NCDC lot 18, diluted 1:64, were used in addition to NCDC lot 1534 histoplasmin, diluted 1:16. Sera that demonstrated 30% hemolysis or less at a particular dilution were considered positive.

Immunodiffusion (ID) tests. Agar gel double-diffusion tests were performed to detect cryptococcal antigens and antibodies in clinical specimens. The agar-gel medium and slide test procedures were essentially the same as those described in a previous paper (9). Antigens were 5× concentrated acetone-precipitated supernatant fluids from 1-week-old Sabouraud dextrose broth cultures of *C. neoformans* strain B-551 grown at 37 C. The antigenicity of these culture filtrates was checked with rabbit anti-*C. neoformans* sera. One or two bands developed, depending upon the rabbit serum used.

In tests for antigen, rabbit *C. neoformans* antiserum lot DR, used at a dilution of 1:128, gave an optimal agar-gel reaction with sera positive for cryptococcal antigen. These antigen-positive sera were obtained from rabbits injected with nonviable *C. neoformans* cells and from human cryptococcosis cases.

Indirect fluorescent antibody (IFA) test. The IFA test was performed by a modification of the method of Vogel (15). FA reagents were prepared by using rabbit antihuman globulin and conjugating it with fluorescein isothiocyanate. Smears of antigen, consisting of intact cells of *C. neoformans* culture B977, were prepared by placing 1 drop of antigen suspension on a clean microscope slide by means of a Pasteur pipette. These slides then were air-dried and heat-fixed. Serum specimens were heated at 70 C for 5 min prior to testing. Three drops (approximately 0.1 ml) of a 1:20 dilution of test serum in phosphate buffered saline (*p*H 7.2) was placed over the dried cells by means of a Pasteur pipette. The slide was incubated in a moist chamber at room temperature for 35 min on a reciprocal platform shaker that moved at a rate of 90 excursions/min. The slide was rinsed for 10 min in phosphate-buffered saline, dipped five times in distilled water and allowed to air dry. The same procedure was followed with fluorescein-labeled antihuman globulin diluted 1:80. The staining reactions were read using a Corning 5113 primary filter in combination with a Wratten 2A barrier filter. Average cell wall staining ranging from 2+ to 4+ intensity was considered positive.

Latex agglutination (LA) test. Latex particle agglutinations were performed according to a modified method of Bloomfield et al. (4). Specimens of serum and spinal fluid were inactivated at 56 C for 30 min, and then serially diluted in *p*H 9.0 glycine-buffered saline that contained 0.1% bovine serum albumin. Equal volumes of a spectrophotometrically standardized suspension of latex particles were mixed with the highest dilution of 4% rabbit *C. neoformans* antiglobulin giving a clear agglutination pattern with a reference serum containing cryptococcal polysaccharide. To perform the test, 0.04 ml of heat-inactivated serum or spinal fluid was placed in a corner of a 2.54-cm square of a paraffin-ruled glass slide. A 0.02-ml amount of the latex-globulin suspension was placed in the opposite corner. The reagents were mixed and agitated for 2 min on a rotary shaker at 100 rev/min. The antigen titer was recorded as the highest dilution of a serum or spinal fluid that showed agglutination equivalent to the positive control. All positive specimens were also tested with latex particles sensitized with normal rabbit globulin to rule out false positives.

Tube agglutination (TA) tests. C. neoformans culture B551, a weakly encapsulated isolate, was used as an agglutinogen (8). Agglutinin titers were determined by a tube test using 0.25 ml of antigen turbidimetrically adjusted to a no. 2 McFarland nephelometer standard. All sera were inactivated by heating at 56 C for 30 min. Twofold dilutions of each serum were made, beginning with an undiluted specimen. Readings were made after 2 hr of incubation at 37 C and overnight refrigeration at 4 C.

RESULTS

The relative sensitivity of the five serological tests for cryptococcal disease was initially determined with 49 serum and CSF specimens from 36 culturally proven cases of cryptococcosis. Of the 46 serum and 3 CSF specimens, 43 sera and all 3 CSF specimens were positive for antigen by the LA test (Table 1). Only 25 sera and 2 of the CSF specimens were positive by the CF test. The least sensitive procedure for detecting antigen was the ID test. None of the CSF specimens and only 15 of the 46 sera were positive with this test. All of the 25 sera and 2 CSF specimens positive by the CF test were positive by the LA test. Of the 15 ID test positive sera, 14 were positive by the LA test. The polysaccharide antigen titers varied from undiluted to 1:4,096 with the LA test and from 1:2 to 1:256 with the CF test.

None of the 3 CSF specimens and very few of

TABLE 1. *Results of serological tests for C. neoformans antibodies and antigens in proven case serum and spinal fluid specimens*

Type	No.	No. of positive tests for antigens			No. of positive tests for antibodies			
		LA	ID	CF	IFA	TA	ID	CF
Serum	46	43	15	25	9	4	0	3
Spinal fluid	3	3	0	2	0	0	0	0

the 46 sera initially screened showed antibody responses (Table 1). The IFA, TA, and CF tests all detected antibody to some degree, whereas all specimens examined with the ID test were negative.

The LA test appeared to be the most sensitive procedure for detecting polysaccharide in the specimens tested. Of the three antibody tests, the IFA procedure was the most sensitive, with the TA and CF test showing about equal reactivity. The TA titers varied from 1:2 to 1:10, whereas the CF titers were all 1:2. The CF test is complex to perform, and it is useless when anticomplementary reagents are encountered. Consequently, along with the insensitive ID test, it was eliminated from more extensive evaluations.

The IFA, LA, and TA tests were further evaluated with 120 serum and 21 CSF specimens from 66 proven cases of cryptococcosis (Table 2). Of the 120 sera, the LA test was positive with 63, the IFA with 53, and the TA with 51 specimens. Analysis of the 21 CSF specimens by IFA and TA tests indicated the presence of little antibody in this clinical material. The LA test appeared to be the most effective means of detecting cryptococcal infection where CSF specimens were concerned.

Of the 141 serum and CSF specimens studied, 55% were positive with the LA test, 37% with the TA test, and 38% with the IFA test. By contrast, 87% of the 141 specimens were positive with one or more of the three tests. Analysis of the serum and CSF specimens by these 3 tests enabled a presumptive diagnosis of cryptococcosis in 61 (92%) of the 66 proven cases.

The specificity of the three procedures was determined by testing 42 sera from patients with

TABLE 2. *Serological reactivity patterns demonstrated by 141 serum and spinal fluid specimens from 66 proven cases of cryptococcosis*

Type	Reactivity patterns with tests			Pattern frequency	
	IFA	LA	TA	No.	Per cent
Sera	+	+	+	5	4
	+	+	0	13	9
	+	0	+	27	19
	+	0	0	8	6
	0	+	+	10	7
	0	+	0	35	25
	0	0	+	9	6
	0	0	0	13	9
Spinal fluids	0	0	0	6	4
	0	+	0	14	10
	0	+	+	1	1

TABLE 3. *Reactivity of the cryptococcosis serological tests with 42 sera from normal subjects and patients with systemic fungus diseases other than cryptococcosis*[a]

Clinical category	No. of subjects	Number of positive reactions		
		IFA	LA	TA
Blastomycosis	12	4	0	1
Candidiasis	2	0	0	0
Coccidioidomycosis	9	1	0	0
Histoplasmosis	12	4	0	1
Normal	7	0	0	0

[a] The specificities for IFA, LA, and TA tests were 78.6, 100, and 95.2%, respectively.

blastomycosis, candidiasis, coccidioidomycosis, and histoplasmosis, as well as sera from apparently healthy individuals (Table 3). Our results indicated that the LA and TA tests were more specific than the IFA procedure. No false positives were recorded with the antigen detecting procedure. However, false positives were encountered with the antibody detecting procedures. Cross-reactions were most frequent with sera from patients with blastomycosis and histoplasmosis, and these occurred mainly with the IFA test.

Studies were undertaken to determine whether the cryptococcal IFA reactions could be more specifically interpreted if parallel IFA and CF tests were performed with *H. capsulatum* and *B. dermatitidis* antigens. The assumption was made that the cryptococcal antibody would not react with the heterologous antigens in the CF and IFA tests. Studies were performed with sera from human cases of blastomycosis, cryptococcosis, and histoplasmosis. The data obtained (Table 4) indicated that the IFA test was susceptible to nonspecific reactions. All of the IFA test antigens showed reactions with the heterologous antisera examined. The CF tests for histoplasmosis and blastomycosis were positive with many of the cryptococcosis case sera and were no more discerning than the IFA tests in diagnosing cryptococcosis.

No particular pattern of serological response could be correlated with any one clinical type of cryptococcosis. The IFA staining reactions varied from 2 to 4+ with the standard 1:20 dilution of serum, whereas the TA titers ranged from 1:2 to 1:20. Intense (4+) staining or high agglutinin titers, or both, were not indicative of severe cryptococcal infections. The LA titers varied from undiluted to 1:64,000. In contrast to high antibody titers, high antigen titers reflected progressive disease.

TABLE 4. *Nonspecific reactions noted with IFA and CF tests for blastomycosis, cryptococcosis, and histoplasmosis*

Proven case serum	Code no.	IFA reaction			CF titer		
		C. neoformans	*B. dermatitidis*	*H. capsulatum*	*H. capsulatum*		*B. dermatitidis*
					Yeast	Histoplasmin	Yeast
Cryptococcosis	13	+	+	+	8	0	0
	41	+	+	+	8	8	32
	42	+	+	+	8	0	8
	56	+	0	0	0	0	8
	72	+	+	+	32	32	0
Blastomycosis	SB5722	+	+	0	0	0	0
	SB5728	+	+	+	0	0	8
Histoplasmosis	788-63	+	0	+	128	256	0
	4008-63	+	+	0	256	64	256
	5905-63	+	+	+	32	128	16

TABLE 5. *Antibody and antigen responses in sera and/or spinal fluids from 10 cases of cryptococcosis prior to and after amphotericin B treatment*

Type	Case	Specimen	Shift in serologic response with chemotherapy		
			IFA	TA	LA
Meningeal	G. A.	Sera	3-4+	1:5-1:20	0
	H. W.	Sera	4-3+	1:10-1:2	0
	J. S.	Sera	4-2+	1:2-1:20-1:5	0
	J. B.[a]	CSF	0	0	1:512-1:16
	W. P.	CSF	0	0	1:512-1:8
	W. P.	Sera	3-2+	0	1:512-1:8
Meningeal and pulmonary	G. O.	Sera	3-2+	1:5-1:2	0
	P. S.[b]	Sera	0	0	1:8
	K. A.	Sera	0-3+	0	1:256-1:8192-1:16
Pulmonary	R. B.	Sera	2+-0	0-1:5	1:2-1:64-0
	D. S.	Sera	2+	0	1:128-0

[a] Hypogammaglobulinemia.
[b] Disseminated TB and Hodgkins disease.

The serological reactions of 10 proven cases of cryptococcosis before and after treatment with amphotericin B were determined by the three procedures (Table 5). Our studies indicated that regardless of the type of cryptococcosis, patients may show only an antibody response, only an antigen response, or both. The effects of chemotherapy were not revealed in the IFA or TA reactions. The LA titers, however, declined significantly with the patients' recovery and response to chemotherapy. In some instances, the LA titers moved upward after administration of amphotericin B and then declined (cases K. A. and R. B.). The LA test was particularly valuable with case J. B.; the patient suffered from hypogammaglobulinemia, showed no antibody activity, and was easily diagnosed and followed by antigen titrations.

DISCUSSION

Our study of the sensitivity of five different techniques (LA, CF, ID, TA, and IFA) for the serological diagnosis of cryptococcosis revealed three of them to have greater diagnostic application (Table 1). They are the LA test, for cryp-

tococcal antigens, and the IFA and TA tests for cryptococcal antibodies.

Unlike the results of Walter and Jones (17) our studies indicated that the LA test was more sensitive than the CF test for detection of capsular polysaccharide in clinical materials. Of the 49 serum and CSF specimens from proven cases, 95% were positive with the LA test, 55% were positive with the CF test, and 30% were positive with the ID test. All specimens positive for antigen by the CF and ID tests were also positive with the LA procedure. The differences noted between the sensitivities of our tests for soluble antigens and those of the tests used by Walter and Jones (17) might be attributed to our modifications.

The ability to detect cryptococcal antibodies in proven case sera varied with the test used. The IFA test, as indicated by Vogel and Padula (16), was the most sensitive procedure because the highest percentage of proven case sera were positive (Table 1). Although the TA and CF tests were less sensitive, in many cases sera negative with the IFA test were positive with the TA and CF tests. In addition to variation in test sensitivity, different types of antibodies appeared to be reacting in the various tests used.

In 1966, Vogel (15) demonstrated that the IFA test, with varied serotypic capsular antigens, was positive with sera from 80% of the proven cases examined. Testing for cryptococcal antibody with a bentonite flocculation test [Kimball et al. (11)] demonstrated antibody in 42% of patients with meningeal infections and in 50% of patients with nonmeningeal infections. Gordon and Vedder (7) reported an inverse relationship between the presence of antigen and the presence of antibody in individuals infected with *C. neoformans*. Consequently, they recommended that a test for antigen be performed in conjunction with one for antibody. Using CF, LA, and slide agglutination tests to assay sera for cryptococcal antigens and antibodies, Walter and Jones diagnosed 28 (70%) of 40 patients with clinically suspected or culturally proved cryptococcosis (17). Recent studies with IFA tests both for cryptococcal antigens and for antibodies indicate that such tests provide presumptive evidence for cryptococcosis in approximately 90% of suspected cases (12). Our studies (Table 2) indicate that the parallel use of LA, TA, and IFA tests permits the maximal sero-diagnosis of cryptococcosis. The application of this battery of tests to CSF or serum specimens, or both, from 66 proven cases permitted a presumptive diagnosis of cryptococcosis in 92% of the patients. No single test provided this level of sensitivity. Our studies also revealed that the LA test was the most useful for detecting cryptococcal meningitis (Table 2). Of the 21 proven case CSF specimens, 71% were positive when the LA test was used, in contrast to less than 0.5% with the TA test and none with the IFA tests for antibody.

In routine applications of the three tests, less than 8% of the sera from proven cases were negative (Table 2). A diagnosis of cryptococcosis should not be excluded on the basis of negative serology. Intensive cultural and biochemical studies are advised in cases in which clinical symptoms are consistent with cryptococcosis and no etiology has been established.

Of the three tests extensively evaluated, the most specific was the LA test (Table 3), which yielded no false positives. Cross-reactions occurred with the tests for antibody, particularly the IFA test which demonstrated a 78.6% specificity. Most of the cross-reactions were with sera from proven cases of blastomycosis and histoplasmosis. These results are contrary to those of Vogel, who reported no cross-reactions with sera from 30 patients with active histoplasmosis and fewer cases of blastomycosis (15). They are, however, in accord with the findings of Kimball et al. (10) who also noted cross-reactions with sera from patients with systemic mycotic infections, particularly blastomycosis.

For satisfactory interpretation of these serological tests, laboratory workers must be aware that the IFA test is more susceptible to cross-reactions than the other procedures evaluated. The performance of parallel IFA tests with *B. dermatitidis* and *H. capsulatum* antigens further demonstrated the spread of common antibodies among patients with cryptococcosis, blastomycosis, and histoplasmosis (Table 4). Attempts to utilize CF tests with *B. dermatitidis* antigens and histoplasmin and yeast-form antigens of *H. capsulatum* to distinguish cryptococcosis case sera from blastomycosis and histoplasmosis case sera also failed. The cryptococcosis case sera showed nondifferential CF titers ranging from 1:8 to 1:32 with all the heterologous antigens. The attribution of these reactions to subclinical heterologous infections appears to be remote. The antigenic relationship of *C. neoformans* to *B. dermatitidis*, *H. capsulatum*, and other fungi has also been demonstrated by Atkinson and Bennett (2), using cryptococcin and other skin test antigens in guinea pigs sensitized with various mycotic pathogens, and by Kaufman and Blumer (8) in other studies. These observations are contrary to those of other investigators (13) who reported no antigenic relationship between the cryptococci and *H. capsulatum*, *B. dermatitidis*, and other agents of the systemic mycoses.

Our studies revealed no correlation between the clinical types of cryptococcosis and reactivity with any particular serologic test. The level of the TA titer or the intensity of staining in the IFA test was not related to the severity of infection. We agree with Kimball et al. (10) that no clear relationship could be established between antibody reactivity and the response to amphotericin-B treatment (Table 5). Cryptococcal antibodies were rarely detected in CSF specimens; however, patients with central nervous system involvement did contain in their serum demonstrable antibodies (Tables 2, 5). Antibodies tended to persist for long periods even after cessation of chemotherapy. We interpret positive TA or IFA reactions as presumptive evidence for cryptococcosis. However, particularly with the IFA test, a positive reaction could also reflect a cross-reaction or past infection. We are in agreement with others (4, 7) that the LA test is diagnostically and prognostically applicable. Any LA antigen titer appears diagnostic of cryptococcosis. Increasing titers reflect progressive infection, and declining titers indicate response to chemotherapy and progressive recovery. The LA test could prove especially valuable where antibody responses are not possible, as in the case with hypogammaglobulinemia (Table 5). Peak antigen titers, unlike those observed by Walter and Jones (17), were sometimes observed after the initial diagnosis was made and even after administration of antiboitics. With extensive chemotherapy, the antigen titer tended either to decline to a low level or become negative (Table 5).

At the time of admission, some patients demonstrated only antibody, some only antigen, and others both substances in various body fluids. For early, accurate diagnosis of cryptococcosis, all three serological tests must be performed for the detection of cryptococcal antigens or antibodies, or both.

ACKNOWLEDGMENTS

We thank Maxine Clark for performing the complement fixation and agar gel precipitin tests.

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