SEROLOGICAL CHARACTERIZATION OF PATHOGENIC FUNGI BY MEANS OF FLUORESCENT ANTIBODIES

I. Antigenic Relationships between Yeast and Mycelial Forms of *Histoplasma capsulatum* and *Blastomyces dermatitidis*

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Abstract

Kaufman, Leo (Communicable Disease Center, Atlanta, Ga.) and William Kaplan. Serological characterization of pathogenic fungi by means of fluorescent antibodies. I. Antigenic relationships between yeast and mycelial forms of *Histoplasma capsulatum* and *Blastomyces dermatitidis*. J. Bacteriol. **85**:986–991. 1963.—Four antiglobulins prepared against the yeast and mycelial forms of *Histoplasma capsulatum* and *Blastomyces dermatitidis* were labeled with fluorescein isothiocyanate. Cross-staining and adsorption techniques enabled the discernment of five antigenic factors in the fungi studied. The yeast-phase cells of *H. capsulatum* and *B. dermatitidis* contained the respective distinct antigens B and D, and a common factor E. In addition, the yeast-phase cells of these two species shared antigen C with the mycelial elements of *H. capsulatum*. The mycelial cells of *H. capsulatum* and *B. dermatitidis* shared antigen A with the yeast-phase cells of these two organisms and contained no distinct antigens.

Recently, we reported the development of fluorescein isothiocyanate-labeled antiglobulins specific for the yeast phase of *Histoplasma capsulatum* (Kaufman and Kaplan, 1961) and *Blastomyces dermatitidis* (Kaplan and Kaufman, Mycopathol. Mycol. Appl., *in press*). These conjugates proved to be valuable for the rapid detection of *H. capsulatum* and *B. dermatitidis* in clinical materials and in yeast-phase cultures. However, further studies showed that these reagents would not react with the mycelial growth produced by conversion from homologous yeast-phase cells.

In nature, the etiological agent of histoplasmosis, and most probably that of North American blastomycosis, exists in the mycelial form. The mycelial colonies of these two species grossly resemble one another. They may be differentiated microscopically when *H. capsulatum* cultures producing tuberculate spores are encountered; however, this is a phenomenon which cannot always be depended upon.

Cross reactions due to the presence of common antigens and the difficulties they present in the use of serodiagnostic procedures are well-documented in the literature (Salvin, 1949; Campbell, 1960; Seeliger, 1962).

This investigation was undertaken to determine whether fluorescent antibody (FA) reagents specific for the mycelium of *H. capsulatum* and *B. dermatitidis* could be developed. In addition, with FA procedures, we hoped to further characterize the serological relationships between the yeast and mycelial forms of these two pathogenic fungi.

Materials and Methods

Antisera and antiglobulins. Mycelial antigens used for the production of antisera were prepared from mycelium that had been grown in Sabouraud dextrose broth. The cultures were shaken for 3 weeks while incubating at 25 C. All cultures were formalinized (1.0%) and, after appropriate sterility tests, were washed three times in formalinized (0.5%) 0.85% sodium chloride. The washed cells were broken up in a Virtis "45" blender and standardized so that cell turbidity yielded 42% light transmission at 550 mμ in a Coleman spectrophotometer (model 6A). Dosages of 1, 2, and 5 ml were injected intravenously into rabbits on 3 consecutive days. This course of inoculations was repeated 1 week later; 2 weeks later, each rabbit received only the 1- and 2-ml injections.

Each rabbit was bled 7 days after the final injection. When the schedule of inoculations was continued beyond that described, some of the animals suffered an immediate fatal shock. Antisera against yeast-phase cells were prepared as described in an earlier paper (Kaufman and Kaplan, 1961). The antiglobulin fractions were obtained from each antiserum by three precipitations with half-saturated ammonium sulfate.

Conjugation of antiglobulin fractions. Antiglobulin fractions adjusted to 2.0 g of protein/100 ml were labeled with fluorescein isothiocyanate according to the method of Riggs et al. (1958). Labeled mycelial *H. capsulatum* antiglobulin was purified by gel filtration by passage through a column of medium G-50 Sephadex (Goldstein, Slizys, and Chase, 1961). The labeled mycelial *B. dermatitidis* antiglobulin was purified by dialysis at 4 C against phosphate-buffered saline (pH 7.2). Yeast-phase conjugates were prepared as described in earlier publications (Kaufman and Kaplan, 1961; Kaplan and Kaufman, *in press*). The staining titer of each conjugate was determined by treating smears of the homologous organisms with serial twofold dilutions of the reagent. Smears were made directly from Sabouraud dextrose agar or Brain Heart Infusion (Difco) agar cultures and heat-fixed.

Smears treated with the labeled antiglobulins were examined with a Reichert microscope employing as a light source a Reichert Flourex unit containing an HBO 200 maximal-pressure mercury vapor arc. A 5113 exciter filter was used in combination with a Wratten 2A barrier filter.

Adsorption procedure. Mycelial antigens for adsorption of antiglobulins were prepared by cultivation in Sabouraud dextrose broth shaken at 25 C. After 3 to 4 weeks of incubation, the growth was harvested, formalinized (1.0%), and checked for sterility. The cells were washed three times in phosphate-buffered saline (pH 7.2) and packed by centrifugation for addition of the conjugated antiglobulin. Yeast-phase antigens were prepared by a previously described procedure (Kaufman and Kaplan, 1961). Antiglobulins were adsorbed with half-volumes of packed cells at 37 C for periods of 2 hr. Adsorptions were carried out until staining of the adsorbing strain was eliminated.

Antigens studied. Cultures (25) were routinely stained. These comprised six yeast-phase cultures of *H. capsulatum* (strains 28, 105, A222, A811, A827, and L261) and seven mycelial-phase cultures (strains 28, 105, 681, A222, A811, A827, and L261). Six strains of *B. dermatitidis* (295, 1442, B414, B415, B417, and B418) were studied in both phases. Only yeast- and mycelial-phase cultures of *H. capsulatum* strain 28 and *B. dermatitidis* strain B417 were used for antibody production.

RESULTS

Serological properties of unlabeled antisera and labeled antiglobulins. The serological properties of the various antisera were determined by the complement-fixation (CF) technique. From the data (Table 1), it is evident that the four antisera cross-reacted strongly with the heterologous antigens. It is also noteworthy that the homologous titers were not significantly higher than those observed with the heterologous reactants. The highest CF titers were obtained with the antisera produced against yeast cells of *H. capsulatum*. The effects of fractionation, conjugation, and adsorption on these antisera could not be determined by the CF technique because, in most instances, these sera became anticomplementary after such treatment.

All 25 cultures were studied with the four labeled antiglobulins. The conjugates prepared against the yeast phases of *H. capsulatum* and *B. dermatitidis*, and the mycelial phase of *H. capsulatum* were used at dilutions of 1:4, whereas the conjugate of the mycelial-phase *B. dermatitidis* antiglobulin was used at a 1:2 dilution. As shown in Table 2, all 25 cultures were stained by

TABLE 1. *Serological properties of unconjugated fungal antisera*

Antisera	Complement-fixation titers with antigens		
	Histoplasmin	*H. capsulatum* (YP*)	*B. dermatitidis* (YP)
Histoplasma capsulatum, MP*	256	128	512
H. capsulatum, YP	256	2,048	1,024
Blastomyces dermatitidis, YP	32	128	256
B. dermatitidis, MP	64	64	512

* Symbols: YP, yeast phase; MP, mycelial phase.

TABLE 2. *Staining reactions of unadsorbed and adsorbed fluorescein-labeled Histoplasma capsulatum and Blastomyces dermatitidis antiglobulins with homologous and heterologous antigens*

Labeled antiglobulins	Adsorbing antigen	Staining reactions*				Antigens homologous to antibodies
		Yeast phase		Mycelial phase		
		H. capsulatum (6 strains)	*B. dermatitidis* (6 strains)	*H. capsulatum* (7 strains)	*B. dermatitidis* (6 strains)	
YP† *Histoplasma capsulatum*‡	None	+	+	+	+	A
YP *H. capsulatum*	YP *Blastomyces dermatitidis*	+	−	−	−	B
YP *H. capsulatum*	MP† *H. capsulatum*	+	−	−	−	B
YP *H. capsulatum*	MP *B. dermatitidis*	+	+	+	−	C
MP *H. capsulatum*‡	None	+	+	+	+	A
MP *H. capsulatum*	YP *H. capsulatum*	−	−	−	−	—
MP *H. capsulatum*	YP *B. dermatitidis*	−	−	−	−	—
MP *H. capsulatum*	MP *B. dermatitidis*	+	+	+	−	C
YP *B. dermatitidis*‡	None	+	+	+	+	A
YP *B. dermatitidis*	YP *H. capsulatum*	−	+	−	−	D
YP *B. dermatitidis*	MP *H. capsulatum*	+	+	−	−	E
YP *B. dermatitidis*	MP *B. dermatitidis*	+	+	−	−	E
MP *B. dermatitidis*‡	None	+	+	+	+	A
MP *B. dermatitidis*	YP *H. capsulatum*	−	−	−	−	—
MP *B. dermatitidis*	MP *H. capsulatum*	−	−	−	−	—
MP *B. dermatitidis*	YP *B. dermatitidis*	−	−	−	−	—

* Reasonable staining = +; poor to negative staining = −.

† Symbols: YP = yeast phase; MP = mycelial phase.

‡ Showed no staining after adsorption with homologous antigens.

the nonadsorbed conjugates, thus indicating a serological relationship between the diphasic species. In some instances, higher dilutions of the conjugates reacted strongly with many of the antigens studied, indicating quantitative differences in the common antigenic factor(s). However, in no instance were the FA titers comparable to the CF titers. The group antigenic factor(s) common to both phases of *H. capsulatum* and *B. dermatitidis* was assigned the letter A. Homologous and heterologous adsorptions were used to elucidate further the antigenic relationships among these diphasic forms. The results of these adsorptions follow, and are summarized in Table 2.

Serological properties of yeast-phase H. capsulatum conjugate. Adsorption of the labeled antiglobulin twice with yeast-phase cells of *B. dermatitidis* produced a fluorescent antibody specific for yeast cells of *H. capsulatum*. No staining occurred with any of the strains of yeast-phase *B. dermtitidis* or the mycelial cultures of *H. capsulatum* and *B. dermatitidis*. These results indicated the presence of a specific antigenic factor in the yeast cells of *H. capsulatum*. This factor was designated by the letter B.

The presence of antibody to antigen B was demonstrated also in labeled antiglobulins adsorbed three times with the mycelium of *H. capsulatum*.

A third antigenic factor, designated by the letter C, was revealed by adsorption of the labeled antiglobulin with the mycelium of *B. dermatitidis*. Such an adsorption produced a conjugate that stained the yeast-phase cells of *H. capsulatum* and *B. dermatitidis* and the mycelial-phase cells of *H. capsulatum*. Thus, antigen C appeared to be distributed among all the fungi studied except the mycelial-phase cells of *B. dermatitidis*.

Serological properties of mycelial-phase H. capsulatum conjugate. This conjugate possessed staining titers that varied from 1:4 to 1:32, depending upon the strains and phase of the fungus stained. After adsorption with homologous yeast-phase cells, it did not stain any of the fungi studied. These results indicated that the mycelial

elements of *H. capsulatum* lack a specific antigen and that the antigens present are shared with the yeast-phase elements.

An attempt was made to render the nonadsorbed conjugate specific for both the yeast and mycelial forms of *H. capsulatum* by adsorption with *B. dermatitidis* yeast-phase cells. This conjugate lost its staining reactivity for both the homologous and heterologous antigens (Table 2).

Table 2 shows data on the staining properties of the labeled mycelial-phase *H. capsulatum* antiglobulin adsorbed three times with mycelial elements of *B. dermatitidis*. This conjugate did not stain any of the mycelial-phase strains of *B. dermatitidis*. It did, however, stain both the yeast and mycelial cultures of *H. capsulatum* and the yeast-phase cells of *B. dermatitidis*. The staining titers dropped significantly as a result of antibody adsorption. The organisms stained best with only undiluted or 1:2 diluted conjugate. These results also indicated that both forms of *H. capsulatum* and the yeast cells of *B. dermatitidis* shared antigen C.

Serological properties of yeast-phase B. dermatitidis conjugate. This FA reagent stained *B. dermatitidis* yeast-phase cells with titers that ranged from 1:4 to 1:32, depending upon the strain tested. However, it exhibited lower staining titers (1:4 to 1:6) with the mycelial elements of the various homologous strains examined.

The *B. dermatitidis* conjugate reacted strongly with elements of both phases of the *H. capsulatum* strains studied. Titrations revealed staining titers of 1:4 to 1:16 for the yeast-phase cells and 1:4 to 1:8 for the mycelial phase of the heterologous strains.

Two adsorptions of the yeast-phase *B. dermatitidis* conjugate with yeast-phase cells of *H. capsulatum* completely eliminated the cross staining of the various diphasic *H. capsulatum* strains and the mycelial elements of *B. dermatitidis*. This demonstrated that *B. dermatitidis* yeast-phase cells contain a specific antigen, designated by the letter D.

Multiple adsorptions of labeled yeast-phase anti-*B. dermatitidis* globulins with mycelial elements of either *H. capsulatum* or *B. dermatitidis* produced a FA reagent that stained only the yeast phase of *H. capsulatum* and *B. dermatitidis* (Table 2). These staining reactions indicated the presence of another antigen in the yeast cells of *B. dermatitidis* and *H. capsulatum*. It was designated factor E. This factor was readily demonstrated in the adsorbed labeled yeast-phase *B. dermatitidis* antibody, but could not be demonstrated in labeled yeast-phase anti-*H. capsulatum* globulin adsorbed with homologous mycelium.

Furthermore, adsorptions of the yeast-phase *B. dermatitidis* conjugate with the mycelium of *B. dermatitidis* resulted in a conjugate that failed to stain the mycelium of *H. capsulatum*. These results indicated either the absence of factor C or our inability to detect its presence in the adsorbed *B. dermatitidis* antiglobulin.

Serological properties of mycelial-phase B. dermatitidis conjugate. This FA reagent contained only antibodies for common antigen A. Adsorptions of this reagent with the yeast phase of *H. capsulatum* or *B. dermatitidis*, or the mycelium of *H. capsulatum* eliminated all staining capacity (Table 2).

DISCUSSION

FA and adsorption techniques were used to demonstrate discrete antigenic components in yeast and mycelial cells of *H. capsulatum* and *B. dermatitidis* (Table 2). The use of these procedures has helped elucidate the antigenic relationships between these dimorphic organisms and has enabled the development of basic antigenic formulas for the cellular elements studied.

It is important to stress that the antigens revealed by our procedures probably represent only the minimal number of antigens present in the cell and that the antigenic structure of these fungi is probably more complex than is apparent. This view appears to be supported by the variation in the degree of staining noted with the various strains studied. The production and use of higher-titered reagents might reveal the presence of other antigenic factors. The possibility that serotypes occur among the various fungus species studied has yet to be investigated.

The antigenic formulas proposed for the fungi studied are presented in Table 3. From these formulas, it is evident that all the organisms share antigen A. The brilliant intensity of staining (3 to 4 +) and the high titers shown by the nonadsorbed reagents indicated this factor to be predominant among the cellular antigens.

The yeast-phase elements of *H. capsulatum* and *B. dermatitidis* were shown to contain the respective specific antigens B and D. These factors appeared to be present in fair quantity, since

TABLE 3. *Antigenic formulas for the yeast and mycelial phases of Histoplasma capsulatum and Blastomyces dermatitidis*

Species	Growth phase	Antigenic components	Specific antigens
H. capsulatum...	Yeast	A B C - E*	B
H. capsulatum...	Mycelial	A - C - -	None
B. dermatitidis..	Yeast	A - C* D E	D
B. dermatitidis..	Mycelial	A - - - -	None

* Not detected in homologous antiserum.

staining of homologous species by the specific adsorbed reagents was of a reasonable intensity (2 to 3 +).

Antigens C and E proved to be interesting factors. Antibody to antigen C was demonstrated after appropriate adsorptions only in the antiglobulins elicited by the yeast and mycelial cells of *H. capsulatum*. These adsorbed antiglobulins stained the yeast cells of *B. dermatitidis*, suggesting the presence of factor C in the somatic make-up of the cell. However, antibody produced in response to yeast-phase cells of *B. dermatitidis* and adsorbed with *B. dermatitidis* mycelium failed to show the presence of antibody to antigen C.

Similarly, antigen E in the yeast-phase cells of *H. capsulatum* was stained by appropriately adsorbed labeled yeast-phase *B. dermatitidis* antiglobulins (Table 1). However, this factor, like C, could not be demonstrated in antiglobulins produced in response to the stained organisms, the yeast cells of *H. capsulatum*. From these results, it is apparent that the antigenicity, or the capacity of factors C and E to elicit antibody, varies with the organism studied. The exact reasons for the scarcity or absence of antibodies to these factors in the antiglobulins studied is presently not resolved.

Although these studies were carried out with the FA technique, it is of interest to compare the results obtained with those observed by other investigators who used similar or different serological techniques.

Salvin and Hottle (1948), using the CF test, examined the relationships between yeast- and mycelial-phase antigens of *H. capsulatum*. They concluded that the antigens were immunologically similar, although they had been derived from two morphologically different growth phases.

Later, Lindberg (1950), who also used the CF procedure, demonstrated two antigenic entities in *H. capsulatum*: a cellular antigen present in both phases of the fungus, and a specific yeast-phase antigen.

The present study verifies the findings of Lindberg. However, it further indicates that the yeast and mycelial cells of *H. capsulatum* share at least two antigens (A and C) and that the yeast cells contain a specific antigen (B) and an additional antigen in common with the yeast cells of *B. dermatitidis* (E).

Gordon (1959), using FA methods, observed that adsorption of a labeled antiyeast-phase *H. capsulatum* globulin with cells of *B. dermatitidis* produced a reagent that failed to stain homologous as well as heterologous yeast-phase cells. In contrast, our investigation revealed that the yeast-phase cells of *H. capsulatum* share at least three antigens (A, C, E) with the yeast-phase cells of *B. dermatitidis* and, furthermore, that each species also has a distinct specific antigen. These specific factors have recently begun to be used in diagnostic work (Kaufman and Kaplan, 1961; Kaplan and Kaufman, *in press*).

Our results showed that the antigens in the mycelium existed in lower quantity than those found in the homologous yeast-phase cells. It would appear that the mycelial phase, in contrast to the more fastidious yeast phase, possesses a less complicated cellular antigenic structure.

The absence of antigen C in the mycelial phase of *B. dermatitidis* permits the differentiation of the mycelium of this organism from those of *H. capsulatum* by the FA procedure.

Although the CF test is useful in the antigenic analysis of fungal cells, the fact that multiple adsorptions tend to make experimental antiglobulins anticomplementary has limited its use. Similarly, the Ouchterlony agar-gel procedure has its own limitations in the analysis of adsorbed fungal antiglobulins (Kaufman and Kaplan, 1961).

The FA test is extremely sensitive and theoretically is applicable to any system in which a specific antigen-antibody reaction occurs, regardless of whether such a reaction is demonstrable by any other serological method. It is apparent from the data presented that the FA technique is a useful and practical tool with potentially wide application in antigen analyses and diagnostic mycoserology.

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