A Non-toxic Method for the Demonstration of Gliosis

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Abstract. Neuropathology laboratories have traditionally relied upon the Holzer method for demonstration of gliosis. However, concerns about the toxicity of aniline oil have markedly reduced the application of this method in recent times. Immunostains for glial fibrillary acidic protein (GFAP) are excellent for showing gliosis in grey matter but cannot distinguish normal from abnormal astrocytes in the white matter. The traditional phosphotungstic acid hematoxylin (PTAH) method stains myelin as well as glial fibers and, thus, is not useful for recognizing areas of gliosis. Here we present a method for the routine demonstration of gliosis based on a modification of Mallory’s PTAH method. The staining of myelin is eliminated by increasing the concentration of potassium permanganate to 5% (from 0.25–1% in traditional methods). The use of aminoisobutylamine adhesive ensures that the sections do not detach during the procedure. Areas of gliosis stand out against a pale background because only abnormal astrocytes and their processes are stained, both in grey and white matter. The method minimizes the use of toxic chemicals and can be completed within an eight hour work day in any routine neurohistology laboratory, using formalin fixed, paraffin-embedded tissue.

Key words: Astrocytes; GFAP; Gliosis; Holzer; Methods; Myelin; PTAH.

INTRODUCTION

The common response to diverse forms of injury to the central nervous system is proliferation of astroglial nuclei, accompanied by hypertrophy and altered composition of the astrocytic cytoplasm. The latter changes, although incompletely understood, have long been exploited by neuropathologists to demonstrate gliosis.

The accumulation in the astrocytic cytoplasm of glial fibrillary acidic protein (GFAP) is a prominent component of the processes of reactive gliosis. Immunohistochemical techniques with antibodies against GFAP are sensitive and specific for the demonstration of gliosis in the grey matter, where GFAP is not normally expressed. However, these techniques do not work well in the white matter, where astrocytes constitutively express GFAP.

The traditional Holzer method is able to detect gliosis in both grey and white matter (1). However, its utilization has been curtailed by recent awareness of the toxicity of aniline oil. Aniline oil is a skin and eye irritant. Inhalation or ingestion of aniline oil is poisonous as it forms methemoglobin, resulting in depression of the central nervous system and prolonged anoxemia; minimal exposure results in red cell hemolysis, followed by stimulation of bone marrow. Long-term exposure may affect the liver, resulting in jaundice, and has been associated with malignant bladder growths (2).

Although Mallory’s phosphotungstic acid hematoxylin (PTAH) method stains reactive astrocytes, the usefulness of this procedure to demonstrate gliosis is diminished by its simultaneous staining of myelin sheaths in a similar, but not identical, hue. Because of this lack of differential staining textbooks recommend the Holzer over the PTAH technique (3). We sought modifications of the PTAH method which would eliminate the myelin staining while retaining the dye binding to reactive astrocytes.

MATERIALS AND METHODS

Modified PTAH for Glia

Brains were fixed in 20% neutral buffered formalin for a minimum of one week prior to gross sectioning. Tissue blocks were processed and paraffin-embedded. Sections cut at 8 micrometers were mounted on aminoisobutylamine (Fluka Chemical Corp., Ronkonkoma, NY; product number 09324) slides (4) and dried overnight in a 50°C hot air oven. The use of a strong adhesive is critical to the success of this method. Solutions were prepared as directed in Table 1.

Staining Procedure

Deparaffinized sections were taken to water and mordanted in Zenker’s-acetic fluid (i) at 50°C for 60 minutes. Slides were then gently washed in running tap water for 15 minutes and oxidized in Lugol’s iodine (ii) for 15 minutes. Iodine staining was bleached by placing slides in several changes of 95% ethanol for a minimum of 60 minutes. Following the bleach, the slides were rinsed in three changes of distilled water, then oxidized in 5% acidified potassium permanganate (iii) for 5 minutes. Without rinsing, drained slides were placed in 5% oxalic acid (iv) for 5 minutes. Again, without rinsing, drained slides were placed in PTAH stain (v) for 60 minutes at room temperature followed by 60 minutes at 50°C. After 2 hours staining, slides were individually drained of excess PTAH stain, quickly dehydrated in two changes of absolute alcohol, cleared in xylene and mounted with Permount. It should be noted that the staining intensity of a freshly stained tissue section does fade on storage.

Several modifications of this staining procedure (see Table 2) were performed to isolate the critical step(s) in achieving optimal results.

Paraffin sections were also immunostained with Vectastain (Vector Labs, Burlingame, CA) ABC technique using DAKO-
TABLE I
Solutions for Use in the Modified PTAH Stain for Gla.
All Reagents, Other than those Specifically Noted, are
Obtained from Fisher Scientific, Pittsburgh, PA.

(i) Zenker’s-acetic fluid
   Stock: Mercuric chloride 5.0 g
   Potassium dichromate 2.5 g
   Sodium sulfite 1.0 g
   Distilled water 100 mL
   Prepare fresh before use:
   To: 9.5 mL Zenker’s stock
   Add: 0.5 mL Glacial acetic acid

(ii) Lugol’s iodine: Iodine 1.0 g
    Potassium iodide 2.0 g
    Distilled water 100 mL

(iii) Acidified potassium permanganate
    To: 50 mL of freshly prepared 5% potassium
    permanganate
    Add: 0.5 mL of 3% sulfuric acid

(iv) 5% Oxalic acid

(v) PTAH staining solution
    Hematoxylin 0.1 g
    (Chroma 5B 535 C.I. 75290 Roboz Surgical
    Instrument Co., Inc., Washington, DC)
    Phosphotungstic acid 2.0 g
    1.79% Potassium permanganate 1.0 mL

Dissolve the hematoxylin in 50 mL of distilled water. Dissolve
the phosphotungstic acid in 50 mL of distilled water, using gentle
heat if necessary. When completely cool, combine and mix well.
To this combined solution, add 1 mL of 1.79% potassium per-
manganate and mix well. Let stand at room temperature over-
night before using. Stored in a brown bottle, the stain is stable
at room temperature for 4–6 weeks. Filter before use. Do not
re-use the PTAH staining solution; heat alters the ratio of red : blue components producing unsatisfactory results.

PATTS rabbit anti-cow GFAP antibody (DAKOPATTS, Glo-
strup, Denmark) in a 1:800 dilution overnight at 4°C, as previ-
ously described (5).

RESULTS

This modified PTAH method selectively demonstrated
abnormal astrocytes. Blue bundles of glial fibers criss-
crossed the perikaryon without filling it, and extended
along the processes of reactive and neoplastic astrocytes
but not of normal white matter astrocytes (Fig. 1). In
contrast, GFAP immunostaining did not discriminate be-
tween reactive or neoplastic and white matter astro-
cyes—in all these cells perikarya and processes showed
fairly diffuse immunoreactivity. The modified PTAH
stained blue fibrin within blood vessels and, inconsis-

ding 0.5% potassium permanganate; glia are stained but the con-
current staining of myelin does not allow visual contrast of the
area of gliosis (×3). (C) Modified PTAH using 5% potassium
permanganate blocks myelin staining, thus allowing abnormal
glia to be clearly identified against a pale background (×3).

Fig. 1. Cavernous angioma of medulla interrupting olivo-
cerebellar fibers illustrating the effectiveness of 5% potassium
permanganate in eliminating myelin staining. (A) SCR stain
demonstrating myelin (×3). (B) Traditional PTAH method us-

Fig. 2. GFAP immunostaining and PTAH demonstrate different astrocytic populations. The staining pattern in the internal capsule in this case and in control cases was identical. Thus, this case was chosen to illustrate in a single section the absence of staining of "normal" white matter astrocytes by the modified PTAH, even if the internal capsule may not be strictly normal in Huntington's chorea. Central Panel: Low power of GFAP immunostained basal ganglia from a case of Huntington's Chorea for orientation (×3). (A) GFAP demonstrates: (i) both abnormal glia in the putamen (×315) and (ii) normal glia in the internal capsule (×315). (B) Modified PTAH demonstrates: (i) only abnormal glia and their processes in the putamen (×315); (ii) with no staining of normal glia in the internal capsule (×315).

ently, neuronal and glial nuclei. Myelin staining was completely suppressed, thus demarcating regions of gliosis (Fig. 2). Other tissue elements, notably neurons, were demonstrated by this method in contrasting salmon color.

The modified PTAH method intensely stained the core of Rosenthal fibers, leaving a thick hyaline rim unstained. Rosenthal fibers showed no GFAP immunoreactivity, although they were often surrounded by a GFAP-positive ring (Fig. 3).

Comparison of the results obtained with this PTAH
method with those produced by methodology variations is presented in Table 2.

TECHNICAL COMMENTS

Post-mordanting of formalin fixed paraffin sections with Zenker's-acetic fluid enhances the PTAH staining (6). The residual mercury deposits are removed by treating sections with Lugol's iodide, thus forming mercuric iodide which is then soluble in 95% alcohol, a preferable bleaching reagent to traditional sodium thiosulfate, which impairs PTAH staining (7, 8). This sequence also aids differential staining (9). Our test panel results showed that acidification of 5% potassium permanganate enhanced reactive astrocyte staining.

Staining results using dilute concentrations of potassium permanganate, as traditionally recommended (10), resulted in myelin being more lightly stained rather than blocked. A higher concentration of potassium permanganate was required to block myelin staining, probably acting by oxidizing unsaturated lipids.

DISCUSSION

The component of reactive astrocytes responsible for bonding with PTAH components has not been identified, but it is unlikely to be GFAP, since this protein is equally well expressed in normal white matter astrocytes not demonstrated by PTAH. Furthermore, the subcellular distribution in astrocytic perikarya and Rosenthal fibers of the component stained by PTAH is different from GFAP. It remains to be investigated whether zB-crystallin, a major protein constituent of Rosenthal fibers (11), is the component recognized by PTAH in astrocytes. In any case, our modified PTAH method offers advantages over GFAP immunostains in demonstrating areas of gliosis involving grey or white matter.

The demonstration of gliosis achieved by this method is comparable to that of the Holzer stain, but does not require the use of toxic aniline oil. Using formalin fixed, paraffin-embedded tissue, this modified PTAH stain can be completed within an eight hour work day in any routine neurohistology laboratory.

REFERENCES


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