FLUORESCENT STAINING OF *HISTOPLASMA CAPSULATUM*[1]

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In an earlier paper from this laboratory (Gordon, 1958) it was demonstrated that fluorescein-labeled specific antiserum employed as a cellular stain will differentiate *Candida albicans* and *Candida tropicalis* from other yeasts and yeastlike organisms, including the remaining members of the genus *Candida*. The present report describes the preparation and use of a similar reagent for detection and identification of the yeast phase of *Histoplasma capsulatum*, which must be differentiated from morphologically similar fungi and protozoa that invade the human body.

Although typical forms of the pathogenic yeasts are usually separable, it is often difficult to identify them specifically or to differentiate them from artifacts. Many reports, for example, of ambiguous large forms of *H. capsulatum* and small forms of *Blastomyces dermatitidis* have appeared, as have instances of proved or possible dual infection (Binford, 1955; Weed, 1955; Starr *et al.*, 1955; Moore, 1955). *Histoplasma duboisii* characteristically produces forms similar to those of *B. dermatitidis* both in tissue and in culture, as do some strains of *H. capsulatum* to a lesser extent (Drouhet and Schwarz, 1956). The latter may also be simulated by *Cryptococcus neoformans* (Weed, 1955; Baker, 1957; Seabury, 1958), *Sporotrichum schenckii* (Kligman and Baldridge, 1951), *Torulopsis glabrata* (Lopez Fernandez, 1952), and certain protozoa, particularly *Leishmania donovani* (Starr *et al.*, 1955). The periodic acid-Schiff stain (Pillsbury and Kligman, 1951) is an excellent tool for detecting fungi in tissues, but often is less satisfactory than the routine hematoxylin and eosin for species identification. *H. capsulatum* and *S. schenckii*, for instance, may look very much alike when stained with the Schiff reagent. The fluorescent antibody reagent differentiates among species of fungi.

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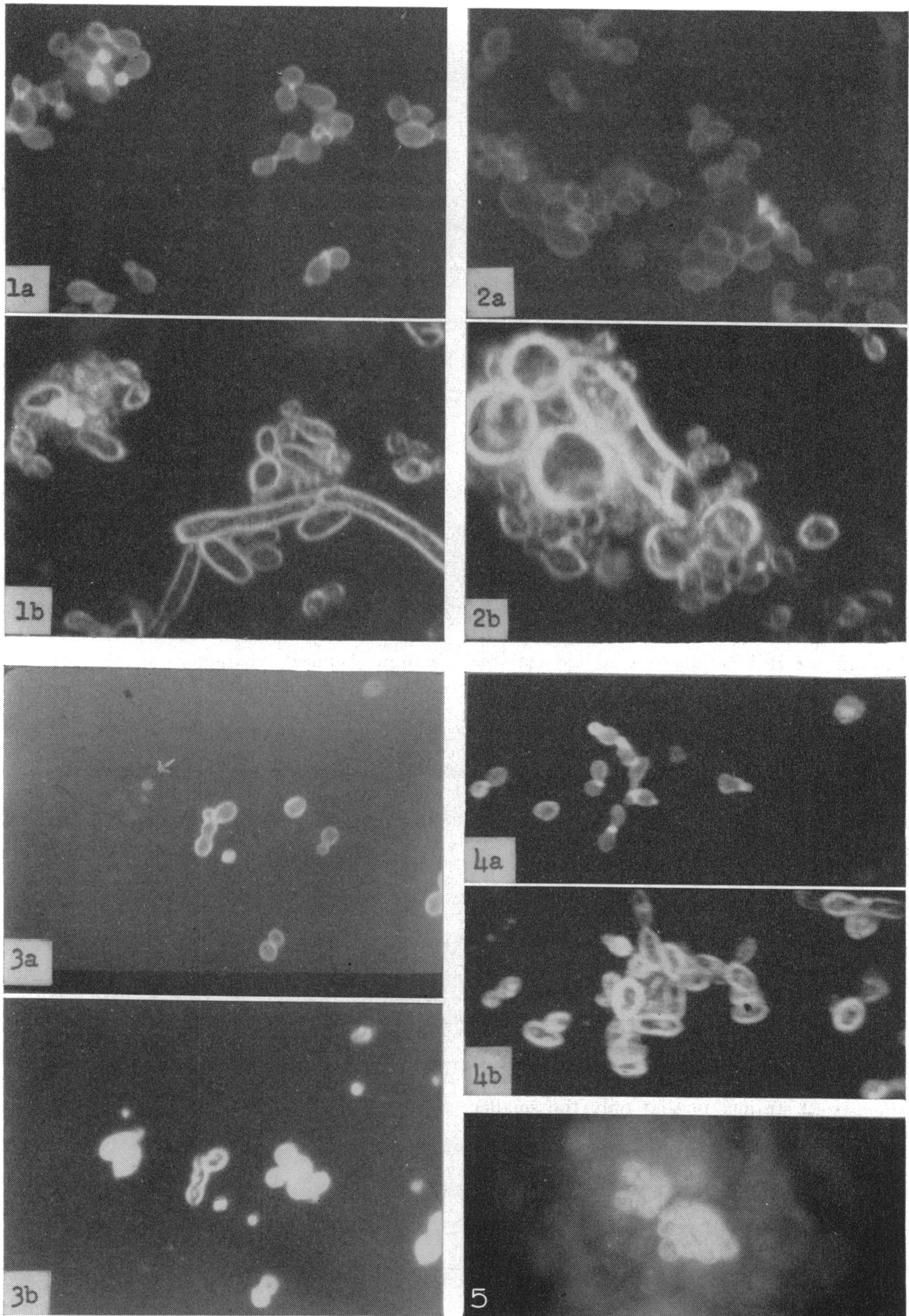
EXPERIMENTAL METHODS

Production of antiserum. Whole yeast-phase cells of *H. capsulatum* were injected into pairs of albino rabbits (4 to 7.5 lb) according to several different schedules, to find a method of high yield of antibody. Among the procedures employed were those of Benham (1931), Saslaw and Campbell (1948), and Salvin (1950). Subcutaneous inoculation of yeast-phase cells was also tried, with and without Freund's adjuvant or phosphorylated hesperidin, neither of which appeared to influence the agglutinin titer. Antibody content was measured by slide and tube agglutination procedures developed in this laboratory, utilizing a washed, formalin-killed, whole yeast-phase cell antigen. Highest titers were obtained by injection of live yeast-phase cells. A suspension of these cells was made up to the turbidity of tube no. 3 of the MacFarland nephelometer and two 1-ml doses were given intravenously, 1 month apart. On the 7th and 11th days following the final inoculation, the rabbits were bled by cardiac puncture, yielding maximal agglutination titers of 1:320 and 1:640 respectively. Complement-fixation titers determined by Dr. Kenneth W. Walls, Communicable Disease Center, Atlanta, on some of our sera were similar to those of the agglutination test. Our observations indicate that staining intensity also is related to the agglutinin titer.

Preparation and application of conjugate. H. capsulatum antiserum with agglutinin titer of 1:640 and normal rabbit serum were fractionated by ammonium sulfate precipitation and the globulin portion of each adjusted to a concentration of 2 g per cent in distilled water. Labeling of globulins with fluorescein isocyanate was accomplished according to methods detailed in the previous paper (Gordon, 1958), as were the preparation and examination of stained slides. The staining ability of the specific conjugate appeared undiminished following storage either in the refrigerator (0 to 4 C) or the deepfreeze (−20 C)



Figures 1–4. Histoplasma capsulatum mixed with *Candida albicans*, *Blastomyces dermatitidis*, *Histoplasma duboisii*, and *Sporotrichum schenckii*, each mixture stained with fluorescent *H. capsulatum* antiglobulin. In each figure, *a* and *b* show the same field photographed first under ultraviolet and then under tungsten illumination. In every case only the cells of *H. capsulatum* take the stain. The arrow in figure 3*a* indicates a fluorescent bud of *H. duboisii*, its mother cell remaining unstained. Magnification 1000×.

Figure 5. Endospores of *Coccidioides immitis* in pus from experimentally infected guinea pig; unstained, photographed under ultraviolet light to demonstrate intense autofluorescence. Magnification 1000×.

for 14 months. Photomicrographs were made on Kodak 35 mm Tri-X film with exposure of 3 sec for darkfield and 30 sec for fluorescence, or on Superanscochrome 35 mm with exposures of 6 sec and 2 min, respectively.

RESULTS

Undiluted H-3 (specific) conjugate caused the cells of the yeast phase to fluoresce to an intensity of 4+ in each of 5 strains of *H. capsulatum*, with the entire wall of every cell staining brilliantly. A peculiar phenomenon was observed, however, in a strain of *H. duboisii*, and also in an aberrant strain of *H. capsulatum* (no. 60), of unknown origin, which formed many large, Blastomyces-like cells similar to those of *H. duboisii*. In these two strains there was minimal or no fluorescence of the walls of mature cells, but the tiniest buds shone brilliantly (2 to 4+). Further trials demonstrated this to be a constant characteristic of *H. duboisii*. Four strains of *B. dermatitidis* treated with undiluted H-3 conjugate exhibited variable fluorescence, ranging in intensity from 0 to 3+. The cells of two strains of *C. albicans* did not stain at all. Absorption of H-3 conjugate with cells of *B. dermatitidis* in an attempt to eliminate staining of the latter resulted in loss of reactivity with the homologous yeast-phase cells as well.

Dilution of H-3 conjugate 1:4 with phosphate-buffered saline (pH 7) resulted in elimination of almost all heterologous activity while maintaining homologous staining at a high level, as illustrated in figures 1 to 4. Five typical strains of *H. capsulatum* stained 3 to 4+, whereas 2 of *H. duboisii* and the atypical no. 60 showed fluorescence only in the buds, the tiny ones staining 4+ while larger structures were as bright as 2 to 3+. A similar phenomenon was observed in *Blastomyces brasiliensis* (2 strains) in that only the smaller buds exhibited significant fluorescence. The meaning of this in terms of serological relationships and antigenic topography remains to be determined. Of 15 strains of *B. dermatitidis*, 10 showed no fluorescence of the cell walls whereas 5 varied from 0 to 1+, with an occasional cell in 2 of the strains being as bright as 2+. None of the yeast-phase cells of the following organisms fluoresced following treatment with H-3 conjugate (diluted 1:4): *Candida albicans* (5 strains); *Cryptococcus neoformans* (5); *Cryptococcus* sp., encapsulated, avirulent (3); *Cryptococcus albidus* (1); *Sporotrichum schenckii* (2); *Torulopsis glabrata* (1); *Trichosporon cutaneum* (1); *Geotrichum candidum* (1). Likewise, 2 protozoan species that might be confused with *H. capsulatum* in conventionally stained smears or tissue sections failed to acquire fluorescence. These were *Toxoplasma gondii* in smears from infected mouse peritoneal fluid and *Leishmania donovani* in hamster spleen impression smears.

Attempts to determine the reactivity of spherules of *Coccidioides immitis*, obtained from an experimental guinea pig abscess, were unsuccessful, since these structures and their endospores possessed a brilliant autofluorescence under ultraviolet light, indistinguishable in the majority of cells from that of fluorescein-stained material (figure 5). Some of the cells, however, were bright orange rather than greenish yellow. A similar autofluorescence was observed in the arthrospores of *C. immitis* (but not in those of *Geotrichum candidum*) and the tuberculate spores of *H. capsulatum*.

Control experiments designed to confirm the immunochemical character of the successful staining of *H. capsulatum* gave the following results: (a) antiserum conjugate diluted 1:4 stained *H. capsulatum* intensely whereas normal serum conjugate in the same dilution did not stain it at all; (b) staining of *H. capsulatum* by H-3 conjugate was inhibited by simultaneous application of unlabeled antiserum but not by unlabeled normal serum.

DISCUSSION

Schwarz (1958) states that "With increasing experience, we have learned that one never should attempt to make a diagnosis based on the morphologic appearance of organisms alone because of the enormous variety in size that can be observed in tissue." The confusion of *H. capsulatum* with *B. dermatitidis* presents an especially difficult problem, one which has not heretofore been approached by means of differential stains. It is hoped to apply the results reported here to the differential staining of the pathogenic yeasts in tissues. Practical applications of the fluorescent antibody technique to the diagnosis of histoplasmosis, in addition to this, might include use of the fluorescence inhibition test of Goldman (1957).

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SUMMARY

Fluorescein-labeled globulin derived from rabbits infected with *Histoplasma capsulatum* was found capable of differentiating this species by cellular stain from tissue forms of other human pathogens, including *Blastomyces dermatitidis*, *Cryptococcus neoformans*, *Sporotrichum schenckii*, and *Leishmania donovani*. *Coccidioides immitis* exhibited striking autofluorescence in the tissue phase. *Histoplasma duboisii* and a duboisioid strain of *H. capsulatum* were peculiar in that only the small buds fluoresced, to the exclusion of more mature cells. Strains of *Blastomyces brasiliensis* reacted similarly.

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