Acta Histochemica 116 (2014) 292–296

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Short communication

Quick staining technique for myeloperoxidase using potassium iodide and oxidized pyronine B

Wan-Xin Chen, Hong-Lin Zhu*, Mei Xue, Hao Zhou, Fei Zhao, Ni Yan, Yan Chen

Department of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

ARTICLE INFO

Article history:
Received 25 June 2013
Received in revised form 31 July 2013
Accepted 1 August 2013

Keywords:
Cytochemistry
Methodology
Myeloperoxidase
Potassium iodide
Pyronine B

ABSTRACT

Myeloperoxidase (MPO) staining has been important for the cytomorphological diagnosis and classification of leukemia. A novel staining method for MPO and its clinical application are presented in the report. Pyronine B (PyB), serving as a chromogenic reagent, was pre-oxidized to obtain stable oxidized Pyronine B solution. The MPO working solution for oxidized pyronine B method consisted of phosphate buffer solution, potassium iodide (KI) solution, and oxidized Pyronine B solution. The positive products of the oxidized Pyronine B method of MPO staining were vibrant red particles located in cytoplasm and the nucleus was stained bluish green. Bone marrow smears from 229 patients with acute leukemia or with grossly normal bone marrow were stained by both oxidized Pyronine B method and the conventional Washburn benzidine staining and a comparison revealed no significant difference in the positive detection rate between the two techniques. The new method eliminates the influence of the varying amount of H_2O_2 on MPO staining. With this method, the reagents were more stable and the staining procedure was simple and time-saving. This MPO staining technique is a better alternative than the conventional benzidine-based methods.

Introduction

Although numerous methods such as flow cytometry, molecular hybridization, chromosome and gene detection can be employed for the diagnosis and classification of leukemia, cytomorphological and cytochemical staining remain the main methods and in particular, myeloperoxidase (MPO) staining is particularly important (Borowitz et al., 2008; Al-Seraihy et al., 2009; Tan et al., 2009; Zhao et al., 2010).

For a long time, benzidine has been used as a chromogenic reagent for MPO staining (Platt, 1979; Ghosh et al., 2003; Casal and Orós, 2007; Tan et al., 2009; Singh et al., 2011). Since benzidine and its derivatives are potentially carcinogenic (Sheibani et al., 1981; Iizuka and Murphy, 1985; Josephy, 1985; Greer et al., 2004; Latger-Cannard et al., 2010), many laboratories employ less toxic chromogenic reagents, such as 3,3′-diaminobenzidine (DAB) (Sheibani et al., 1981; Angermüller et al., 2009; Hu et al., 1993; Jain et al., 1987; Scott et al., 1993; Shibata et al., 1985; Beutler et al., 1995), 3-amino-9-ethyl carbazole (Shibata et al., 1985; Beutler et al., 1995; Krieg et al., 2000; Greer et al., 2004; Pan, 2006), 3,3′,5,5′-tetramethylbenzidine (Liem et al., 1979; Mesulam et al., 1980), 4-chloro-1-naphthol (Elias, 1980; Greer et al., 2004), and alpha-naphthol/pyronine (Latger-Cannard et al., 2010) instead of benzidine. Moreover, the International Committee for Standardization in Haematology (ICSH) recommended three MPO staining methods to replace benzidine techniques (Scott et al., 1993; Shibata et al., 1985). Some scholars tried to use 4-chloro-1-naphthol, tetramethylbenzidine, 4-aminoantipyrine and α-naphthol substrate for the staining, but found that they were no better than benzidine for the MPO staining (Zhao, 2006). What is more, the derivatives of benzidine might also be carcinogenic (Iizuka and Murphy, 1985).

Nevertheless, the bezindine staining has been the most commonly used method for MPO staining. Currently, many laboratories, including ours, are still employing the method (Tan et al., 2009; Singh et al., 2011). Several recently published monographs still describe the benzidine method for MPO staining (Lu and Gong, 2008; Wu, 2009; Cui, 2010; Zhou, 2010; Han et al., 2010). Two reasons may explain the reluctance to abandon the benzidine method. First, the positively stained particles with the benzidine method are clear and easy to identify and second, Wright-Giemsa counterstaining used in the method renders the blasts easy to identify.

Pereira (1984) introduced the potassium iodide (KI) method for the staining of MPO, and due to the non-toxic nature of the reagents used in the method, soon became popular. Some scholars made efforts to improve the technique (Chen, 1987; Wang and

* Corresponding author at: Department of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.
E-mail address: zhuhlin68@163.com (H.-L. Zhu).

0065-1281/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.acthis.2013.08.001

Wang, 1989; Chen et al., 2002), but the results were not very satisfactory (Wang and Wang, 1989). We, when trying to use different new chromogenic reagents to replace Leishman's stain used in the Pereira method, found that Pyronine B could better visualize MPO. On the basis of this finding, we developed a novel Pyronine B-KI method by artificially pre-oxidizing PyB to achieve optimal oxidization. The new method is time-saving and sensitive since addition of H_2O_2 is not necessary during the preparation of the working solution and the positive products are presented as vibrant red particles. Like a number of other reports on the modified MPO staining techniques (Sheibani et al., 1981; Iizuka and Murphy, 1985; Tan et al., 2009; Latger-Cannard et al., 2010), this study compared the new method with benzidine techniques (Washburn staining method in this study) (Platt, 1979) in terms of the staining result of 229 smears from the subjects with acute leukemia and those with grossly normal bone marrow.

Materials and methods

Study subjects

A total of 229 specimens of bone marrow were from 37 cases of acute lymphocytic leukemia (ALL), 149 patients with acute myeloid leukemia (AML), and 43 subjects with grossly normal bone marrow. The 149 AML patients, according to the FAB classification, included 38 cases of AML-M1, 42 cases of AML-M2, 25 cases of AML-M3, 19 cases of AML-M4, and 25 cases of AML-M5. Each smear was stained to show MPO using both our oxidized Pyronine B method and the Washburn benzidine method. Two hundred nucleated cells were counted and the percentages of MPO-positive cells/the total nucleated cells were calculated. Informed consent was obtained from all patients before the study and the study was approved by the Ethical Committee of Union Hospital of the Tongji Medical College, Wuhan, China.

Chemical reagents

Wright's stain, Giemsa's stain, and methyl green were obtained from Shanghai SSS Reagent Co. (Shanghai, China). Benzidine, Pyronine B, and Pyronine G were purchased from Shanghai Chemical Reagent Procurement supply station subpackage plant (Chroma, Standard Fluka). Potassium iodide (KI) and hydrogen peroxide were derived from Wuhan Chemical Reagent Factory (Wuhan, China).

Preparation of reagents

Buffered formaldehyde–acetone solution was made by mixing 1/15 mol/L Na_2HPO_4 11.25 mL, 1/15 mol/L KH_2PO_4 18.75 mL, acetone 45 mL, and 40% formaldehyde 25 mL. The concentration of phosphate buffered solution was 0.67 mol/L (pH = 5.8). For the preparation of the KI solution, 500 mg potassium iodide was dissolved in 50 mL distilled water and then stored in a brown glass bottle at room temperature or 4 °C (The solution is good for 1 year). To prepare oxidized Pyronine B solution, 250 mg pyronine B was completely dissolved in 50 mL 50% ethanol; then, 50 μL 30% H_2O_2 was added to the ethanol and then stored in a brown glass bottle at room temperature or 4 °C (The solution is good for at least 1 year. It is important to use 50% ethanol, 50% methanol as solvent for preparation of oxidized Pyronine B solution to achieve oxidation). Methyl green solution was obtained by dissolving 500 mg methyl green in 50 mL distilled water. Working solution was prepared just before use by mixing 1 mL phosphate buffer solution, 50 μL KI solution, 100 μL oxidized Pyronine B solution and was used within 2 h.

Staining procedures

Air-dried blood or bone marrow smears were fixed by buffered formaldehyde–acetone solution for 30–60 s, and then washed with tap water and dried in air or with filter paper. Working solution was added and allowed to stand for 2 min (the solution should be mixed just before use and used within 2 h). Solution on the surface was discarded without washing, and then the smears were dried on filter paper. The samples were counterstained for 5 s in methyl green solution. After discarding the solution, without washing, the slides were re-dried on filter paper and observed under a light microscope.

The conventional Washburn benzidine method

The film preparation was covered with sufficient 0.3% benzidine solution for 1 min (0.3 g benzidine dissolved in 99 mL of 88–95% ethanol solution plus 1 mL 360 g/L saturated aqueous solution of sodium Nitroprusside, stored in a brown glass bottle). An equal amount of diluted H_2O_2 solution was added (H_2O_2 solution was prepared just before use by adding 50 μL 30% H_2O_2 to 50 mL distilled water). After 4–5 min, the stained smears were washed with tap water. The smears were counterstained by Wright-Giemsa solution (containing 10 mL glycerine, 1.0 g Wright's stain, and 0.5 g Giemsa's stain dissolved in 500 mL methanol) for 10 min. After washing with tap water and drying, the slides were examined under a light microscope.

Statistical analysis

Data were presented as $\bar{x} \pm S$. Paired-samples t test and Wilcoxon Rank-sum test were used to evaluate the statistical differences between the experimental and control groups. A p value less than 0.05 was considered to be statistically significant.

Results

MPO staining

The positive products of MPO staining with the oxidized Pyronine B method were particles of red or dark red color localized in the cytoplasm. The weakly positive staining appeared as a number of bright-red particles situated in the cytoplasm. With strong positive staining, the products were presented as particles or patches with color ranging from dark-red to purple-black or even black color and tended to fill the entire cytoplasm or even cover the nucleus. When counterstained with methyl green solution, the nuclei were evenly stained pale green. When the reaction was negative, cytoplasm was stained light blue and devoid of red particles (Fig. 1A–D). On the other hand, with the Washburn benzidine method, the positive reaction for MPO was shown as brown-black particles located in cytoplasm (Fig. 1E and F), and upon counterstaining with Wright-Giemsa solution, the color of the nucleus turned to purple red.

Results of MPO staining of 229 bone marrow smears

The 229 bone marrow smears were stained for MPO by separately employing the oxidized Pyronine B method and Washburn benzidine technique. Of these, 37 smears of ALL patients and 4 of 25 smears of AML-M5 patients gave negative results with both methods. Only a small fraction of neutrophils in the smears were strongly MPO-positive. The MPO-positive rates in the other 188 cases are shown in Table 1. The comparison of the two methods showed that the average MPO-positive rate with the oxidized Pyronine B method (60.57%) was very close to that with Washburn benzidine