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Fluorescent-Antibody Reagent for the Identification of *Cryptococcus neoformans*[1]

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A sensitive and diagnostically applicable conjugate for the rapid and accurate detection and identification of *Cryptococcus neoformans* has been developed. *C. neoformans* rabbit antisera were produced by 14 daily intravenous injections of 36 million cells for a total dosage of approximately 500 million cells. Cross-staining reactions with species of *Cryptococcus* other than *C. neoformans*, as well as with *Candida* species, were eliminated by adsorption of the *C. neoformans* conjugate with cells of *C. diffluens* and *C. krusei*.

Although the value of fluorescent-antibody (FA) staining for the detection of *Cryptococcus neoformans* antigens has been adequately demonstrated (2, 4, 5), no reliable reagent, which is sensitive and specific, has hitherto been developed. The purpose of the present study was to produce such a diagnostic reagent for the rapid detection and identification of *C. neoformans*.

MATERIALS AND METHODS

Antigen. *C. neoformans* strain B551, a weakly encapsulated isolate, was used in preparing agglutinogens and immunizing antigens. Antigens were prepared from 120-hr cultures grown in 500 ml of Sabouraud Liquid Medium (BBL) at 27 C. Cultures were rotated at 182 rev/min in a Psycrotherm Incubator Shaker (New Brunswick Scientific Co., New Brunswick, N.J.). All cultures were treated with 0.5% Formalin and, after appropriate sterility tests, were washed three times in 0.85% NaCl containing 0.5% Formalin.

Antisera production. Antigens were injected into at least two rabbits by one of several routes or by a combination of two routes as indicated in Table 1. The total number of cells injected into each rabbit, irrespective of method, was 5.0×10^8 cells.

For all of the immunizations, test bleedings were routinely made at 5-day intervals beginning on the tenth day of injection. Each serum was tested against *C. neoformans* strain B551 for agglutinins.

Agglutination tests. Agglutinin titers were determined by a tube test using 0.5 ml of antigen turbidimetrically adjusted to a no. 2 McFarland nephelometer standard. Twofold dilutions of each serum were made beginning with a 1:5 dilution. Titrations were read after 2 hr of incubation at 37 C and after overnight refrigeration at 4 C.

FA and other procedures. The conjugation, adsorption, and storing methods used were essentially those described by Kaufman and Kaplan (6).

Cultures. The identity of all the fungi examined in this study was confirmed by the Diagnostic Laboratory of the Mycology Section utilizing procedures described in the National Communicable Disease Center mycology manual (1).

RESULTS

Response to immunization. Rabbits were immunized via four immunization routes or by a combination of routes in an attempt to determine the most effective channel for obtaining high-titered antisera. Table 2 shows the maximal agglutinin titers obtained by the various routes on the first day antibodies were detected and on the day of maximal agglutinin response. The maximal titer of 1:640 was obtained with sera from rabbits injected intravenously. Attempts to obtain titers consistently beyond 1:640 by immunization with 500 million cells plus adjuvant via subcutaneous, combined intravenous and subcutaneous, and intraperitoneal routes failed. Immunization by the intravenous route obviously yielded higher titers more quickly than any of the other routes.

C. neoformans antisera with a homologous agglutinin titer of 1:640 were fractionated with half-saturated ammonium sulfate and conjugated to fluorescein isothiocyanate.

Reactivity of the labeled antiglobulins. Several *Cryptococcus* and *Candida* species were used as antigens to determine the staining characteristics

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TABLE 1. *Immunization routes, dosages*[a], *and schedules*

Route	Antigen inoculum	Dosage (ml)	Schedule
Intravenous	3.6×10^7 cells/ml	1	14 consecutive days
Subcutaneous	28.8×10^7 cells/2 ml[b] followed by	2	1st day
	7.2×10^7 cells/ml	1	5th, 8th, and 12th days
Combined:			
Intravenous	3.6×10^7 cells/ml	1	1st, 3rd, 5th, 8th, 10th, and 12th days
Subcutaneous	7.2×10^7 cells/ml[b]	1	1st, 5th, 8th, and 12th days
Intraperitoneal	7.2×10^7 cells/ml[b]	2	1st
		1	3rd, 5th, 8th, 10th, and 12th days

[a] Total dosage of *Cryptococcus neoformans* antigen injected per rabbit, regardless of procedure, totaled 5×10^8 cells.

[b] Suspended in Freund's complete adjuvant.

TABLE 2. *Initial and maximal agglutinin titers in rabbits inoculated by various routes with* 5×10^8 *cells of lot 4, B551*

Inoculation route	Initial response		Maximal response	
	Day	Titer	Day	Titer
Intravenous	10	1:40	19	1:640
Intravenous and subcutaneous	15	1:10	20	1:80
Subcutaneous	15	1:10	20	1:80
Intraperitoneal	20	1:20	30	1:160

TABLE 3. *Homologous and heterologous staining titers demonstrated by the Cryptococcus neoformans B551 conjugate*

Antigen	FA titer
Cryptococcus neoformans (B551)	1:256
C. neoformans var. *uniguttulatus*	0
C. albidus	1:128
C. diffluens	1:256
C. terreus	1:128
Candida albicans	0
C. curvata	1:256
C. humicola	1:128

of the unadsorbed conjugate. As indicated in Table 3, the conjugate not only stained the homologous fungus, but reacted strongly with five of the seven heterologous species tested. Because some of the heterologous titers were equivalent to the homologous ones, specificity could not be achieved through dilution. Of particular interest was the fact that the conjugate failed to stain *C. neoformans* var. *uniguttulatus* and *C. albicans*.

Reactivity of adsorbed conjugates. In an attempt to produce a specific reagent, the conjugate was adsorbed. In view of their intense cross-staining titers, cultures of *C. diffluens* and *C. terreus* were selected for adsorption studies. Adsorptions with cells of *C. diffluens* (Table 4) greatly reduced the homologous staining titer of the conjugate from 1:256 to 1:4, but all of the five cross-reactions were eliminated.

Adsorptions with cells of *C. terreus* removed the cross-reaction with *C. terreus* but did not eliminate the cross-staining of *C. albidus*, *C. diffluens*, *C. curvata*, or *C. humicola* (Table 4). The homologous FA titer was reduced 32-fold, whereas the heterologous FA titers were reduced only four- to 16-fold. Adsorptions of the *C. terreus* adsorbed conjugate with *C. diffluens*, to eliminate further cross-staining, completely eliminated both homologous and heterologous reactions (Table 4).

Evaluation with cultures and clinical materials. These results showed that the *C. diffluens* adsorbed conjugate was the most promising reagent; hence, more extensive studies were carried out to evaluate its specificity and sensitivity. Numerous strains of fungi belonging to the genera *Blastomyces*, *Candida*, *Coccidioides*, *Cryptococcus*, *Histoplasma*, *Rhodotorula*, and *Torula* were examined (Table 5). Of a total of 30 heterologous strains studied, only the three strains of *C. krusei* were cross-stained. This cross-staining, however, could be eliminated by further adsorption of the conjugate with cells of *C. krusei*.

In tests with 34 strains of *C. neoformans* from a variety of sources, all except one (strain no. 369-66) gave 3 to 4+ staining. The exceptional strain gave questionable staining. Thus, 97% of the *C. neoformans* strains were specifically identified with *C. diffluens* adsorbed conjugate.

When used in the diagnostic laboratory, the *C. diffluens* adsorbed conjugate gave strong, specific staining of *C. neoformans* cells whether they were in lesion exudates, tissue sections, or in culture. As shown in Table 6, lesion exudate

and a brain tissue section examined by the direct FA and cultural techniques were positive for *C. neoformans*. Of particular importance is the fact that, with the exudate, the positive FA reaction was demonstrated within 2 hr after the initial smears were made at the patient's bedside. In contrast, the routine isolation and identification of *C. neoformans* from this exudate required more than 2 weeks.

C. diffluens adsorbed conjugate also promises to be useful for the identification of both *C. neoformans* and *C. krusei*, since the morphology of the two organisms is distinct enough to preclude confusion. Indeed, in a limited diagnostic application of the reagent (Table 6), *C. krusei* was identified in a culture with the FA reagent. This result was confirmed by appropriate morphological and biochemical studies.

TABLE 4. *Effects of various adsorptions on the staining reactions o, the Cryptococcus neoformans B551 conjugate*

Antigen	Staining titer of *C. neoformans* conjugate adsorbed with		
	C. diffluens	*C. terreus*	*C. terreus* and *C. diffluens*
Cryptococcus neoformans (B551)	1:4	1:8	0
C. neoformans var. *uniguttulatus*	0	0	0
C. albidus	0	1:16	0
C. diffluens	0	1:64	0
C. terreus	0	0	0
Candida albicans	0	0	0
C. curvata	0	1:16	0
C. humicola	0	1:8	0

DISCUSSION

Our data (Table 2) indicate that 14 daily intravenous injections of 36 million cells, for a total dosage of approximately 500 million cells, were tolerated by the rabbits and yielded maximal agglutinin titers of 1:640. Attempts to attain titers beyond 1:640 regularly by immunization with 500 million cells plus adjuvant via the subcutaneous, intravenous and subcutaneous, and intraperitoneal routes failed to yield higher titers (Table 2). This agrees with the finding of Škařil and Fragner (9) that intravenous immuni-

TABLE 5. *Reactivity of the Cryptococcus neoformans conjugate (adsorbed with cells of C. diffluens) with homologous and heterologous antigens*

Culture (no. of strains)	FA results
Cryptococcus neoformans (33)	3 to 4+
C. neoformans (1)	± to 1+
C. neoformans var. *uniguttulatus* (1)	−
C. albidus (1)	−
C. diffluens (3)	−
C. laurentii (1)	−
C. luteolus (1)	−
C. terreus (1)	−
Candida albicans (3)	−
C. curvata (1)	−
C. humicola (1)	−
C. krusei (3)	2 to 4+
C. parasilosis (1)	−
C. stellatoidea (2)	−
C. tropicalis (2)	−
Coccidioides immitis (endospores) (1)	−
C. immitis (spherules) (1)	−
Blastomyces dermatitidis (1)	−
Histoplasma capsulatum (1)	−
H. duboisii (1)	−
Rhodotorula sp. (2)	−
Torulopsis glabrata (2)	−

TABLE 6. *Comparison of results obtained with the specific FA reagent and cultural studies on cultures and clinical materials from suspected and proven cases of cryptococcosis*

Code	Test material	FA results	Cultural identification
MBD 193-7	Skin lesion exudates	+	*C. neoformans*
MBD	Impression smears of skin lesions	+	Not cultured
MBD	Smears-washings of lesion exudate	+	Not cultured
Columbia	Brain tissue section	+	*C. neoformans*
55-7	Culture	−	*Candida* sp.
162-7	Culture (dry)	+	*C. neoformans*
173-7	Culture	−	*C. diffluens*
198-7	Culture (mucoid)	+	*C. neoformans*
230-7	Culture	−	*Candida* sp.
253-7	Culture	−	*Candida* sp.
292-7	Culture	+	*C. krusei*
293-7	Culture	+	*C. neoformans*

zation of rabbits produces the most desirable antiserum for labeling with fluorescein isothiocyanate. However, Rezai and Haberman (8) obtained higher agglutinin titers in rabbits immunized via a combination of the intravenous and intramuscular route plus adjuvant (1:160) than in rabbits immunized via the intravenous route without adjuvant (1:32).

Previously, Kaufman and Blumer (5), using the same strain of *C. neoformans*, the same route of immunization, and a similar dosage and number of injections as were used in the present study, also obtained agglutinin titers of 1:640. The major differences between their study and the present one was the schedule of injections. Peak titers in the present study were reached on the fifth to eighth day after the series of 14 injections, whereas the peak titers in Kaufman and Blumer's study were reached 7 days after the 19-day schedule used for the 15 injection series. This difference in immunization schedules may account for the greater specificity of the unadsorbed conjugate (Table 3) in the present study (3).

The greater specificity of the present conjugate is indicated by the absence of cross-staining of *C. neoformans* var. *uniguttulatus* and *C. albicans*. This was also noted by Kaufman and Blumer (5) in their study with unadsorbed conjugate.

The present findings are in agreement with those of Kase and Marshall (4), who, using a shorter immunization schedule, reported that their conjugate did not stain eight strains of an unspecified number of *Candida* sp. These results suggest that a more specific unadsorbed conjugate may possibly be attained through use of an even shorter immunization schedule than our 14-day schedule. However, a direct comparison cannot be made with Kase and Marshall's results since they failed to list the species of *Candida* used in their study.

Since *C. neoformans* and *C. neoformans* var. *uniguttulatus* are not only antigenically distinct but differ in their assimilation reactions and temperature requirements (7), we believe, as did Kaufman and Blumer (5), that *C. neoformans* var. *uniguttulatus* merits species rank.

The cross-staining reactions demonstrated by the unadsorbed conjugate for *C. curvata*, *C. humicola*, and *C. diffluens* were consistent with the staining reactions of the unadsorbed reagent observed by Kaufman and Blumer (5). In the present study, a single adsorption of the *C. neoformans* conjugate with cells of *C. terreus* eliminated the cross-staining for *C. terreus*, and also greatly reduced the staining of *C. neoformans*. It is interesting, however, that other *Cryptococcus* and *Candida* species still stained well (Table 4). Hence, *C. terreus* and *C. neoformans* seem to share an antigen or antigens not shared with the other cryptococci tested.

An effective and practical diagnostic FA reagent was produced by the adsorption of the *C. neoformans* conjugate with cells of *C. diffluens* (Tables 5 and 6). *C. diffluens* seems to be more complex antigenically than is *C. terreus*. This organism not only adsorbed its homologous cross-staining antibodies, but it also adsorbed the heterologous cross-staining antibodies of *C. terreus* and the other *Candida* and *Cryptococcus* species, with the exception of *C. krusei*. Studies showed that this cross-staining could be eliminated by adsorption with *C. krusei* cells without impairing the staining intensity of the homologous antibody. However, the *C. diffluens* adsorbed conjugate can be used for the identification of both *C. neoformans* and *C. krusei*, since the micromorphological characteristics of these organisms are distinct.

The diagnostic usefulness of the specific *C. neoformans* conjugate was effectively demonstrated by the results obtained with lesion exudates and tissue sections from infected humans and with numerous strains of *C. neoformans*. Presently, the reagent is a valuable adjunct to cultural studies. Accompanied by adequate evaluation in a diagnostic situation, a positive result with the reagent would be confirmatory rather than presumptive evidence for the presence of *C. neoformans* in clinical material or cultures. The reagent permits a diagnosis minutes after staining the specimen, and it immediately provides the physician with data essential for the proper treatment of the disease.

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