The Congo Red Stain Revisited*

M. TAREK ELGHETANY, M.D.,
ABDUS SALEEM, M.D.,
and KAYE BARR, H.T., A.S.C.P.

Departments of Pathology,
Baylor College of Medicine,
and
The Methodist Hospital,
Houston, TX 77030

ABSTRACT

The Congo red stain has undergone several modifications since it was first used by Bennhold in 1922 in order to increase the specificity for staining amyloid. Most of the laboratories in the United States use the method of Puchtler which uses alkaline Congo red solution. Some of the variables associated with the procedure were investigated by us. Our results showed the following: (1) amyloid showed green birefringence at all levels between 4 to 12 μ thick sections with better visualization of small deposits with increased thickness. Best results were obtained with 8 μ thick sections; (2) omission of the pretreatment with alkaline alcoholic solution of sodium chloride (NaCl) did not affect the sensitivity of the method; (3) the use of polar mounting media had no effect on amyloid and collagen birefringence; (4) 50 percent saturation of the Congo red staining solution with NaCl caused strong staining of collagen, elastic fibers and eosinophilic granules. In addition, collagen showed green birefringence and dichroism and its differentiation from amyloid became difficult; and (5) using the staining solution fully saturated with NaCl, no positive staining was seen with tissues other than amyloid. Collagen and elastic fibers showed red fluorescence which was of less intensity than amyloid. It is our conclusion that the method of Puchtler for detecting amyloid gives better results if the staining solution is fully saturated with NaCl. The pretreatment step may be deleted without compromising the quality of staining. Improved staining of amyloid enhances the specificity of green birefringence, dichroism, and red fluorescence.

Introduction

The term amyloidosis describes a heterogeneous group of diseases character-
lary quaternary structure with electron microscopy. The Congo red stain was first used by Bennhold in 1922 for the detection of amyloid in tissues. Despite several modifications of the stain, the procedure has not been standardized. Variables such as thickness of the section, saturation of the staining solution with sodium chloride (NaCl) and the type of the mounting media could interfere with the quality and specificity of the staining. This work was done to investigate some of the variables to get optimal staining of amyloid.

Materials and Methods

Three cases of systemic amyloidosis, including two cases of primary systemic amyloidosis and one case of myeloma-related amyloidosis, were found in the autopsy files of The Methodist Hospital between 1979 to 1986. A total of 20 samples of different organs, including heart, liver, spleen, kidney, lung, tongue, uterus, prostate, pancreas, peripheral nerves, bowel, and lymph nodes, were included in the study. All specimens were fixed in four percent buffered formaldehyde solution, routinely processed in ethyl alcohol and xylene, and embedded in paraffin. The following staining solution was prepared: alkaline alcoholic solution saturated with NaCl in which two grams of NaCl were dissolved in 20 ml of distilled water followed by 80 ml of absolute alcohol. The solution was alkalinized just before use by the addition of one ml of one percent sodium hydroxide (NaOH). Staining solution: 0.2 g of Congo red* was added to the previous solution.

Staining procedure (modified from Puchtler and Sheehan and Hrapchak was as follows: (1) deparaffinize and hydrate sections through graded alcohol to distilled water; (2) stain with Mayer’s acid hemalum for 10 minutes; (3) wash in three changes of distilled water; (4) stain in freshly alkalinized alcoholic solution of Congo red for 50 minutes; and (5) dehydrate quickly in three changes of absolute alcohol, clear in xylene and mount. In our laboratory, sections are routinely mounted in Harleco. This mounting medium contains the neutral resin ethyl methacrylate.

Sections were examined under light and polarized microscopy with and without red compensation for birefringence and dichroism using American Optical microscope 110 with built-in analyzer and attachable polarizer and red compensator (AO 1153). Fluorescence was examined using Microphot, FX, Nikon.

Objectives, condensers, glass slides, and cover glasses were tested to be strain free as follows: with the light source turned on, glass slides and covers were examined with both polarizer and analyzer in crossed position. The field was completely dark without any light spots.

Dichroism was examined using two polarizing plates as follows; the polarizer and analyzer are placed in crossed position. Either of them is rotated a small angle alternately clockwise and counterclockwise from the crossed position. The background and non-dichroic birefringent objects will brighten symmetrically as one of the polarizing plates is rotated in either direction while a dichroic object will brighten when polarizer is rotated in one direction and darken as it is rotated in the opposite direction.

Variables examined include: (1) thickness of the section: serial sections of 4, 6, 8, 10, 12 μ in thickness were stained with the above procedure and mounted.

---

* C.I. 22120, Sigma Chemical Company, St. Louis, MO.

† EM Diagnostic System Inc., NJ.
in Harleco medium; (2) pretreating the tissue with alkaline alcoholic solution saturated with NaCl before staining (pretreatment step): An 8 µ thick section was pretreated for 20 minutes before staining in the Congo red solution; (3) 50 percent saturation of Congo red solution with NaCl: An 8 µ thick section was stained as previously stated except for using Congo red staining solution which was 50 percent saturated with NaCl, i.e., one g of NaCl instead of two g in the alkaline alcoholic solution; (4) effect of mounting media: Sections of 8 µ thickness were stained using Congo red solution with full and 50 percent saturation with NaCl. The sections were gradually rehydrated in 70 percent alcohol, 50 percent alcohol, and distilled water then mounted in Aqua Mount. This medium contains a high polar synthetic resin, polyvinyl alcohol. Sections were compared with those mounted in the neutral mounting medium; and (5) unstained birefringence and fluorescence: An 8 µ thick section was deparaffinized and mounted unstained in Harleco medium and examined.

For controls, normal tissues were selected from the surgical and autopsy files representing fibrin (thrombi in arterial repair), nerves (sympathetic and peripheral), smooth muscles (prostate, uterus, gall bladder), collagen (fibrocystic disease of breast, Dupytren's contracture, fibrous thickening of pericardium), and elastic fibers (aorta and pulmonary artery from young autopsies). Several sections of the control tissues were examined for each of the variables.

Results

THICKNESS OF THE SECTION

The green birefringence and dichroism were seen at all examined levels between 4 to 12 µ in thickness. Collagen did not stain and showed a white-blue birefringence and no dichroism. With sections, between four and eight µ thick, more intense staining and green birefringence of small deposits were seen with increasing thickness. No appreciable effect on dense deposits was detected. Sections of more than 8 µ in thickness were technically cumbersome and did not add any advantage in detecting amyloid. The white-blue birefringence of collagen was increased with thicker sections.

EFFECT OF THE PRETREATMENT STEP

No appreciable difference was detected in staining or birefringence with or without pretreatment step. Occasional sections showed slight increase in collagen birefringence and slight decrease in Congo red staining of amyloid with no effect on green birefringence if the pretreatment step was used.

EFFECT OF SALT SATURATION

When the Congo red staining solution was used with 50 percent saturation with NaCl, collagen, elastic fibers, eosinophils, and amyloid were stained. Collagen and amyloid showed green birefringence and dichroism. Fibrin, smooth muscles, and nerves did not stain. When the Congo red staining solution was fully saturated with NaCl, no positive staining was seen with tissues other than amyloid. Collagen showed a white-blue birefringence and no dichroism.

EFFECT OF MOUNTING MEDIA

The use of a water soluble polar mounting medium required the sections to be rehydrated and washed in distilled water, resulting in apparent decrease in the intensity of staining of amyloid. Nevertheless, green birefringence and
dichroism of amyloid and the white-blue birefringence of collagen were still detected. When compared with neutral mounting media, polar media showed no difference in birefringence of collagen and amyloid.

**Unstained Sections**

Both unstained amyloid and collagen have a white positive birefringence. With the red compensator, both have purple-blue color when parallel to the slow axis of the red compensator and orange-red when perpendicular to it. Unstained elastic fibers, smooth muscles, fibrin, sympathetic and peripheral nerves did not show birefringence.

**Fluorescence**

The Congo red powder showed red fluorescence. Unstained collagen and elastic fibers had white-blue fluorescence. Unstained amyloid gave blue fluorescence. Congo red-stained amyloid had a bright red fluorescence. Collagen and elastic fibers stained with Congo red solution fully saturated with NaCl had white-blue fluorescence. With the use of 50 percent saturation of NaCl in the staining solution, elastic fibers and separate collagen bundles showed red fluorescence which was of lesser intensity than Congo red stained amyloid.

**Discussion**

The use of the Congo red stain is still the mainstay for the identification of amyloid in tissues. The method most commonly used is the alkaline Congo red described by Puchtler. This method required pretreating tissues with alkaline alcoholic solution saturated with NaCl followed by staining in alkaline alcoholic solution of Congo red saturated with NaCl. Amyloid stains red and shows green birefringence, dichroism, and red fluorescence. Neural tissue, collagen, exogenous polysaccharide material such as vegetable material and cotton fibers are stained by Congo red and show green birefringence. Eosinophilic granules, elastic fibers, and fibrin are congophilic with no green birefringence. Our results confirm that saturation of the staining solution with NaCl is an important step in the staining technique to increase the specificity of the method. The current authors agree with Carson and Kingsley that with full saturation of the staining solution with NaCl, no false positive staining, green birefringence and dichroism was seen and collagen showed white-blue birefringence. Using 50 percent saturation with NaCl introduced false positive staining of elastic fibers, collagen and eosinophilic granules. In addition, collagen showed green birefringence and dichroism, and its distinction from amyloid became impossible. The addition of salt had been known to have an effect on the staining properties of some dyes. The binding of Congo red to amyloid occurs by non-ionic bond. The ionizing groups of amyloid exert a repelling force against the anionic Congo red. This repulsive effect is neutralized by the addition of NaCl allowing the linear amyloid molecule to align itself along the filaments of amyloid.

It had been suggested that the hydrogen bonding is the major way of linking amyloid to Congo red. However, dye bath manipulation indicates that a combination of van der Walls forces, hydrophilic and hydrogen bonding are responsible for the dye-amyloid binding. The red fluorescence had been described to be specific for Congo red stained amyloid. Unstained collagen and elastic fibers have white-blue fluorescence. False red fluorescence of collagen and elastic fibers occurs with false positive
staining. The intensity of the false red fluorescence was less than with amyloid. The red fluorescence may be a function of the Congo red stain itself since Congo red powder had red fluorescence. Wolman and Bubis\textsuperscript{24} reported that the green birefringence of amyloid was only detectable in tissue sections between five and 10 \( \mu \) thick.

In this study, green birefringence was seen at all levels between four and 12 \( \mu \) thick. Increase in the thickness between four and eight \( \mu \) was accompanied by increase staining and green birefringence of small amyloid deposits but had no effect on heavy deposits. Sensitivity was not increased with section of more than eight \( \mu \) in thickness. The eight \( \mu \) thickness is the one used in our lab and gives excellent results. Adding the pretreatment step did not have an appreciable effect on the staining of normal and amyloid-containing tissues. Occasional sections showed slight decrease in the intensity of staining of amyloid with no effect on green birefringence. The use of polar mounting media had no effect on the birefringence of amyloid and collagen. The disadvantage of water soluble polar medium was that the sections were rehydrated with some degree of stain "wash out". There is disagreement between us and Romhanyi\textsuperscript{20,21} who reported that collagen birefringence was avoided by the use of polar mounting media.

It is our conclusion that selecting the staining solution is a critical step in the specific identification of amyloid in tissues. Better staining greatly increases the specificity of green birefringence, dichromism, and red fluorescence.

References


