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Serotyping *Cryptococcus neoformans* by Immunofluorescence

WILLIAM KAPLAN,[1]* SANDRA L. BRAGG,[1] STANLEY CRANE,[1] AND DONALD G. AHEARN[2]

Centers for Disease Control, Atlanta, Georgia 30333,[1] and Department of Biology, Georgia State University, Atlanta, Georgia 30303[2]

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Four serotypes of *Cryptococcus neoformans* designated A, B, C, and D are currently recognized. Although an agglutination test is most often used to serotype *C. neoformans* in cultures, this test is not appropriate for typing the fungus in fixed tissues. A study to prepare fluorescent-antibody reagents for typing *C. neoformans* in cultures and to determine whether they can be used to type this fungus in fixed tissues was carried out. Antisera to one strain belonging to each of the four serotypes were prepared in rabbits by intravenous injection of whole Formalin-killed cryptococci. Each antiserum was labeled with fluorescein isothiocyanate and then adsorbed with cells of each of the heterologous serotypes. The adsorbed conjugates were then tested against six serotype A isolates and five isolates of each of the other three serotypes. Labeled serotype A or D antiserum adsorbed with either B or C cells stained the A and D, but not the B or C, isolates. Labeled serotype B antiserum adsorbed with A cells stained the B and C, but not the A or D, isolates. Labeled A antiserum absorbed with D cells differentiated A from D; labeled C antiserum absorbed with B cells differentiated C from B. Of the 21 test isolates, 17 could be serotyped in paraffin sections of tissues of experimentally infected mice.

In 1949, Evans divided isolates of *Cryptococcus neoformans* into three serological types, A, B, and C, on the basis of the antigenic makeup of their capsules (3). In a later study, Wilson et al. (14) distinguished a fourth serotype which was designated type D. All four serotypes can cause disease; however, they differ in their geographic distribution (1). Serotype A has a cosmopolitan distribution. Serotype D is common in some parts of Europe, but it is infrequently encountered in the United States. Serotypes B and C cause cryptococcosis infrequently, except in southern California. Recently, isolates of group B or C have been found to be important causes of this disease in residents of southeastern Oklahoma (11). The four serotypes also differ in their natural habitats (1). Serotypes A and D are commonly found in association with pigeon droppings, whereas the natural reservoir of serotypes B and C is unknown. These differences indicate that serotyping of *C. neoformans* isolates would be of value from an epidemiological standpoint.

Agglutination is the technique most often used to serotype *C. neoformans*. This procedure is suitable for use with cultures, but not with fixed tissues or other clinical materials. It would be desirable to be able to type *C. neoformans* in histological specimens, because the results obtained from such analyses would be of epidemiological and ecological value. Also, recent findings suggest that the serotype of the isolate causing cryptococcosis might have therapeutic implications (D. K. Henderson, J. E. Edwards, Jr., W. E. Dismukes, and J. E. Bennett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, F11, p. 315). The fluorescent-antibody (FA) technique has been used successfully to detect and identify *C. neoformans* in fixed tissues (8, 9) and in cultures (7, 13), suggesting that the FA technique might also be suitable for typing *C. neoformans* in such specimens. The present study was undertaken to develop FA reagents for serotyping this fungus in cultures and to determine their value for carrying out such an analysis with fixed tissues.

MATERIALS AND METHODS

Cultures. Twenty-one isolates of *C. neoformans*, whose serotypes had been established by the agglutination procedure, were studied. Six of these cultures (B551, B3166, B3168, B3169, B3170, and B3270) belonged to serotype A, five (B3171, B3172, B3173, B3268, and B3271) belonged to serotype B, five (B3182, B3183, B3184, B3267, and B3269) belonged to serotype C, and five (B3176, B3177, B3178, B3179, and B3266) belonged to serotype D. With the exception of isolate B551, these cultures were obtained from the collection maintained by the Department of Biology, Georgia State University, Atlanta. Isolate B551 was obtained from the culture collection maintained by

the Mycology Division, Centers for Disease Control, Atlanta, Ga.

Antisera. The antigens used for the production of antisera were prepared from four isolates of *C. neoformans* representing each of the serotypes (B551, B3172, B3182, and B3179). These cultures were selected on the basis of smallness of capsule when compared with the other isolates in their respective serological group when grown on Sabouraud dextrose agar slants for 72 h at 35°C. This criterion for selection was based on earlier observations that highest serum antibody titers are obtained when lightly encapsulated *C. neoformans* cells are used as immunization antigens (12). Antigens used for immunization were prepared by growing cultures B551, B3172, and B3179 in Sabouraud dextrose broth. The inocula for these broth cultures consisted of two loopfuls of surface growth from Sabouraud dextrose agar slants incubated for 96 h at 25°C. The cultures were shaken at 180 rpm for 72 h while they were incubated at 25°C. Culture B3182 was grown on modified Sabouraud dextrose agar, pH 5.0, containing 2.9% NaCl for 72 h at 35°C to reduce the size of its capsule (2). The inoculum consisted of one loopful of growth on a Sabouraud dextrose agar slant that had been incubated for 96 h at 25°C. The cultures were killed with Formalin (1.0%) and then washed three times with Formalin-treated (0.5%) physiological saline. The washed cells were resuspended in Formalin-treated (0.3%) 0.01 M phosphate-buffered saline, pH 7.4. Vaccines were prepared by adjusting the suspensions of the B551, B3172, and B3179 cells to the turbidity of a no. 10 and the suspension of B3182 cells to the turbidity of a no. 8 McFarland nephelometer standard.

Four rabbits per immunization antigen were used for the production of antisera to strains B551 (type A), B3172 (type B), and B3179 (type D). However, seven rabbits were used for the production of antiserum to strain B3182 (type C). Before immunization, each rabbit was bled and the preimmunization serum was used as a control for its corresponding antiserum. Each rabbit was injected intravenously with 10 3-ml doses of vaccine spread over a period of 4 weeks. The vaccine was administered at 3- or 4-day intervals. Starting 1 week after the 10th injection, the rabbits were bled once each week for 5 weeks. Doses of 1 and 2 ml of the vaccine were administered intravenously during this period to maintain the titer.

Conjugation of sera. Each preimmunization serum and antiserum was labeled with fluorescein isothiocyanate at a ratio of 3 mg of dye per ml of serum. The whole-serum conjugates were prepared as follows. The dye was dissolved in 0.1 M Na_2HPO_4 (1 mg of fluorescein isothiocyanate per ml of buffer). The fluorescein isothiocyanate solution was then added to the serum, and the pH was adjusted to 9.5 with 0.1 M Na_3PO_4. The mixture was then allowed to stand for 3 h at room temperature. Unreacted fluorescein isothiocyanate was removed by gel filtration with Sephadex G-25. Conjugates were diluted with 0.01 M phosphate-buffered saline containing 1.0% bovine serum albumin and Merthiolate (1:5,000) adjusted to pH 8.5 to 9.0. The titers of these conjugates were based on the volume of conjugate relative to the volume of unlabeled serum used to prepare the reagent. The conjugates were stored at 4°C.

Testing of conjugates. Tests for the sensitivity and specificity of the conjugates were carried out on smears of suspensions of Formalin-killed *C. neoformans* cells that had been cultivated on Sabouraud dextrose agar at 37°C for 5 days. The ability of the conjugates to stain their respective immunization strain was the basis for selecting a conjugate of each serotype for detailed study. The conjugates selected were tested for sensitivity with all strains of the same serotype as the immunization antigen. These conjugates were then tested for specificity with all strains belonging to the other serotypes. In all of these tests, reagents were used at dilutions of 1:10, 1:40, 1:160, and 1:640.

Adsorption procedure. The cells used for adsorptions were prepared from cultures grown in brain heart infusion broth. The broths were inoculated with two loopfuls of growth from Sabouraud dextrose agar slants that had been incubated for 96 h at 25°C. The cultures were shaken for 72 to 96 h while they were incubated at 25°C. They were treated with Formalin (1.0%), washed twice in Formalin-treated (0.5%) physiological saline, and stored at 4°C in Formalin-treated (0.5%) saline. Conjugates diluted at 1:10 were adsorbed with half volumes of packed cells at 37°C in a water bath for 2 h. Adsorptions were then continued overnight at 4°C. Each conjugate selected for detailed study was adsorbed with cells of the homologous immunization strain and with cells of the other three heterologous strains. Adsorptions were repeated until staining of the adsorbing strain had been eliminated.

Staining procedure and visualization of stained preparations. The procedure used to stain the preparations was the one described by Kaplan and Ivens (5). All preparations were examined with a Leitz Ortholux II incident-light fluorescence microscope. Criteria similar to those of Goldman (4) and Moody et al. (10) were used to rate the intensity of staining. Results were recorded as −, ± (no reaction), 1+, 2+, 3+, and 4+ (reaction).

RESULTS

Staining properties of unadsorbed labeled sera. The labeled preimmunization sera did not stain the *C. neoformans* strains. Antisera obtained from all four rabbits immunized with the B551 antigen (serotype A), when labeled, intensely stained cells of the homologous strain. One conjugate, prepared from serum collected from one of the rabbits 8 weeks after immunization was begun, gave the brightest staining. It was selected for further study and was designated A-1.

Antisera from only one of the rabbits immunized with the B3172 antigen (serotype B), when labeled, brightly stained the homologous organism. The conjugate prepared from serum collected 8 weeks after immunization had begun gave the best staining reactions. It was selected for further study and was designated B-1.

Antisera obtained from only one of the seven rabbits immunized with the B3182 antigen (serotype C), when labeled, stained the homologous strain. The conjugate prepared from serum collected 5 weeks after immunization had begun appeared to give the best staining reactions. It was selected for further study and designated C-7.

Antisera obtained from all of the rabbits immunized with the B3179 antigen (serotype D), when labeled, brightly stained the homologous strain. The conjugate prepared from serum collected 8 weeks after immunization had begun gave the brightest staining. It was selected for further study and was designated D-4. The staining properties of these four conjugates were then studied with all of the strains belonging to each of the respective serotypes and with those of the heterologous serotypes. The staining titers obtained are presented in Table 1.

Staining properties of adsorbed labeled antisera. Portions of each of the labeled antisera selected for further study were adsorbed with cells of the homologous immunization strain and cross adsorbed with cells of each of the three heterologous immunization strains. The staining properties of each of the adsorbed conjugates were studied with all 21 typed strains. The staining reactions observed are summarized in Table 2.

Use of adsorbed conjugates to serotype *C. neoformans* in paraffin sections of Formalin-fixed tissue. Groups of three mice were experimentally infected with each of the 21 serotyped strains. Infections were induced by intraperitoneal injection of 0.5 ml of saline suspensions of each culture that had been grown on Sabouraud dextrose agar for 96 h at 25°C. These suspensions were prepared by dispersing the surface growth from one agar slant in 5.0 ml of physiological saline. The mice were sacrificed 1 to 2 weeks later, and portions of all organs with visible lesions were fixed in 10.0% neutral Formalin and then embedded in paraffin. Sections of these tissues were stained with hematoxylin and eosin and by the Gomori methenamine-silver nitrate procedure. An examination of these preparations disclosed the presence of cryptococci in the lesions. Replicate sections were then tested with selected adsorbed conjugates to determine whether these reagents could be used to serotype the cryptococci present. The procedures used to prepare the sections for FA staining and to stain them were those described by Kaplan and Kraft (6).

The serotyping was carried out in two steps with four adsorbed conjugates. In the first step, the sections were tested with the A-1 conjugate that had been adsorbed with B cells and also with the B-1 conjugate that had been adsorbed with A cells. The purpose of this first step was to determine whether the cryptococci belonged to either serotype A or D or serotype B or C. In the second step, the sections were tested with the A-1 conjugate that had been adsorbed with

TABLE 1. *Staining titers of selected unadsorbed fluorescein-labeled antisera to strains of C. neoformans belonging to serotypes A, B, C, and D*

Conjugate	Staining titer[a] to serotype:			
	A (6)[b]	B (5)	C (5)	D (5)
A-1	160–640	160–640	40	≤640
B-1	>640	≤640	≤640	≤640
C-7	160–≤640	>640	160–≤640	40–640
D-4	160–640	40–160	40–160	>640

[a] Reciprocal of dilution.
[b] Number of strains.

TABLE 2. *Staining properties of adsorbed fluorescein-labeled C. neoformans antisera with homologous and heterologous serotype antigens*

Conjugate	Adsorbing antigen	Staining reaction[a] of *C. neoformans* serotype:			
		A (6)[b]	B (5)	C (5)	D (5)
A-1	Serotype A cells	–	–	–	–
	Serotype B cells	+	–	–	+
	Serotype C cells	+	–	–	+
	Serotype D cells	+	– (4)[b] + (1)	–	–
B-1	Serotype A cells	–	+	+	–
	Serotype B cells	–	–	–	–
	Serotype C cells	+	+	–	+
	Serotype D cells	+	+	+	–
C-7	Serotype A cells	–	+ (3) – (2)	+	–
	Serotype B cells	–	–	+	–
	Serotype C cells	–	–	–	–
	Serotype D cells	+ (3) – (2)	+	+	–
D-4	Serotype A cells	–	–	–	–
	Serotype B cells	+	–	–	+
	Serotype C cells	–	–	–	+
	Serotype D cells	–	–	–	–

[a] –, Negative reaction; +, staining that ranged from 1+ to 4+.
[b] Number of strains.

D cells to separate A from D, if they had been found in the first step to belong to group A or D, or with the C-7 conjugate adsorbed with B cells to separate C from B, if they had been found in the first step to belong to group B or C.

In this preliminary study, in all cases we were able to determine whether the cryptococci in the fixed tissues belonged to either group A or D or group B or C. With the exception of one serotype C strain, we were able to separate the C strains from the B strains. In tissues, this particular type C strain did not react with the C-7 conjugate that had been adsorbed with type B cells. It was also possible in most cases to differentiate the type A strains from the type D strains. In one case, however, a type A strain was stained equivocally with the A-1 conjugate that had been adsorbed with D cells; in two cases involving type D strains, some of the fungal cells in the tissue sections were observed to react equivocally with the adsorbed A-1 conjugate.

Use of adsorbed conjugates to serotype *C. neoformans* cultures of unknown serotype. Sixty *C. neoformans* cultures of unknown serotype were typed by the FA technique. Of these, 47 were obtained from various investigators in the United States and 13 were obtained from foreign laboratories. Thirty-five of the cultures from the United States had been isolated from clinical materials, five had been isolated from environmental samples, and the source of seven was unknown. Six of the cultures submitted by foreign laboratories were from Israel, five were from Colombia, and two were from Spain. The conjugates used for typing were those used with the sections of fixed tissues. As was done with the tissue sections, the typing was carried out in two steps.

The reagents and procedure used enabled us to serotype all 60 cultures. A total of 41 were found to be type A, 2 were type B, 5 were type C, and 12 were type D.

DISCUSSION

The purpose of this study was to develop FA reagents for serotyping *C. neoformans*, and this goal was met. By reciprocal cross staining and cross adsorption procedures, FA reagents were produced that, when used in appropriate combination, make possible the serotyping of this fungus. We found that labeled antiserum to serotype A or D, when adsorbed with either B or C cells, differentiated types A and D from types B and C. Conversely, labeled antiserum to serotype B, when adsorbed with A cells, differentiated B and C from A and D. Separation of A from D was done by using labeled antiserum to A that had been adsorbed with D cells; separation of C from B was done by using labeled antiserum to C that had been adsorbed with B cells.

Serotyping by means of these reagents can be carried out in one or two steps. We prefer to use a two-step procedure because it saves reagents. In the first step we determine whether the isolate belongs to group A or D or to group B or C. In the second step the group is separated into its respective components.

In this study we did not encounter any difficulties in serotyping *C. neoformans* in cultures. The staining results were clear-cut and unequivocal. In preliminary tests with sections of fixed tissues, we could also readily establish whether the *C. neoformans* cells present belonged to the A or D or the B or C group. In some cases, however, it was not possible to differentiate the components in these two groups. Further studies are needed to resolve this problem.

The agglutination procedure has been successfully used for serotyping *C. neoformans* cells from cultures. Thus, the question can be raised as to the need for immunofluorescence to type such cells. Immunofluorescence has several important advantages over agglutination. First, contaminated cultures can be typed by the FA technique, whereas this may not be possible by agglutination, without time-consuming purification procedures. Also, nonviable cultures with scant growth can be typed by the FA technique, whereas this might not be possible by agglutination.

In most laboratories FA reagents are prepared from globulin fractions of sera. This is done to reduce presumed nonspecific staining and to obtain more intense specific staining due to the concentration of antibodies. In the present study whole-serum conjugates were used. Our decision to use such FA reagents was based upon the fact that they are easy to prepare and can be made in 1 day. Furthermore, we did not encounter nonspecific staining with these conjugates.

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