New, Special Stain for Histopathological Diagnosis of Cryptococcosis

KYUNG J. KWON-CHUNG,* WILLIAM B. HILL, AND JOHN E. BENNETT

Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases,
Bethesda, Maryland 20205

The Masson-Fontana silver stain for melanin was employed for the differentiation of pathogenic fungal species in human or mouse tissues. The fungi studied were *Candida albicans*, *Candida tropicalis*, *Candida glabrata* (*Torulopsis glabrata*), *Cryptococcus neoformans*, *Cryptococcus bacillisporus*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, *Rhizopus rhizopodiformis*, and *Aspergillus fumigatus*. The tissue sections stained with Masson-Fontana silver stain showed a dark brown to black color in the wall of cryptococci, whereas the walls of remaining fungal species were hyaline, except for those of *S. schenckii*. The yeastlike cells of *S. schenckii* showed faint brown pigment on the wall. Cultures of these fungi showed staining characteristics identical to those of the in vivo results. Cultures of four nonpathogenic *Cryptococcus* species, *Cryptococcus unitulatus*, *Cryptococcus laurentii*, *Cryptococcus terreus*, and *Cryptococcus luteolus*, were also tested for staining by the Masson-Fontana procedure. Of these, only *C. laurentii* stained positively, and the pigment on the cell wall was as intense as that of the cells of *C. neoformans*. These results indicate that the Masson-Fontana silver stain can be used as a specific stain in the histological diagnosis of cryptococcosis.

The identification of a fungal etiological agent which is seen in tissue should be confirmed by culture. In practice, however, pathologists are often confronted with situations in which they have to make a diagnosis only with fixed histopathological materials. The Gomori methenamine-silver stain and the periodic acid-Schiff reaction are the two best and the most widely used stains for detecting fungi in tissue. When the fungal cells are typical and the histological techniques are adequate, a pathologist with experience can make with confidence a diagnosis of several diseases on the basis of fungal morphology in tissue. Cryptococcosis (2) is one of the fungal diseases which can be diagnosed histopathologically when the fungus is typical. However, cells of *Cryptococcus neoformans* in tissue can be confused with those of *Blastomyces dermatitidis* or other yeastlike fungi. Mayer mucicarmine stain is a useful method (2) for differentiating cryptococci from other fungi of similar size and appearance. Mucicarmine stains the mucopolysaccharide of the cryptococcal capsules. Over the years, however, we have seen several cases in which the results of mucicarmine stain were not decisive although the clinical picture and the morphology of fungal cells were those of *C. neoformans*. *B. dermatitidis* cells usually are not stained by mucicarmine, but

occasionally some cells are lightly colored by the method (2) and can be confused with lightly stained cryptococcal cells.

The Masson-Fontana melanin staining method was developed by Fontana in 1912 (3) and modified by Masson in 1928 (8) to detect melanin and other silver-reducing granules. *C. neoformans* produces a melanin-like pigment when grown on a substratum containing di- or polyhydroxy phenols (10). Bearing this phenomenon in mind, we studied the effect of Masson-Fontana melanin staining on the cells of *C. neoformans* in tissue and compared it with the results obtained with various important systemic fungal pathogens.

MATERIALS AND METHODS

Fungal cultures and animal inoculation. Yeast cells (48 to 72 h old) from five species of yeastlike fungi (*Candida albicans*, *Candida tropicalis*, *Candida glabrata* [*Torulopsis glabrata*], *Cryptococcus neoformans*, and *Cryptococcus bacillisporus*) and four species of dimorphic fungi (*B. dermatitidis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii*) were suspended in physiological saline and diluted to yield an inoculum containing ca. 10^5 cells per ml. Arthrospores from *Coccidioides immitis*, conidia from *Aspergillus fumigatus*, and sporangiospores from *Rhizopus rhizopodiformis* were obtained from 1- to 2-week-old cultures and

suspended in saline (ca. 10^6 /ml). Female white mice (18 to 20 g, obtained from the National Institutes of Health [NIH] breeding colony) were injected with 0.5 ml of the inocula into a lateral tail vein. The *C. neoformans* cells were also injected into white rats (obtained from Taconic Farms, Germantown, N.Y.). The animals were killed after 2 to 3 weeks, and spleen, lung, liver, and kidney or brain (depending on the fungus) tissues were prepared for histological staining (4). At least six sections were cut from each block; three were stained with Gomori methenamine-silver stain, and the remainder were stained with Masson-Fontana silver stain.

Tissue sections from human mycoses cases. Paraffin-mounted tissue sections prepared from autopsy materials were obtained from Surgical Pathology and Postmortem Service (Pathological Anatomy Department, Clinical Center) of NIH. The tissues were from culturally proven cases of *B. dermatitidis* and *C. neoformans* as well as from histologically diagnosed cases of aspergillosis and mucormycosis. The staining was the same as for the mouse tissue sections.

Masson-Fontana silver stain. Fontana silver solution (8) is made of 10% silver nitrate in aqueous solution (100 ml), ammonium hydroxide, and distilled water (100 ml). The solution is prepared as follows. Ammonium hydroxide is added to 10% silver nitrate solution drop by drop until a faint opalescence appears. This is best accomplished by continuous stirring while adding the ammonia. The odor of ammonia should not be present. The ammoniated silver nitrate solution is mixed with distilled water, filtered, and then stored overnight at room temperature in the dark. The solution remains stable for up to 1 month when it is stored in the dark. The solution should not be reused. The procedure for staining was slightly modified from the Masson method (1, 8) as follows. (i) Place sections in xylol, and then pass through a series of xylol-alcohol to absolute alcohol and through a series of alcohol-water to water. (ii) Wash in distilled water. (iii) Transfer to Fontana silver solution and store in a covered dish in the dark for 18 h. (iv) Wash well in two changes of distilled water. (v) Fix sections in 5% sodium thiosulphate for 5 min. (vi) Wash in tap water for 5 min. (vii) Counterstain in 1% neutral red for 2 min. (viii) Quickly rinse with distilled water. (ix) Dehydrate, clear, and mount in permount.

Staining of fungal cultures with Masson-Fontana silver. Nine yeast-like fungi and four dimorphic organisms were studied. The fungal cultures included *Candida albicans*, *C. tropicalis*, *C. glabrata*, *Cryptococcus neoformans*, *C. bacillisporus*, *C. uniguttulatus*, *C. luteolus*, *C. terreus*, *C. laurentii*, *H. capsulatum*, *B. dermatitidis*, *P. brasiliensis*, and *S. schenckii*. The yeastlike fungi were cultured on malt extract agar slants for 48 h. The dimorphic fungi were first converted into yeast form and maintained on blood-glucose-cysteine agar and brain heart infusion agar for 4 days. A small loopful of cells was placed on a microscope slide with a drop of distilled water, the cell mass was pressed with another slide, and the slides were drawn apart to give a thin preparation. The preparation was air dried before staining.

Other staining for melanin. The brain sections

of mice infected with *C. neoformans* were also stained with the Warthin-Starry silver method modified by Warkel et al. (11). The method is believed to be more specific for melanin than the Masson-Fontana procedure. Unlike the Masson-Fontana method, the Warthin-Starry procedure requires silver nitrate, gelatin, and hydroquinone. The modification of Warkel and his co-workers used a pH 3.2 solution and incubation of the slides in the silver nitrate solution for 30 min.

RESULTS

Fungal cells in animal tissue. All of the sections stained with Gomori methenamine-silver stain showed darkly stained fungal cells with typical morphology of each species. In Masson-Fontana-stained tissues, however, only *C. neoformans* and *C. bacillisporus* cells were stained dark brown to almost black (Fig. 1 and 2). The pigment was on the cell wall and less intensely on the capsular material of the cells. The cytoplasm appeared hyaline or pink due to the counterstain. The cell walls of all the other fungi (Fig. 3) except *S. schenckii* were hyaline. The counterstain was taken up by cytoplasm more vividly in *C. albicans*, showing a pink color. The nucleus of host cells was also pink due to the counterstain. The cell wall of *S. schenckii* was faintly brown (Fig. 4), but the intensity of the color was not comparable with that seen in the cryptococcal cells regardless of the type of host tissues. The shape of cells and the total absence of capsular material in *S. schenckii* were additional differences between this fungus and the cryptococci. The cells of *C. neoformans* stained by the modified Warthin-Starry silver method showed a faintly brown wall with hyaline inside. The extracellular capsule was not detectable by the stain.

Fungal cells in human tissue. Gomori methenamine-silver-stained sections showed deeply stained typical fungal cells of each species. The results of the Masson-Fontana silver staining were identical to those found in mouse tissue. *C. neoformans* was the only pathogen positively stained, showing a reddish brown wall and pink or hyaline cytoplasm (Fig. 5 and 6). The intensity of staining seemed to vary, depending upon the age of the autopsy blocks and the therapy record of the patients (Table 1). The autopsy blocks prepared from the patients were from 5 to 19 years old at the time of sectioning, and the duration of amphotericin B or flucytosine treatment before their death varied from 0 to 109 days. The *C. neoformans* cells in the sections tended to stain weakly when the autopsy block was older than 15 years or when the patients had received the antimycotic treatment for longer than 5 weeks.

Fungal cultures stained with Masson-

FIG. 1 and 2. *C. neoformans* cells in mouse brain ($\times 1,200$). Stained with Masson-Fontana silver stain.

FIG. 3. *B. dermatitidis* cells (see arrow) in mouse spleen section ($\times 1,200$). Stained with Masson-Fontana silver stain.

FIG. 4. *S. schenckii* cells (see arrow) in mouse spleen ($\times 600$). Stained with Masson-Fontana silver stain.

FIG. 5. *C. neoformans* cells in lung section of a patient after 37 days of amphotericin B treatment ($\times 1,200$). Stained with Masson-Fontana silver stain.

FIG. 6. *C. neoformans* cells in a brain section of a patient who received 9 days of amphotericin B treatment ($\times 1,200$). Stained with Masson-Fontana silver stain.

TABLE 1. Tissue sections prepared from humans or animals with cryptococcosis (stained with Masson-Fontana silver stain)

Tissue source	Yr of autopsy	Therapy ^a	Days of therapy	Intensity of staining ^b
Human				
1	1961	A	3	+
2	1965	A	6	+++
3	1967	A	3	+++
4	1968	—	0	+++
5	1974	A, F	9	+++
6	1974	A, F	37	++
7	1975	A	109	++
Mouse				
1	1980	—	0	++++
2	1980	—	0	++++
Rat				
1	1980	—	0	++++

^a A, Amphotericin B; F, flucytosine; —, no therapy.

^b +, Faintly brown; ++, brown; +++, reddish brown; +++++, black to dark brown.

Fontana silver. The only fungal cultures positively stained with Masson-Fontana silver were three *Cryptococcus* species (i.e., *C. neoformans*, *C. bacillisporus*, and *C. laurentii*) plus one dimorphic fungus (*S. schenckii*). There was no difference in the intensity of dark brown color on the cell wall of the three *Cryptococcus* species. Yeastlike cells of *S. schenckii*, however, were stained in light brown, as was the case in vivo (mice).

DISCUSSION

Fontana (3) used ammoniacal silver nitrate in staining spirochetes. Masson (8) adapted Fontana's method to stain melanin and other silver-reducing granules such as those in argentaffin cells. Melanin and polyphenolic compounds are known to combine with silver salts, reducing them to a black metallic state (8). The duration of the immersion in a silver bath is critical for revealing melanin, and Masson (9) recommended 8 h as the maximum time. When the immersion is prolonged beyond 8 h, the reaction loses its specificity for melanin, and other silver-reducing objects will be stained black. The staining results showed that the cells of *C. neoformans* and *C. bacillisporus* in tissue or from cultures grown on malt extract agar stained dark brown when they were immersed in the silver bath overnight (18 h). When they were removed from the silver bath before 8 h, the pigment on the cell wall was faintly brown. Therefore, 18 h of incubation was preferred for differentiating *C. neoformans* and *C. bacillisporus* from other

fungi.

According to Warkel et al. (11), various substances such as Formalin, iron, and lipofuscin also reduce silver prepared by the Masson-Fontana procedure. The Warthin-Starry silver method modified by Warkel et al. revealed *C. neoformans* with a faintly brown wall in mouse tissue. If the cells contained melanin, they would appear black by the method. Since the cells of *C. neoformans* and *C. bacillisporus* cultured on malt extract agar are white to cream in color and still stain dark brown by the Masson-Fontana technique, it is unlikely that the silver-reducing compound of the cell wall is melanin. It is interesting to note that the yeastlike cells of *S. schenckii* were light brown when stained by the Masson-Fontana procedure. Mycelial cultures of *S. schenckii* turn dark with age due to the formation of many deeply brown-walled conidia. Yeastlike cells of *S. schenckii* are dull white to cream in color. It is possible that the yeast form of *S. schenckii* and the cells of *C. neoformans* and *C. bacillisporus* contain a precursor of melanin such as polyphenols.

It is significant that *C. laurentii* was the only other cryptococcus among the four nonpathogenic species tested which reduced silver. The cell wall of *C. uniguttulatus*, which was formerly considered to be a variety of *C. neoformans* (7), did not contain silver-reducing components. These results suggest that *C. laurentii* may be more closely related to *C. neoformans* and to *C. bacillisporus* than is *C. uniguttulatus*. It has been recently shown that the perfect state of *C. uniguttulatus* belongs to the genus *Filobasidium* (6), whereas that of *C. neoformans* belongs to the genus *Filobasidiella* (5).

ACKNOWLEDGMENT

We thank Jose Costa, Head of Surgical and Postmortem Section, Laboratory of Pathology, National Cancer Institute, for supplying tissue sections from autopsy cases.

LITERATURE CITED

1. Bancroft, J. D., and A. Stevens. 1975. Histopathological stains and their diagnostic uses. Churchill Livingstone, New York.
2. Emmon, C. W., C. H. Binford, J. P. Utz, and K. J. Kwon-Chung. 1977. Medical mycology. Lea & Febiger, Philadelphia.
3. Fontana, A. 1912. Verfahren zur intensiver und raschen Färbung des *Treponema pallidum* und anderer Spirochäten. Dermatol. Wochenschr. 55:1003-1004.
4. Kwon-Chung, K. J. 1968. *Gymnoascus demonbreunii* Ajello & Cheng: evidence that it is not the perfect state of *Histoplasma capsulatum* Darling. Sabouraudia 6: 168-175.
5. Kwon-Chung, K. J. 1975. A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. Mycologia 67:1197-1200.
6. Kwon-Chung, K. J. 1977. Perfect state of *Cryptococcus uniguttulatus*. Int. J. Syst. Bacteriol. 27:293-299.

7. **Lodder, J., and N. J. W. Kreger-van Rij.** 1952. The yeasts, a taxonomic study. North-Holland Publishing Co., Amsterdam.
8. **Masson, P.** 1928. Carcinoids (argentaftin-cell tumors) and nerve hyperplasia of the appendicular mucosa. *Am. J. Pathol.* **4**:181-211.
9. **Masson, P.** 1970. Human tumors, 2nd ed. (translated by S. D. Kobernick). Wayne State University Press, Detroit.
10. **Shaw, C. E., and L. Kapica.** 1972. Production of diagnostic pigment by phenoloxidase activity of *Cryptococcus neoformans*. *Appl. Microbiol.* **24**:824-830.
11. **Warkel, R. L., L. G. Luna, and E. B. Helwig.** 1980. A modified Warthin-Starry procedure at low pH for melanin. *Am. J. Clin. Pathol.* **73**:812-815.