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Development and Use of a Polyvalent Conjugate to Differentiate *Histoplasma capsulatum* and *Histoplasma duboisii* from Other Pathogens

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Previous investigations have demonstrated the existence of five *Histoplasma capsulatum* serotypes. Available specific fluorescent-antibody reagents stain only four of the five serotypes. Antibodies produced against the most complete *H. capsulatum* serotype were labeled with fluorescein isothiocyanate to develop a reagent specific for *H. capsulatum* that was reactive with all the known serotypes. The unadsorbed reagent not only stained all the *H. capsulatum* serotypes, but it also stained cultures of *Blastomyces dermatitidis*, *H. duboisii*, several *Candida* species, and a variety of other fungi. Adsorption of the conjugate with antigens of *C. albicans* produced a reagent that intensely stained only *H. capsulatum*, *H. duboisii*, and *B. dermatitidis*. Differentiation of *B. dermatitidis* from the *Histoplasma* species was accomplished by application of a *B. dermatitidis* specific fluorescent antibody to antigens positive with the *H. capsulatum* reagent. At present, differentiation of *H. capsulatum* from *H. duboisii* may be accomplished only by animal inoculation. Our data substantiate the antigenic relationships hypothesized earlier, and they indicate that *H. capsulatum* shares at least two antigens with the other fungi that were studied.

In 1961, Kaufman and Kaplan (5) described a fluorescent antibody (FA) specific for the identification of the yeast form of *Histoplasma capsulatum*. Later, in an extensive evaluation of this reagent, an isolate of *H. capsulatum* (strain B703) was encountered that did not stain. Subsequently, additional isolates of this fungus were obtained which also failed to stain. Prompted by these observations, Kaufman and Blumer (4) carried out an antigen-analysis of these and other isolates of *H. capsulatum* whereby they illustrated the existence of five serotypes among the *H. capsulatum* isolates studied. Listed in Table 1 are the five serotypes (*1:2*; *1:4*; *1:2:3*; *1:2:4*; and *1:2:3:4*), the percentage of 113 isolates representing each serotype, and their geographical source. Of these serotypes, only one, the *1:4* type, consistently did not react with the FA reagent produced by Kaufman and Kaplan (5). Its inability to stain the *1:4* serotype rendered the conjugate ineffectual for maximal diagnostic service. Demonstration that the specific FA reagent contained antibodies homologous only to antigens *2* and *3* explained the inadequacy of the reagent (4).

The purpose of this study was to develop a FA reagent that would allow specific detection of *H. capsulatum* regardless of its antigenic makeup. Thus, our approach was to prepare a conjugate against *H. capsulatum* serotype *1:2:3:4*. Adsorption of this conjugate with an organism such as *Candida albicans* that contained only antigen *1* (4) in common with *H. capsulatum* and *H. duboisii* should remove only antibody *1* and eliminate the cross-staining of *Candida* species and other fungi. The resultant polyvalent conjugate would contain antibodies *2:3:4*, essential for the detection and identification of all known serotypes of *H. capsulatum*. Development of such a reagent would allow maximal diagnostic coverage.

MATERIALS AND METHODS

Antiglobulins, conjugation, and adsorptions. Rabbits were immunized intravenously with Formalin-treated yeast-form cells of *H. capsulatum* strain B646. We selected this strain because it represented our most complete serotype consisting of antigens, *1:2:3:4*. The protocols concerning antigen preparation, schedule of injections, harvesting of antisera, extraction of antiglobulins, and conjugation and adsorption techniques were reported in a previous paper (4). Adsorptions were performed with antigens of *Blastomyces dermatitidis* isolate B414, *C. albicans* isolate B612, *H. capsulatum* isolates A811, B580, B703

TABLE 1. *Distribution of 113 Histoplasma capsulatum isolates by serotype and geographical source*

Serotype	Percentage of total no.	No. of isolates grouped by geographical source[a]				
		USA	C-SA	Europe	Asia	Africa
1:2	15.9	15	2	—	1	—
1:4	50.4	7	43	1	3	3
1:2:3	26.5	23	7	—	—	—
1:2:4	1.8	—	2	—	—	—
1:2:3:4	5.3	1	5	—	—	—

[a] USA, United States; C-SA, Central and South America.

(representing serotypes *1:2:3*, *1:2*, and *1:4*, respectively), and *H. duboisii* isolate 1085.

Antigens studied. Five isolates of *H. capsulatum* (A811, B580, B646, B703, and IHM 1522) representing each of the serotypes, three isolates of *H. duboisii* (936, 1085, and B193), and five of *B. dermatitidis* (A295, A373, A490, B52, and B414) were routinely studied.

Complement-fixation (CF) test. CF titers of the rabbit anti-*H. capsulatum* sera were determined by the National Communicable Disease Center Laboratory Branch Complement Fixation Test (7) with optimally diluted homologous antigens.

RESULTS

Serological properties of unlabeled antisera and labeled antiglobulins. Antisera demonstrating CF titers of 1:2,048 or more were fractionated, and the antiglobulins obtained were conjugated to fluorescein isothiocyanate. Preliminary studies indicated that a 1:20 dilution of this FA consistently stained the *H. capsulatum* strains with an intensity of 3 to 4+. Table 2 gives the staining reactions of the 1:20 diluted nonadsorbed conjugate with the homologous and heterologous serotypes of *H. capsulatum*, with yeast-form isolates of *H. duboisii*, *B. dermatitidis*, and with various other fungi. The nonadsorbed conjugate exhibited cross-staining with all the heterologous fungi studied (Table 2). Therefore, all the species were characterized by the sharing of at least one antigen.

To free the conjugate from the unifying factor(s), thus obtaining a polyvalent reagent comprised of antibodies homologous to *H. capsulatum* antigens *2*, *3*, and *4*, we adsorbed the conjugate with cells of *C. albicans*. Table 2 presents the staining reactions of the conjugate after adsorption with *C. albicans*. In contrast to the unadsorbed conjugate, the adsorbed conjugate did not stain any of the *Candida* species nor did it stain *Coccidioides immitis* (spherules), *Cryptococcus neoformans*, *Torulopsis glabrata*, *Geotrichum candidum*, *Rhodotorula* sp., and *Sporotrichum schenckii*. However, this conjugate exhibited pronounced staining of all the *H. capsulatum* serotypes, as well as isolates of *H. duboisii* and *B. dermatitidis*. Our data indicated

TABLE 2. *Staining reactions of unadsorbed and adsorbed fluorescein-labeled Histoplasma capsulatum isolate B646 antiglobulins[a] (serotype 1:2:3:4) with homologous and heterologous antigens*

Adsorbing antigen (serotype)	*H. capsulatum* isolates (serotype)[b]					*H duboisii* (three isolates)	*Blastomyces dermatitidis* (five isolates)	*Candida albicans* and other fungi[c]	Serum factors
	B580 (*1:2*)	B703 (*1:4*)	A811 (*1:2:3*)	IHM 1522 (*1:2:4*)	B64 (*1:2:3:4*)				
None	+	+	+	+	+	+	+	+	*1:2:3:4*
C. albicans	+	+	+	+	+	+	+	0	*2:3:4*
H. capsulatum (*1:4*)	+	0	+	+	+	0	0	0	*2:3*
H. capsulatum (*1:2:3*)	0	+	0	+	+	+	+	0	*4*
H. capsulatum (*1:2*)	0	+	+	+	+	+	+	0	*3:4*
H. duboisii (*1:4*)	+	0	+	+	+	0	0	0	*2:3*
B. dermatitidis (*1:4*)	+	0	+	+	+	0	0	0	*2:3*
C. albicans and *H. duboisii* or *B. dermatitidis*	+	0	+	+	+	0	0	0	*2:3*

[a] Showed no staining after adsorption with homologous antigen (strain); +, reasonable FA staining ranging from 1 to 4+.

[b] Based upon earlier study (5).

[c] Other fungi are *C. krusei*, *C. stelladoidea*, *Coccidioides immitis* (spherules), *Cryptococcus neoformans*, *Torulopsis glabrata*, *Geotrichum candidum*, *Rhodotorula* sp., and *Sporotrichum schenckii*.

the presence of two extra generic factors—one shared by *H. capsulatum* and all the heterologous fungi studied, which we refer to as factor *1*, and the second shared among *H. capsulatum*, *H. duboisii*, and *B. dermatitidis*, which we refer to as factor *4*.

Although adsorption of the labeled *H. capsulatum 1:2:3:4* factor serum with cells of *C. albicans* resulted in a polyvalent reagent which stained all the known serotypes of *H. capsulatum*, it nevertheless failed to remove those antibodies that allowed the cross-staining of *H. duboisii* and *B. dermatitidis*. The latter reactions have been attributed to common antigen *4* (4). To test further the theoretical antigenic pattern, additional adsorptions of the *Candida* adsorbed conjugate were performed—in one instance with *B. dermatitidis* and in the other with *H. duboisii* (Table 2). These adsorptions produced conjugates with identical staining patterns. Concurrent with the loss of staining for *B. dermatitidis* and *H. duboisii* was the loss of staining for *H. capsulatum* serotype *1:4*. The retained staining was attributed to the occurrence of antibodies to factors *2* and *3*.

Labeled *1:2:3:4* antiglobulins, adsorbed with cells of *H. capsulatum* isolate 703 (serotype *1:4*), *H. duboisii*, or *B. dermatitidis*, gave identical staining patterns characteristic of antibodies *2* and *3*.

Adsorption of the conjugate with *H. capsulatum* isolates B580 (serotype *1:2*) and A811 (serotype *1:2:3*) resulted in reagents that contained only serum factors *3:4* and *4*, respectively.

These data suggest that only the *C. albicans* adsorbed conjugate can be used to stain all of the *H. capsulatum* serotypes. In spite of the *H. duboisii* and *B. dermatitidis* cross-staining, we thought that use of this conjugate in conjunction with the specific *B. dermatitidis* conjugate (3) would permit the differentiation of *H. capsulatum* and *H. duboisii* from *B. dermatitidis*. To establish whether this was possible, we treated duplicate smears of numerous isolates representing all the serotypes of *H. capsulatum*, as well as isolates of *H. duboisii* and *B. dermatitidis*, with the *C. albicans* adsorbed *H. capsulatum* conjugate and the *B. dermatitidis* specific conjugate (Table 3). These tests indicated that, despite the cross-staining exhibited by the labeled *2:3:4* factor serum, differentiation of *B. dermatitidis* from *H. capsulatum* and *H. duboisii* can be accomplished by the use of a *B. dermatitidis* specific FA reagent in conjunction with the polyvalent *Histoplasma* conjugate. Thus far, *H. capsulatum* and *H. duboisii* cannot be distinguished through the use of FA reagents.

TABLE 3. *Fluorescent antibody staining reactions for differentiating Histoplasma species and Blastomyces dermatitidis strains*

Organism tested	Serotype	No. of isolates	Fluorescent-antibody staining reaction	
			Histoplasma polyvalent conjugate	*Blastomyces* conjugate
H. capsulatum..	*1:2*	11	+	0
H. capsulatum..	*1:4*	33	+	0
H. capsulatum..	*1:2:3*	18	+	0
H. capsulatum..	*1:2:4*	2	+	0
H. capsulatum..	*1:2:3:4*	4	+	0
B. dermatitidis.		5	+	+
H. duboisii.....		11	+	0

DISCUSSION

Reciprocal cross-staining and adsorption procedures have revealed conclusively that serotypes exist among yeast-form isolates of *H. capsulatum* (4). Knowledge of the antigenic makeup of these serotypes and of their relationship to other fungi contributes not only to our understanding of the successes and failures of previously described investigations involving development and application of *H. capsulatum* fluorescent antibodies, but it also assists us in the development of the present new FA reagent and staining protocol.

The data (Table 2) indicate that *H. capsulatum* shares with other fungi two antigens, *1* and *4*. Adsorption of *H. capsulatum* isolate B646 antiserum that contains serum factors *1:2:3:4* with cells of *C. albicans* eliminated antibody *1* and left an FA reactive with all the *H. capsulatum* serotypes, plus *H. duboisii* and *B. dermatitidis*. Adsorption of the conjugate with *B. dermatitidis* or *H. duboisii* not only made the conjugate specific for *H. capsulatum* but it also eliminated the factor *4* antibody. Such an FA, containing factors *2* and *3*, is identical to the specific FA produced by Kaufman and Kaplan in 1961. Apparently, the absence of factor *4* antibody from this reagent was responsible for its limited diagnostic application.

Despite its cross-reacting antibodies, the labeled polyvalent *2:3:4* factor serum, produced by adsorption with *C. albicans* cells, can be employed diagnostically. This is accomplished through the use of a *B. dermatitidis* specific FA reagent in conjunction with the polyvalent *Histoplasma* conjugate (Table 3). Isolates of *B. dermatitidis* are stained with both of these FA reagents, but *Histoplasma* species stain only with the polyvalent conjugate. The polyvalent

FA is useful in the identification of the yeast form of *H. capsulatum* and *H. duboisii* and is advantageous over the previous conjugate produced by Kaufman and Kaplan (5) in that it may be diluted 1:20 without diminution of its staining intensity. In contrast, the earlier reagent had to be used undiluted.

H. duboisii and *H. capsulatum* cannot be differentiated from each other by use of FA reagents. However, when necessary, differentiation of these two species can be achieved by animal inoculation and demonstration of the large yeast forms typical of *H. duboisii*. We have been unable to confirm those studies that have reported that *H. capsulatum* and *H. duboisii* can be differentiated by enzymatic tests (S. Blumer and L. Kaufman, Sabouraudia, *in press*).

In 1959, Gordon (2) produced a *H. capsulatum* conjugate which cross-stained *H. duboisii* and *B. dermatitidis*. Adsorption of this FA with *B. dermatitidis* cells caused the loss of staining for both the homologous and heterologous organisms. Our antigenic studies indicated that these negative reactions could possibly have been due to the use of *H. capsulatum* labeled antiglobulin containing only factors *1* and *4*.

In 1962, Lynch and Plexico (6) observed that their *H. capsulatum* conjugate, which had been adsorbed with *Candida* yeast cells, still stained *B. dermatitidis*. In the same year, Carski, Cozad, and Larsh (1) essentially verified the work of Lynch and Plexico (6), but because of the persistent cross-staining of *B. dermatitidis* yeasts they urged caution in relying upon such FA reagents for the diagnosis of histoplasmosis. This study substantiates the observations of these investigators and demonstrates that the cross-reactivity is due to factor *4*.

Our polyvalent adsorbed conjugate contains, in addition to antibodies *2* and *3*, antibodies homologous to antigen *4* and consequently lacks absolute specificity. However, its use with the specific FA for *B. dermatitidis*, combined with knowledge of the geographical origin of the material under study, is of practical value.

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