Double staining method: Jones' periodic acid silver methenamine technique plus Masson's trichrome stain

Método de doble tinción: técnica del ácido periódico metenamina de plata de Jones más tinción tricrómica de Masson

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Abstract

The methodical, frequent and simultaneous use of Jones' periodic acid silver methenamine technique and Masson's trichrome stain, prepared independently, have been of great help to us for histopathological diagnosis. We wholly acknowledge that individual results of both techniques are excellent and that they can complement each other. Nevertheless, we intended to perform the two methods in a single histological preparation, expecting to combine their respective tinctorial virtues. After multiple trials it became clear to us that Jones' silver methenamine should be used as the base stain and Masson's trichrome as a counterstain. By adding to Masson's trichrome stain the capacity to visualize basal membranes and basal laminae as well as elastic fibers, a foreseen natural effect of silver methenamine action, the information that the trichrome stain can provide is greatly enhanced. Some of many splendid results that this double stain can provide were found to be in the study and diagnosis of neoplasias and vascular diseases. This new double stain serves as a complement to the two techniques that gave rise to it, in no way is it intended to replace them.

KeyWords: Jones' periodic acid silver methenamine technique; Masson's trichrome stain; Double histologic staining.

Resumen

La práctica metódica, frecuente y simultánea de las tinciones del ácido periódico metenamina de plata de Jones y la tricrómica de Masson, practicadas por separado, ha resultado de gran ayuda para establecer el diagnóstico histopatológico. Reconocemos plenamente que los resultados individuales de ambas técnicas son maravillosos y que pueden complementarse entre sí. No obstante, intentamos montar las dos técnicas en una sola preparación histológica, esperando combinar sus respectivas virtudes tintoriales. Después de múltiples pruebas se encontró que la metenamina de plata de Jones debe usarse como la tinción de base y la tricrómica de Masson como una tinción de contraste. Agregándole a la tinción de Masson la capacidad de visualizar las membranas, láminas basales y las fibras elásticas, un resultado natural predecible por la acción de la metenamina de plata, se enriquece mucho la información que la tricrómica puede brindar. Algunos de los muchos espléndidos resultados que esta doble tinción puede ofrecer se encuentran en el estudio y diagnóstico de las neoplasias y de las enfermedades vasculares. Esta nueva tinción sirve de complemento para las dos técnicas que le dieron origen, de ninguna manera se pretende que las sustituya.

PALABRAS CLAVE: Tinción del ácido periódico metenamina de plata de Jones; tinción tricrómica de Masson; doble tinción histológica.

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BACKGROUND

Recently, an unjustifiable tendency of supporting histopathological diagnoses based primarily or exclusively on results of immunohistochemical studies has been adopted. In doing so, four fundamental facts of pathology practice are disregarded. First, the most important advances in anatomic pathology were made during the first 70 years of the 20th century, with only routine staining and tinctorial histochemistry, while with the help of immunohistochemistry, mainly adjustments were made to the original descriptions and interpretations. Not having any intention to minimize such numerous valuable advances. Second, for immunohistochemical studies to yield its best results, it is necessary planning the application of corresponding antibodies based upon a differential diagnosis oriented towards a probable correct diagnosis, which is always based on studies carried out with routine staining and tinctorial histochemistry. Third, devotees of immunohistochemistry pretend to ignore that this technique can give completely unexpected positive results (such as cytokeratins in multiple myeloma or epithelioid angiosarcoma, or HMB-45 in angiomyolipoma); that by using combinations of multiple and different antibodies that exist in the market you can get results that, even being positive, can be confusing and very difficult or impossible to interpret. Four, immunohistochemistry also has the intrinsic potential to give false positive results (with very concentrated dilutions of antibodies and lack of controls), and deceptively negative results (with poorly preserved tissues affected with autolysis or by using denatured antibodies due to caducity or deficient refrigeration), especially in inexperienced hands. Immunohistochemistry has not replaced traditional histopathological morphology, stained with routine and special stains, in general complements and refines it. However, is also fair to recognize that there are numerous cases in which the results of immunostains are

unappealable for a correct diagnosis, especially when the technique is planned, practiced and interpreted orthodoxly.

Our working group truthfully considers tinctorial histochemistry techniques as a great help to establish definitive diagnoses or, at least, to propose reasonable differential diagnoses. Consequently, over the years we have tried to modify some classical techniques of tinctorial and enzymatic histochemistry:¹ simplifying and/or improving them or looking for other applications.²⁻⁶ Among such projects, we have tried to improve Masson's trichrome stain,⁷ with very little expectation of doing so. However, luckily, we were able to make some changes, which enriched the results that Masson's technique can provide in some applications. The technique proposed here is not a substitute for classical Masson's trichrome stain, to some extent complements it, and the simultaneous use of the two techniques is advisable. Basically, we tried to combine Masson's trichrome stain⁷ with Jones' periodic acid silver methenamine technique,^{8,9,10} in order to accomplish the advantage of merging the main tinctorial properties of each technique in a single histological preparation.

MATERIALS AND METHODS

To practice in a single histological preparation the tinctorial techniques of Masson's trichrome stain and Jones' periodic acid silver methenamine stain, the chemical reagents used were those originally recommended by Luna in 1968 in the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology.¹¹ Thus, the main chemical solutions for Masson's trichrome stain, according to the modified AFIP technique, were: Bouin's solution, Weigert's iron hematoxylin solution, Biebrich scarlet-acid fuchsin solution, phosphomolybdic-phosphotungstic acid solution, aniline blue solution, light green solution and glacial acetic acid solution. The

chemical solutions for Jones' periodic acid silver methenamine technique, in accordance with the modified AFIP technique by Avalone,¹¹ were: periodic acid solution 0.5%, methenamine solution 3%, silver nitrate solution 5%, borate buffer solution pH 8.2, gold chloride solution 0.2%, and sodium thiosulfate (hypo) solution (5%). Likewise, initially the technical procedures recommended by the same Manual¹¹ were followed. Once performed independently both histochemical procedures, with very good results, and after testing several combinations of them, it was found that the basic stain had to be Jones' periodic acid silver methenamine technique, and Masson's trichrome stain should be used as a counterstain. Needless to say, therefore, that counterstaining Jones' periodic acid silver methenamine technique with Harris hematoxylin and eosin is irrelevant for this new method.

Beforehand, in order to obtain good technical results it is of outmost importance to follow some recommendations: a) to process optimally fixed tissues in order to obtain very thin histological sections (2-4 microns); b) to add gelatin to the water bath for good adhesion of sections onto the slides; c) to chemically clean all glassware employed for staining; d) and to utilize good quality laboratory distilled-demineralized water for the preparation of aqueous solutions and second rinses of sections. In order to carry out the combination of both techniques, there were several problems to solve. First, we found that 70-75 °C temperature and 90 minutes incubation time, in silver methenamine solution, for practicing Jones' periodic acid silver methenamine technique was adequate, that is without blackening of all tissue or been so clear as to be obliterated by Masson's trichrome stain. The desired color of structures suitable for staining, such as basement membranes, should be a very dark tobacco color or frankly black (without significant nonspecific background staining, mainly of collagen). However, as a means to obtain adequate results, according to Jones:¹⁰ "sections can be removed

from the silver bath at any time, rinsed in distilled water (at 70-75 °C) and examined under the microscope. If further stain is needed, sections may be replaced in the silver bath. (This procedure can be repeated several times until adequate staining is obtained. Contrariwise), if the slides became overstained, an extremely diluted solution of potassium ferricyanide may be used to uncover the sections to the desired intensity". Second, it was indispensable to determine an appropriate temperature and incubation time for Masson's trichrome stain, which, by requiring a second incubation in a mordant such as Bouin's solution, did not detach the histological sections off the slides. The classic method advises to incubate sections overnight in Bouin's solution at room temperature (18 °C), which is satisfactory to us; or, as an alternative, recommends incubating at 56 °C for 60 minutes. Instead, we concluded that incubating at 90 °C for 60 mins was quite suitable for our purposes. With the repeated practice of the procedure most problems were solved, and the results obtained seemed to be excellent, beautiful as well as useful.

RESULTS

For the initial experiments we used sections of normal rat kidney and different types of normal and diseased human tissues. Particularly helpful resulted those tissues containing different types of vessels (**Figures 1,2 and 3**), because of their richness in smooth muscle and elastic fibers (*vide infra*). In the regular practice of anatomic pathology, the results of double staining were very useful in those cases which presented mixtures of different types of epithelial and mesenchymal tissues, for example: carcinoma in situ (**Figure 4**) versus infiltrating carcinoma (**Figure 5**) or malignant tumors of myoepithelial cells (**Figure 6**). Moreover, the results were splendid in all vascular diseases, whether congenital (**Figure 7**), inflammatory, reactive, neoplastic (**Figures 8 and 9**) or degenerative. Various degrees of arteriosclerosis (**Figures 10 and 11**) and different

stages of vascular thrombosis (**Figure 11**) became very beautiful and easier to interpret. In lesions that include vessels masked by the pathological process, such as severe inflammation and eosinophilic or tumoral necrosis, double staining was useful because of its property of better revealing remains of smooth muscle, elastic fibers and fibrosis of affected vessels (**Figures 9 and 12**). Such abnormal vessels are better unveiling by double

staining than by simply staining for elastic fibers, because there are many types of vessels which lack elastic fibers in their composition, nonetheless they can retain in their structure basement membrane and basal laminas of smooth muscle, and sometimes even elastic fibers as well.

Figure 1. Longitudinal and oblique sections of normal arterioles. Jones' silver methenamine-Masson's trichrome method.

Figure 2. Cross section of a normal arteriole. Jones' silver methenamine-Masson's trichrome method.

Figure 3. Longitudinal section of a proximal normal lymphatic vessel. Jones' silver methenamine-Masson's trichrome method.

Figure 4. Mammary ductal carcinoma in situ surrounded by elastosis. Jones' silver methenamine-Masson's trichrome method.

Figure 5. Multiple foci of infiltrating squamous cell carcinoma enclosed by basal membrane. Jones' silver methenamine-Masson's trichrome method.

Figure 7. Cross section of a coronary artery with fibromuscular dysplasia. Jones' silver methenamine-Masson's trichrome method.

Figure 6. Prominent foci of myoepithelial cells of a mixed malignant tumor are encompassed by basal membrane. Jones' silver methenamine-Masson's trichrome method.

Figure 8. Continuous basal membranes of endothelial cells encircle the vessels of a lobular capillary hemangioma. Jones' silver methenamine-Masson's trichrome method.

Figure 9. Within necrotic tissue, the lumen of a vein is completely occupied by carcinomatous cells. Jones' silver methenamine-Masson's trichrome method.

Figure 11. Wall of an abdominal arterial aneurysm and part of an organized luminal thrombus. Jones' silver methenamine-Masson's trichrome method.

Figure 10. Oblique and cross sections of pulmonary arteries with different degrees of arteriosclerosis and thrombosis. Jones' silver methenamine-Masson's trichrome method.

Figure 12. Recent thrombosis of multiple reactive vessels immersed within eosinophilic necrosis. Jones' silver methenamine-Masson's trichrome method.

DISCUSSION

The usefulness and beauty of Masson's trichrome stain always seemed unbeatable to us, after practicing it countless times over many years, in normal and diseased tissues. However, after independently and simultaneously using Jones'

periodic acid silver methenamine technique for diagnostic purposes and having also found it of great utility and beauty, we decided to try to combine the tinctorial properties of both techniques in the same histologic section. In the end, the technical problem was not as complicated as we expected to be. After multiple trials we

found that the best way to obtain the desired results was to use Jones' periodic acid silver methenamine technique,^{8,9,10} according to Luna's modification,¹¹ as the base stain and Masson's trichrome stain⁷ as a counterstain. Always in that order, although trying to balance the tinctorial characteristics of each one of them. If the combination of both techniques is used in opposite order, they simply do not work.

It is necessary to recognize that independent results of the two techniques are aesthetically wonderful and of great diagnostic value. Moreover, it is indisputable that the information they provide can be complemented with each other. The main problem is that it is required to mentally integrate the results given by each of the techniques, when practiced separately, in order to obtain good results. It should be realized that when Masson published his technique in 1929,⁷ he did not have access to important information which was obtained eventually from electron microscopy. In such a way that Masson did not have a complete and objective histological knowledge of the existence of basal membranes of tissues [such as those of epithelia, endothelia, myoepithelium, mesothelium and glomeruli (today type IV collagen and laminin)], or of cellular basal laminas (like in smooth and striated muscle cells, Schwann cells, sustentacular cells and nevus cells), which were frequent and erroneously interpreted as reticulum (today collagen type III). The intrinsic chemical characteristics of Masson's trichrome stain lack the capability to stain these structures. However, Jones' periodic acid silver methenamine technique^{8,9,10} perfectly and beautifully stains all basal membranes and basal laminas, because they all have polysaccharides in their composition (**Figures 1,2 and 3**). Precisely, it was Gomori¹² who in 1946 created the silver methenamine solution and the corresponding histological technique, originally devised to stain glycogen and mucopolysaccharides in tissues. However, Gomori's method uses chromic acid

(CrO₃) and not periodic acid (H₅IO₆) as Jones' stain does it,⁸⁻¹⁰ so as to oxidize polysaccharides to produce aldehydes. These important radicals are required to specifically combine with silver methenamine. On the other hand, neither could Masson know that his technique stained perfectly well those cells that have in their structure cytoskeletons rich in intermediate filaments, which for a long time were interpreted as "myogenic cytoplasm". Consequently, if tinctorial attributes of both techniques are intelligently combined in one histological preparation, the possibilities of correctly identifying the characteristics of numerous histological structures, both normal and pathological, are increased. Mainly those counting in their structure with basal membranes and basal laminas, as well as cells endowed with cytoplasm rich in different types of intermediate filaments, which in fact are many (**Figures 1,2 and 3**). In addition, with the double staining technique, elastic fibers are also stained distinctly (**Figure 2**).

It is fair to comment now that the technique described here has been a significant result of our permanent intention to try to modify, improve or simplify classical laboratory procedures.¹⁻⁶ The technique was born without prior knowledge that it had already been described before. In fact, it has not been formally described, as it is not present in any formal book of histological staining methods¹¹⁻¹³ or in any medical journal or available in any pathology textbook. However, it is appropriate and necessary to confess that when reviewing the different applications and variants of the silver methenamine stain described by Gomori,¹² such as Grocott's¹⁴ and Churukian-Schenk's¹⁵ stains, we found that in the original description in 1953 of the technique known as Jones' periodic acid silver methenamine technique, Jones⁹ makes mention that as a possibility of counterstaining he considered Masson's trichrome stain. Thus, Jones⁹ simply said: "*Masson's trichrome is useful for*

its differential staining". Once Jones considered such a possibility, he did not refer to Masson's trichrome again as a counterstain in the rest of his articles, mainly on kidney and glomerulonephritis,⁸⁻¹⁰ probably because its results did not seem useful to him for his own work. In fact, Jones advised^{9,10} contrasting his own stain with Harris's hematoxylin and eosin.

At any rate, this new double staining method will serve as a complement to the two techniques that gave rise to it, in no way was intended to replace them. To us: Masson's trichrome stain and Jones' periodic acid silver methenamine technique will continue to be "the queens of tinctorial staining", as Rodolfo Céspedes Fonseca (1917-1994) used to say about hematoxylin and eosin ("the queen of all stains"). Actually, our contribution to histochemistry should be considered as a minor tribute to the importance of both stains in histopathology and a sincere recognition to three outstanding pathologists: Pierre J. Masson,⁷ György Gömöri¹² and David B. Jones.^{8,9,10}

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