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Combined Methenamine-Silver Nitrate and Hematoxylin & Eosin Stain for Fungi in Tissues

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Initial examination of hematoxylin & eosin-stained tissue from a human brain specimen did not reveal the fungi which were seen in subsequent tissue sections stained with methenamine-silver nitrate. Microabscesses seen in the hematoxylin & eosin-stained sections were not apparent in the methenamine-silver nitrate-stained tissue. Staining with methenamine-silver nitrate and counterstaining with hematoxylin & eosin proved excellent not only for detecting fungus cells, but also for revealing their relationship to the host cellular response in this case and in examples of experimental murine coccidioidomycosis and histoplasmosis.

A brain tissue specimen had been sent to us for study with the information that suspected fungus cells had been seen in histopathological sections and a *Chaetomium* species had been cultured from a blood clot. Although species of *Chaetomium* are known to produce toxic metabolites when growing on dead organic substrates (1, 2, 7, 12, 14), their association with living animals has been no more remarkable than that of other airborne saprobic fungi. They have been found on birds (5, 13), in pigs with respiratory problems in common with other microorganisms (9), and in humans as a possible allergen (8). Rippon mentioned three cases of infection in human nails (11). Although we are unaware of any reports implicating *Chaetomium* species as an etiological agent of systemic mycoses in humans, the increasing frequency of opportunistic mycoses justified considering this potential also for *Chaetomium* because it is a common inhabitant of the human environment.

Examination of sections stained separately with hematoxylin and eosin (H & E), periodic acid-Schiff, Gridley, and the Grocott modification of Gomori's methenamine-silver nitrate (GMS) procedures proved frustrating. Many fungus cells were seen in the periodic acid-Schiff-, Gridley-, and GMS-stained sections, but very few were visualized clearly in the H & E preparations. Furthermore, the fungi did not appear to be associated with obvious host cellular response. This unexpected observation prompted us to attempt a combination staining with the special procedures for fungi and counterstaining with H & E. The initial results with this combined stain demonstrated most effectively that the fungus cells were associated with microabscesses in the brain tissue. A search of the literature revealed that Ford et al. (3) mentioned briefly that the GMS-H & E combination was excellent for demonstrating host cellular reaction as well as spores and hyphae of *Aspergillus* in tissue. Because this staining procedure has received little attention to the best of our knowledge, we report here the excellent results obtained not only with the possible (but not confirmed) *Chaetomium* infection, but also with examples of other systemic mycoses.

MATERIALS AND METHODS

In addition to the human brain specimen, histoplasmosis and coccidioidomycosis were chosen as examples in which fungi would be most difficult and easiest to demonstrate, respectively. Lungs were obtained from mice (dba/2 strain, Jackson Laboratories, Bar Harbor, Maine) which had been used in other experiments. The mice had been infected intranasally by methods described previously (6) and had been sacrificed at varying intervals of time postinfection. Samples of mouse lung with macroscopic lesions were fixed in 10% neutral buffered Formalin and embedded in paraffin. The brain tissue from the human case had been received fixed in Formalin and was embedded in paraffin. Sections for staining were cut at a 5-μm thickness.

Because there have been many variations in the procedure for GMS staining of tissue sections, our method for GMS counterstained with H & E will be presented in detail, although it is basically a combination of procedures reported from the Armed Forces Institute of Pathology (10). The Gridley staining followed by H & E was inferior for differentiating fungi from host cells and is not described. Metal contamination was avoided by using only chemically clean glassware, including glass baskets for multiple slides.

Solutions.

1. Chromic acid, 4% aqueous.
2. Sodium bisulfite, 1% aqueous.

3. Stock methenamine-silver nitrate, made as separate solutions and then mixed.
   a. Silver nitrate, 5% aqueous.
   b. Hexamethylenetetramine, 3% aqueous.
   c. Mix in proportions of one volume of 5% silver nitrate to 20 volumes of 3% hexamethylenetetramine.
   d. A white precipitate forms but dissolves immediately with shaking. The clear solution remains usable for months when stored in a dark bottle at 4°C.
4. Working methenamine-silver nitrate, made fresh, used once, and discarded.
   a. Stock methenamine-silver nitrate, 125 ml.
   b. Distilled water, 125 ml.
   c. Sodium borate, 5% aqueous, 10 ml.
5. Gold chloride, 0.1% aqueous.
6. Sodium thiosulfate, 2% aqueous.
7. Hematoxylin.
   a. Harris hematoxylin solution (Harleco), 250 ml.
   b. Glacial acetic acid, 5 ml.
8. Stock eosin, eosin Y, 1% in 95% ethanol.
9. Stock phloxine, phloxine B, 1% aqueous.
10. Eosin-phloxine working solution.
    a. Stock eosin, 50 ml.
    b. Stock phloxine, 5 ml.
    c. Ethanol, 95%, 390 ml.
    d. Glacial acetic acid, 2 ml.
11. Acid alcohol.
    a. Ethanol, 70%, 500 ml.
    b. Hydrochloric acid, concentrate, 5 ml.
12. Ammonia water.
    a. Ammonium hyroxide, 58% aqueous, 1 ml.
    b. Distilled water, 500 ml.

Procedure

1. Deparaffinize and hydrate to water.
2. Chromic acid, 1 h.
3. Wash in running tap water, 1 min.
4. Sodium bisulfite, 1 min.
5. Wash in running tap water, 5 to 10 min.
6. Rinse in four changes of distilled water.
7. Methenamine-silver nitrate working solution, freshly mixed.
8. Incubate at 58 to 60°C until sections turn golden brown. Inspect after minimum of 1 h. Desired end point usually 2 to 3 h.
9. Rinse with six changes of distilled water.
10. Gold chloride, 4 min.
11. Rinse briefly by dipping several times in distilled water.
12. Sodium thiosulfate, 4 min.
13. Wash in running tap water, 4 to 5 min.
14. Hematoxylin, 30 min.
15. Rinse in running tap water, 30 s.
16. Differentiate by a few quick dips in acid alcohol.
17. Rinse briefly in tap water.
18. Dip in ammonia water until blue.
19. Wash in running tap water, 5 to 10 min.
20. Check differentiation microscopically for distinct nuclei with blue to blue-black color, light to colorless background, and black fungi. Repeat steps 16 to 19 until differentiation achieved.
21. Eosin-phloxine working solution, 30 to 60 s, depending on depth of counterstain desired.
22. Dehydrate, clear, and mount.

Histopathological sections were photographed with a Nikon model AFM microscope with Kodak Ektopan plate film.

RESULTS

Comparisons of results with each staining procedure were made by staining successive tissue sections with H & E, GMS with H & E counterstain (GMS-H & E), and GMS with light green counterstain (GMS-LG). The corresponding field in each tissue section was located by using the corner of the tissue as a reference point and coordinates obtained with a graduated mechanical microscope stage.

Fungus cells in sections from the human brain specimen were recognized readily with the GMS-LG and GMS-H & E staining procedures. Host tissue reactions could not be determined with GMS-LG. In contrast, the GMS-H & E procedure stained fungus cells equally well and, in addition, revealed that these were located in microabscesses (Fig. 1). Details of the host tissue response with GMS-H & E were slightly less satisfactory than with H & E alone. The H & E preparation was least effective because the fungi stained poorly with eosin only. In fact, most fungus cells were found only by careful examination of the microabscesses under high magnification after determining from the GMS preparation that fungus cells were present.

Results with experimental murine coccidioidomycosis and histoplasmosis are illustrated in Fig. 2 and 3, respectively. The endospores escaping from the ruptured spherule were readily apparent with H & E staining (Fig. 2A) but were even more obvious and striking with GMS-H & E (Fig. 2B). The early granulomatous response was apparent with both these procedures but was inapparent with GMS-LG (data not shown). The GMS-H & E staining procedure was less effective with histoplasmosis (Fig. 3A and B). Nevertheless, the yeast cells were more apparent with GMS-H & E than with H & E (data not shown), although they were less obvious than with GMS-LG (Fig. 3C). It should be noted that the black-stained yeast cells seen microscopically in color contrasted more sharply with the dark blue nuclei of tissue cells than is apparent in the black and white photographs. The primarily mononuclear, pneumonic infiltrate was obvious in the GMS-H & E preparation but could not be read with GMS-LG staining.

DISCUSSION

This attempt to improve methods for visualizing fungi in tissues was initiated because of our difficulty with the human brain specimen. Fungus cells were not seen with certainty in sections

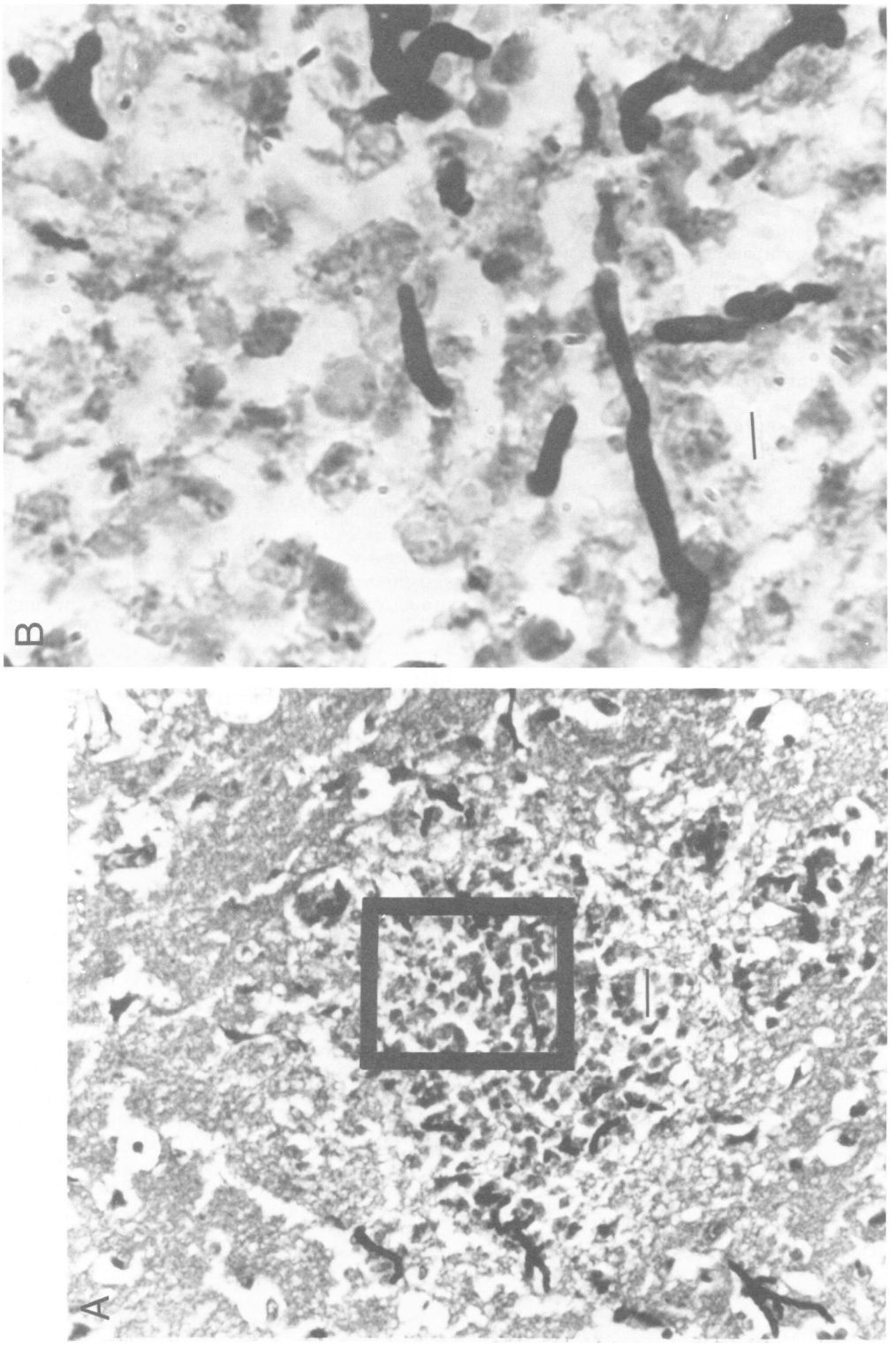

FIG. 1. *Tissue section from human brain stained with GMS-H & E showing a microabscess containing readily visible fungus elements. (A) Low magnification (bar = 40 μm); (B) high magnification of outlined area in A (bar = 10 μm).*

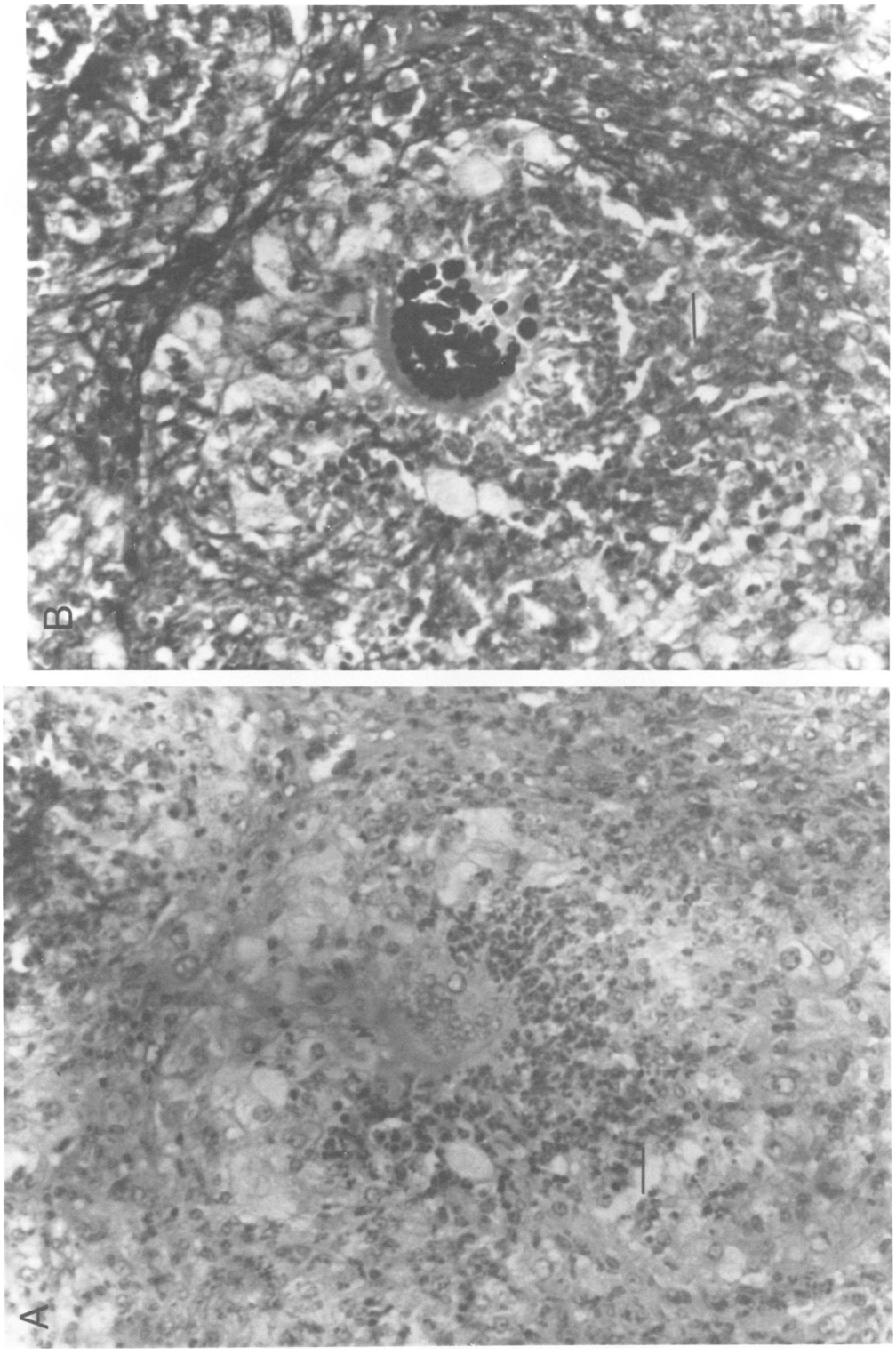

FIG. 2. *Tissue section from lung of mouse infected intranasally with C. immitis (bars = 10 μm): (A) stained with H & E; (B) stained with GMS-H & E.*

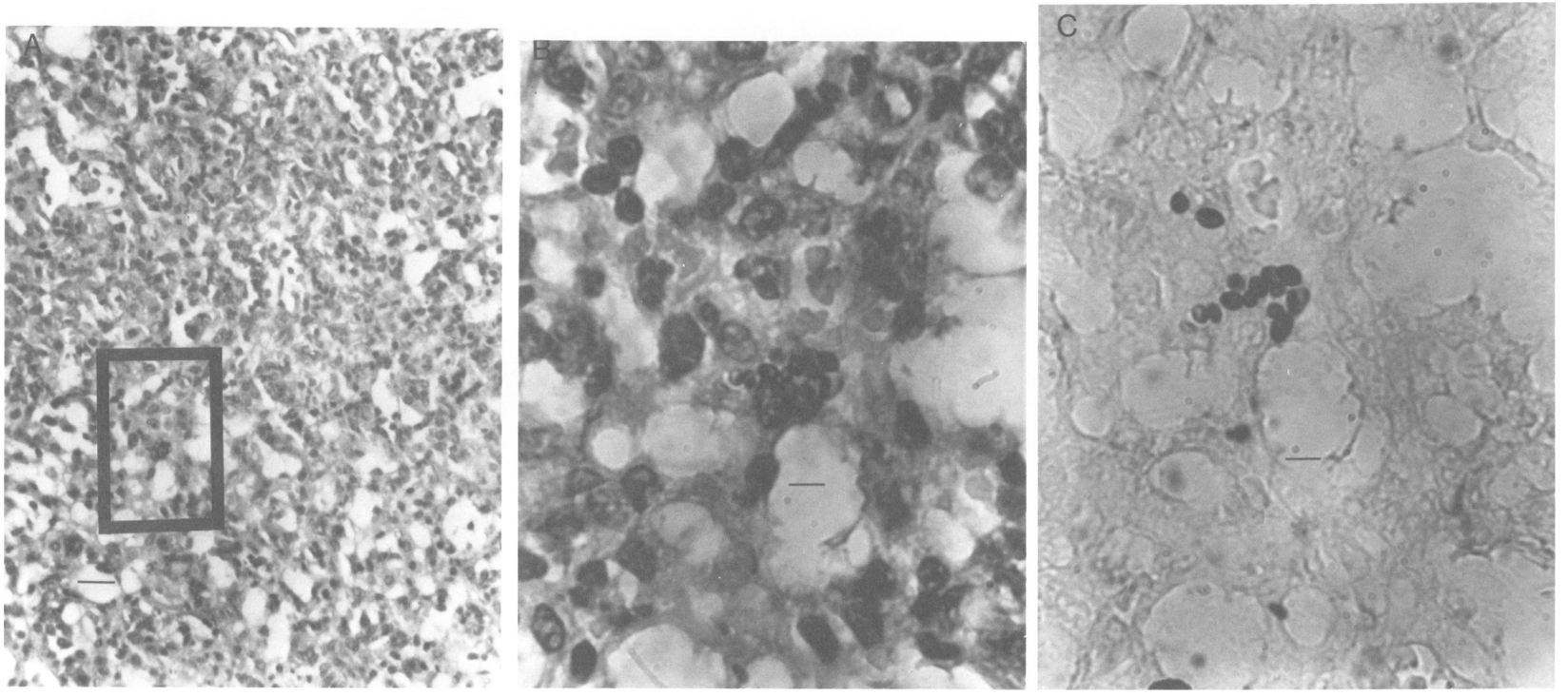

FIG. 3. *Tissue section from lung of mouse infected intranasally with H. capsulatum: (A) stained with GMS-H & E (bar = 40 μm); (B) high magnification of outlined area in A (bar = 10 μm); (C) microscopic field corresponding to that in B but photographed after GMS staining (bar = 10 μm).*

stained with H & E during the initial examination, but they were very obvious in the GMS sections. It was only after matching microscopic fields in serial sections stained alternately with the several procedures that we could demonstrate fungi in H & E preparations and then only with difficulty and some degree of uncertainty. In this particular specimen, the fungi stained only with eosin and no nuclei were apparent. Hence, there was virtually no differential staining from the host cells. A similar problem was encountered when Gridley preparations were counterstained with H & E (data not shown), comparable to Rippon's (11) observation that the light red color of fungus cell walls with periodic acid-Schiff was not differentiated sharply from host tissue components when the periodic acid-Schiff-H & E combination was used. In contrast, fungus cells were observed without difficulty when the tissue was stained with GMS-H & E. The failure of H & E staining alone to clearly differentiate this fungus from host cells raises the question whether the same problem might occur with other cases of opportunistic mycoses and a diagnosis might be missed for lack of satisfactory staining. In this case we do not know whether the fungus seen in tissue was *Chaetomium* because it had not been cultured from the brain specimen. Furthermore, information provided us concerning recovery of *Chaetomium* from the blood clot did not eliminate the possibility that this could have been a contaminant. Therefore, we do not believe on the basis of the information available to us that *Chaetomium* should be included as one of the fungi causing opportunistic systemic mycoses.

Nevertheless, there were fungus cells in the brain specimen, and these could have been overlooked too easily in sections stained only with H & E. These observatios strongly support a recommendation that routine screening of tissue sections stained with H & E must be supplemented by additional special staining procedures whenever mycotic infection is a possibility. If only a single procedure were to be used, the GMS-H & E combination would be the most suitable in our opinion. It has proven satisfactory when fungus cells were not obvious with H & E staining, as with the brain specimen reported here, providing an example in which the etiology could have been missed with an H & E preparation alone. In addition, the GMS-H & E procedure was excellent for demonstrating endospores and spherules of *Coccidioides immitis* and was better than H & E alone for detecting the yeast cells of *Histoplasma capsulatum*. The GMS-LG staining was still the best for revealing fungus cells with startling clarity, but this method has the disadvantage of not showing the relationship of host tissue response to infecting organism. In our opinion, an excellent routine procedure when mycotic infection is included in a differential diagnosis would be staining with the GMS procedure, mounting with nonpermanent fluid (e.g., glycerin-water, 50%, vol/vol), examining microscopically for the presence of fungi, and then counterstaining with H & E. Thus, maximum detection of fungi would be combined with relating fungus cells to the host cellular response.

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