Differentiation of Yeasts by Means of Fluorescent Antibody.* (23849)

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Methods currently employed for classification and identification of yeasts, based upon a combination of morphological and physiological characteristics(1,2) are complex, time-consuming, and require many special media. Conventional serological methods could be applied with advantages of potentially greater specificity and relative simplicity, but difficulties are encountered in preparation of stable and specific antigen systems and a good deal of time and equipment are generally consumed. Several investigators have applied serological technics to classification within the genus *Candida* (3, including a review of the literature; 4), but none of these has reached the status of routine identification procedure.

An entirely new tool for identification of yeastlike organisms became available with development of Coons fluorescent antibody technic(5). Following Goldman's(6) adaptation of the method to differentiation of whole amoeba cells *in vitro*, we became interested in applying it to yeastlike agents of human disease. *Candida albicans*, for example, is commonly identified in the mycology laboratory by formation of characteristic chlamydospores on cornmeal agar. Neither this medium nor any of its numerous substitutes has proved completely reliable for this purpose and, in addition, it is well known that some strains of *C. stellatoidea* form similar spores on these media. Difficulty is often encountered also in precise identification of systemic fungus pathogens in tissue when stained by conventional technics. This applies particularly to the confusion between intergrading forms of *Blastomyces dermatitidis* and *Histoplasma capsulatum*. The present paper deals with the use of specific, fluorescein-labelled anti-*Candida* globulins (conjugates) to identify yeast cells in dried smears.

Materials and methods. Production of fluorescent antibody. Heat-killed saline suspensions (1:200 v/v) of 24 to 48-hour *Candida albicans* cultures (strain Br) on Sabouraud agar were injected intravenously into albino rabbits weighing 5 to 7.5 lb. The immunization schedule was similar to that employed by Benham(7), resulting in serum agglutinin titers of 1:1280 to 1:40,960 against the homologous organism. Globulin fractions of serum from 2 hyperimmunized rabbits, C-1 and C-4, and from a normal rabbit were obtained by ammonium sulfate precipitation. Tube agglutination titers for C-1 and C-4 globulins against *C. albicans* cells were 1:160 and 1:5,120, respectively; protein concentrations (biuret method) 0.43 and 2.72 mg %. The protein content of each was adjusted to 2 mg % for labelling, C-1 being concentrated by evaporation through a dialysis sac in front of fan. Fluorescein isocyanate was synthesized according to the method of Coons and Kaplan(5) and reacted overnight with globulins at 0 to 2°C, C-1 and C-4 globulins being labelled with isocyanate derived from separate lots of amine.† Unreacted fluorescein compounds were removed by dialysis of the conjugate in the cold against phosphate-buffered saline (pH 7). For some staining experiments fluorescent conjugates were absorbed with heterologous yeast cells to increase their specificity. In these cases the sediment from a suspension of fresh yeast cells was resuspended in 1 ml of diluted (1:10) conjugate, the mixture agitated for 2 hours at 37°C on a reciprocating shaker and then refrigerated overnight. Following removal of cells by centrifugation the clear supernatant was employed in staining. *Preparation of slides.* Yeast phases of the diphasic pathogens were cultured upon cystine heart-

* This investigation was supported by research grant from N.I.H., P.H.S.

† Most of the amine was prepared locally by Col. John W. Steedly, The Citadel. The lot from which conjugate C-1 was prepared was kindly contributed by Dr. Morris Goldman, Communicable Disease Center, Atlanta.

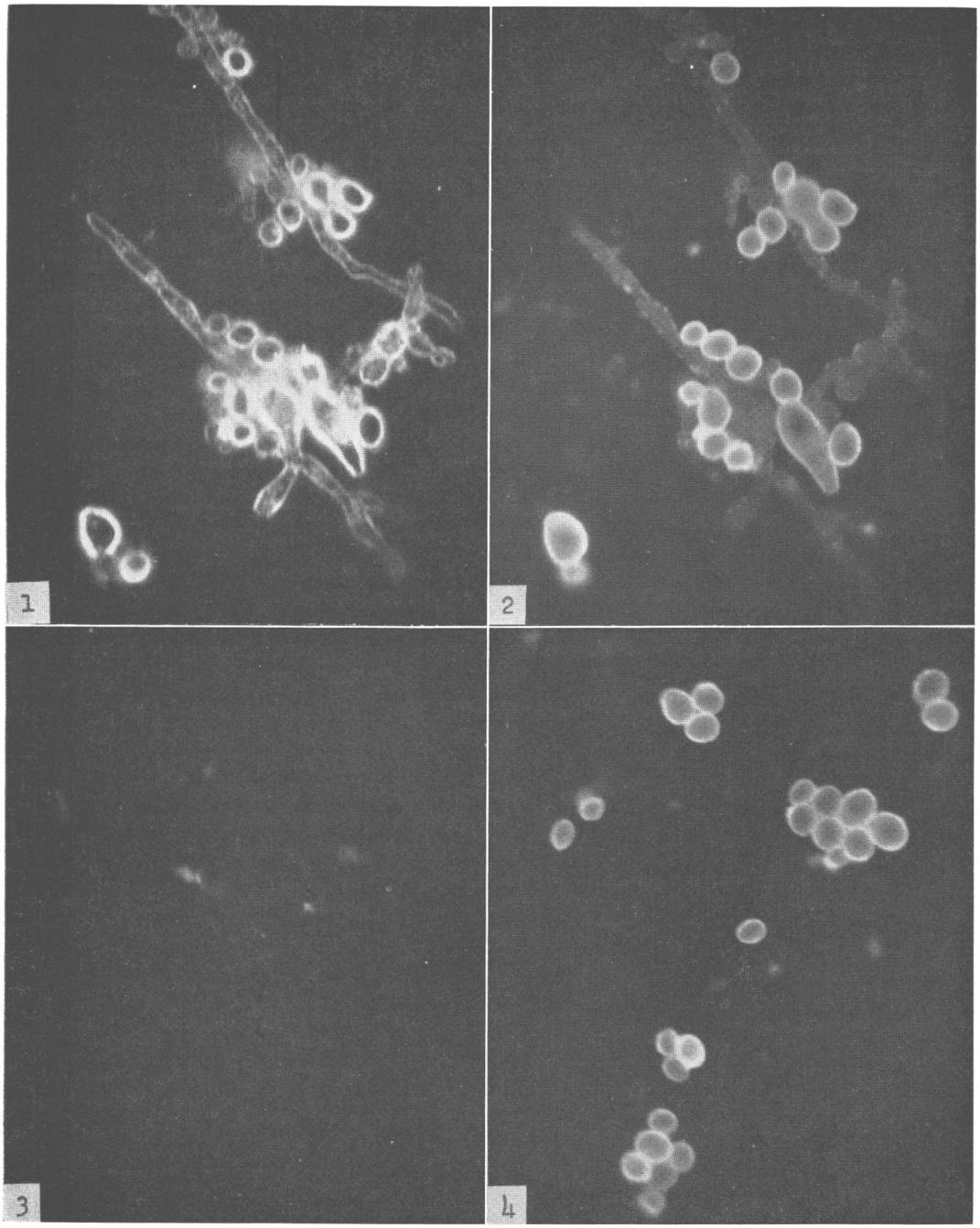
hemoglobin agar slants at 37°C and harvested at 3 to 7 days. All other yeasts were grown on Sabouraud dextrose agar at room temperature for 3 to 4 days. Growth from slants was emulsified directly upon a slide in a drop of distilled water, permitted to air-dry, and gently fixed by heat. Each smear was covered with a drop of labelled globulin and placed in moist chamber at room temperature for 30 minutes. It was then drained, rinsed in 2 changes of buffered saline for 10 minutes, blotted dry, and mounted in a drop of glycerol-saline (9 parts glycerol : 1 part saline, pH 7) under a coverglass. Brightness of fluorescence upon microscopic examination was judged by a system of "plus" symbols similar to that employed by Moody *et al.* (8).

In examination of the slides quantitative readings (estimation of fluorescence) of films prepared from yeast cultures were most consistent when restricted to those areas wherein clusters of cells lay in a single plane rather than those in which scattered cells appeared to be "floating" at different optical levels. *Fluorescence microscopy.* A Reichert Zetopan microscope equipped with cardioid darkfield condenser and Reichert illumination system was used. Illumination consisted of a 30 watt tungsten bulb and a 200 watt, air-cooled, maximum pressure mercury vapor arc lamp, which were instantly interchangeable. A BG 12 3 mm glass filter in front of mercury lamp permitted passage of only near ultraviolet, violet, and some blue light. All this incident light was taken out, after having passed through specimen slide, by a Wratten G gelatin filter fitted into the microscope tube, so that only the secondary, fluorescent rays reached the ocular. Both monocular and binocular heads, fitted with 8X oculars, were employed. Routinely yeast cells were found with a 16mm 10X objective and examined more closely with a 1.8mm 100X apochromat. Photomicrographs were taken on Kodak 35mm Tri-X film, with exposures of 3 seconds for visible light (darkfield) and 30 seconds for fluorescence.

Results. The conjugates were tested against 103 cultures of yeasts or yeastlike organisms, of which 57 were strains of *Candida*. All 28 strains of *Candida albicans* stained 4+ (Fig 1, 2) with a 1:10 dilution of either C-1 or C-4 conjugate, this dilution being employed because it permitted greater specificity of staining without significant sacrifice of sensitivity. No fluorescence was induced by normal globulin conjugate. C-4 1:10 absorbed with cells of *C. parakrusei* (2 successive absorptions with 1:20 v/v suspension of packed cells) stained all but one of the *C. albicans* strains 3+ to 4+, the exception being read as 2-3+. Results of the application of these 3 reagents to the 7 other species of *Candida* are summarized in Table I. It should be pointed out that *C. tropicalis* could not be distinguished from *C. albicans* with this set of conjugates. The remaining species could readily be differentiated from these two with the C-1 stain; likewise with the absorbed C-4, except for 2 strains of *C. stellatoidea.*

Forty-six strains of non-*Candida* yeasts or yeastlike organisms, representing 15 genera and at least 22 species, were exposed to C-1 or C-4 or both. The following species exhibited no fluorescence (0 or ±) (figure in parentheses denotes number of strains tested): *Blastomyces dermatitidis* (12), *B. brasiliensis* (2), *Histoplasma capsulatum* (4), *H. duboisii* (2), *Sporotrichum schenckii* (1), *Cryptococcus neoformans* (2), *C. albidus* (1), *Trichosporon cutaneum* (1), *Trichosporon* sp. (1), *Geotrichum candidum* (3), *Rhodotorula* sp. (4), *Pullularia pullulans* (2), *Hansenula anomala* (1), *H. californica* (1) (ascospores of these two fluoresced, but not the vegetative cells), *Lipomyces starkeyi* (1), *Saccharomyces cerevisiae* (1), *S. veronae* (1) (ascospores fluoresced), *Torulopsis glabrata* (1). One strain of each of the following species exhibited fluorescence of at least 1+ when treated with unabsorbed C-4 (intensity denoted by figure following): *Endomycopsis fibuliger* 1-2+, *Pichia fermentans* 1-3+, *P. polymorpha* 2+, *Hansenula beckii* 2+ (ascospores 4+), *Debaryomyces hansenii* 3-4+. Absorption of the conjugate with *C. parakrusei* eliminated these reactions. Various unidentified cultures of bacteria exposed to the above conjugates exhibited no fluorescence whatsoever.

As a further control on the serological specificity of the observed staining reactions,



All photographs taken at a magnification of 800 ×.

FIG. 1. Mixture of *Candida albicans* and *Histoplasma capsulatum* cells exposed to *C. albicans* (C-4) conjugate for 30 min. Visible light, darkfield.

FIG. 2. Same field as Fig. 1, ultraviolet light. Only the homologous cells fluoresce.

FIG. 3. *C. albicans* exposed simultaneously to C-4 conjugate and unlabelled C-4 serum for one hr. Ultraviolet light. Inhibition of staining.

FIG. 4. *C. albicans* exposed simultaneously to C-4 conjugate and normal serum for one hr. Ultraviolet light. No inhibition of staining.

TABLE I. Fluorescence of *Candida* Species Exposed to Labelled Anti-*C. albicans* Globulins (1:10 Dilution).

Species of *Candida*	No. of strains	Range of fluorescence, each conjugate		
		C-1	C-4	C-4 absorbed
C. tropicalis	8	2+ to 4+	2+ to 4+	2+ to 4+
stellatoidea	7	0 to 1+	2+ to 4+	0 to 3+
parakrusei	3	0 to 2+	2+	0
krusei	4	0	2+ to 4+	0 to 1+
pseudotropicalis	2	0	0 to 2+	0
guilliermondi	4	0 to 2+	± to 4+	0
lipolytica	1		0	

homologous absorption procedures were applied to the C-1 and C-4 conjugates. It was found that a 1:10 dilution of either, when absorbed with an equal volume of a 1:5 v/v suspension of either *C. albicans* or *C. tropicalis*, no longer stained smears of these or any other species of *Candida*. Further, staining of the homologous cells by the unabsorbed conjugates was inhibited by either prior or simultaneous exposure to unlabelled specific serum (Fig. 3, 4).

Some preliminary trials with non-*Candida* conjugates may be reported. Anti-*Histoplasma capsulatum* (H-3) and anti-*Blastomyces dermatitidis* (B-1) globulins obtained from rabbits experimentally infected with the respective organisms were labelled with fluorescein isocyanate as described above. The B-1 conjugates stained yeast cells of both *B. dermatitidis* and *C. albicans* strongly, but exhibited little or no reaction with *Blastomyces brasiliensis* or *H. capsulatum*. H-3 conjugates stained *H. capsulatum* strongly, *B. dermatitidis* to a much lesser extent, and reacted only very weakly with *B. brasiliensis*.

Comments. The fluorescent antibody technic retains the high specificity inherent in serological procedures while offering several advantages over agglutination and precipitation methods for the identification of yeasts. The problem of obtaining a smooth agglutinating antigen is obviated, as are the complex procedures involved in extraction of a soluble and specific precipitin antigen. Large numbers of cells are not required for the reaction, since even a single organism can be identified by stain, and it need not be viable. Additional diagnostic potentials of the fluorescent antibody method for medical mycology are readily apparent. It may be applied, for example, to the serological examination of body fluids by the inhibition procedure described by Goldman(9) for toxoplasmosis and utilized by us herein as a specificity control (Fig. 3, 4). Also, preliminary studies have shown that yeast cells and pseudohyphae of *Candida albicans* may be stained directly in clinical specimens such as tissue sections, smear impressions and material obtained by oral or vaginal swab. This would be of value in differentiating this pathogen from the nonreacting harmless commensals, such as *C. stellatoidea, Cryptococcus,* and *Saccharomyces,* particularly when pseudohyphae are not seen. Our *Candida* conjugates also display no reaction with *Torulopsis glabrata,* whose increasing importance as a possible human pathogen has recently been reported by Wickerham (10). Thus, another potential source of confusion may be eliminated.

Summary. 1) The Coons fluorescent antibody technic is here extended to differentiation and classification of yeasts. The globulin portion of antiserum produced in rabbits against *Candida albicans* was labelled with fluorescein isocyanate and applied as a differential stain to dried smears from various yeastlike cultures. 2) All 28 strains of *C. albicans* tested and all 8 strains of *C. tropicalis* exhibited positive staining reactions with the anti-*C. albicans* conjugates. Twenty-one strains belonging to 6 other species of *Candida,* and 46 strains of yeastlike organisms representing 15 other genera were unstained or only weakly stained, and could readily be distinguished from the preceding 2 species. The staining specificity of high-titered conjugates was greatly increased through absorption with cells of *Candida parakrusei.* 3) Appropriate controls on the serological specificity

of the fluorescent staining, including inhibition and absorption tests, are described. 4) Preliminary results with *Blastomyces dermatitidis* and *Histoplasma capsulatum* conjugates are reported.

I wish to thank the following for contributions: Dr. Morris Goldman, for advice; personnel of Special Projects Laboratory at CDC; Mr. John C. Aull, Chemistry Dept., Med. Coll. of S. Carolina, for protein determinations on globulins; Dr. Norman F. Conant, Duke Univ., Dr. Margarita Silva, Columbia Univ., Dr. Lynferd J. Wickerham, U. S. Dept. of Agriculture, Peoria, Miss Shirley M. McMillen, Hektoen Inst., Chicago, and Dr. R. Vanbreuseghem, Inst. de Medecine Tropicale, Antwerp, for many cultures.

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Received January 13, 1958. P.S.E.B.M., 1958, v97.

Tyrosine, Iodide, and Human Serum Lipoproteins.* (23850)

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Thyroid active substances such as desiccated thyroid(1,2), thyroxine(3) and l-triiodothyronine(4) have all been shown to have a profound effect on human serum lipoprotein concentrations.

Since tyrosine and inorganic iodine are the only 2 known precursors required for thyroidal synthesis of thyroxine, the effect of administration of tyrosine and of iodide on human serum lipoproteins has been investigated.

Tyrosine study; methods. Serial blood samples were obtained from a group of 16 male hospitalized chronic schizophrenic patients, judged by routine laboratory tests and a physical examination to be free of other disease. None of these patients had received any medication or electroshock therapy for several weeks to months before initiation of the present study. Eleven of these patients, ranging in age from 31-47 years, received a daily dose of 30 g of tyrosine suspended in glass of water daily for 18 weeks; the remaining 5 patients, ranging in age from 29-48 years, served as a control group receiving no medication. Five blood samples were obtained in all patients during a 6 month interval preceding tyrosine administration, to serve as a base line, 7 samples were drawn during the 18-week period of tyrosine intake and 3 samples in a 6-week period following the last dose of tyrosine. Serum lipoprotein(5) and total serum cholesterol analyses(6) were done on all blood samples and body weight, blood pressure and pulse rate were recorded for each patient at the time of venipuncture.

Results of complete low density serum lipoprotein analyses and of cholesterol determinations in 240 blood samples and of body weight, blood pressure and pulse rate data are summarized in a greatly condensed manner in Table I. Analysis of all the data shows that a daily dose of 30 g of tyrosine, estimated to be approximately equivalent to 8-10 fold the normal dietary intake(7), has no effect on

* This work was supported by grant from Albert and Mary Lasker Foundation.