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Questions

1. If Nissl stain is used to demonstrate RNA/DNA in the neurons of CNS, what staining technique is used for identifying the same in peripheral neurons?
2. Is Luxol Fast Blue Stain also used to detect demyelination in the PNS? Whatever your answer is, explain the procedure involved in the demonstration of demyelination in the PNS.

Answers

1. Nucleic acids and DNA of the peripheral nervous system are mostly stained using Fuelgen Reaction. Fuelgen reaction procedure is for the demonstration of DNA in tissue sections. It is darkly stained. It depends on acid hydrolysis of DNA, therefore fixating agents using strong acid should be avoided.

**Principle**

This is a relatively new technique that is used for demonstrating DNA in tissue sections. It is a sensitive means of detecting aldehydes, which makes it the ideal method for detecting the presence of DNA. Here, the section is treated with dilute hydrochloric acid in order to remove the bases.

The sugar part that remains reacts as an aldehyde ultimately forming a visible color. Therefore, this method can be said to be divided into two main parts:

1. The first part of the procedure is the hydrolysis phase that involves the of 5N HCL, ambient temperature for 40 minutes. This step is aimed at separately 2 purine bases(adenine and guanine) which are removed from the DNA molecule.
2. The second step is the staining phase. The reagent used is preferred because it is highly selective for DNA rather than RNA. Here, RNA does not react because of the presence of hydroxyl on carbon 2 of ribose, which prevents the acid(HCL) from hydrolyzing sugar. The reaction is also precise for the localization of DNA given that deoxyribose radicals are bound to phosphoric acid of the apurinic acid molecule following the removal of purine bases.

**Reagents**

* Hydrochloric acid solution
* Schiff reagent
* Fixative solution
* Counterstain

**Staining procedure:**

* Prepare Hydrochloric Acid Working solution; combine and mix well.
1. Hydrochloric Acid, 20% Aqueous 16ml
2. Distilled Water 24ml
3. Preheat and maintain Hydrochloric Acid Working solution at 60%C prior to use
* Deparaffinize sections thoroughly in three changes of xylene, 3 minutes each. Hydrate through two changes each of 100% and 95% ethyl alcohols, 10 dips each. Wash well with distilled water
* Hydrolyze sections in hydrochloric Acid working solution at 60 degree celcius for 10 minutes.
* Place slides directly in Schiff Reagent, McManus for 45 minutes.
* Wash in running tap water for 5 minutes; rinse in distilled water.
* Counterstain in Light Green SF Yellowish Stain 0.2%, Aqueous for 1 minute
* Dehydrate in two changes each of 95% and 100% ethyl alcohol. Clear in three changes of xylene, 10 dips each; coverslip with compatible mounting medium.

**RESULTS**

|  |  |
| --- | --- |
| DNA | Red-purple |
| Nuclei | Red-purple |
| Background | Green |

**PROCEDURE NOTES:**

1. Do not allow sections to dry out at any point during staining procedure
2. Bouin fixed tissue is unsatisfactory for use with FUELGEN reaction
3. Drain staining rack/slides after each step to prevent solution carry over
4. Prolonged exposure to hydrochloric acid may over hydrolyze sections with poor staining result.

Some other stains used for both DNA/RNA include:

* Methyl green pyronin stain
* Acridine orange

Methyl green pyronin stain: methyl green pyronin is a classical histological staining used in basic(cationic) dyes for the demonstration and differentiation of DNA and RNA. Methyl green is specific for phosphats radicals in DNA double helix staining it green blue. Pyronin does not possess this affinity and binds to the remaining negatively charged RNA staining it red.

 This method is useful in identifying the distribution of nissl substances in neuronal cell bodies.

1. Luxol Fast Blue Stain is not used to detect demyelination in PNS

**Demonstration of demyelination in PNS**

The disintegration of myelin sheaths of degenerating peripheral nerve occurs in two stages. The early stage is characterized by a physical disruption of myelin sheaths without chemical alteration. The late stage, beginning about 1 week after atonal section, consists of a chemical degradation of myelin lipids. In degenerating peripheral nerves these changes develop within schwann cells, indicating that these cells are capable not only of forming but also of digesting myelin sheaths.

In the central nervous system the breakdown of myelin sheath after atonal degeneration occurs at a much slower pace. This temporal difference suggests that oligodendrocytes, which form the myelin sheaths of central nerve fibers, do not behave like schwaan cells, but that myelin digestion must await the arrival of macrophages.

Myelinated axons can be observed in the fresh state by phase-contest microscopy of teased nerves or of squash or touch preparations of central nervous tissue. Living myelinated fibers can be seen in thin tissue cultures derived from sensory or autonomic ganglia or from immature brain or spinal cord. The nervous tissue is fixed, sectioned and stained to provide permanent preparations.

When a neuron dies, or if its axon has been severed, the part has been cut off from the neuronal cell body becomes beaded and then breaks into a string of fragments which are eventually taken up by phagocytosis cells. This process is known as wallerian degeneration.

There are several methods for demonstrating a degenerating myelin:

1. Swank and Davenport’s Marchi Method for degenerating myelin
2. Adams’s OTAN Method for Normal and degenerating Myelin
3. Polarized light method for degenerating myelin(applicable to central nervous tissue)
* **Swank and Davenport’s Marchi Method for degenerating Myelin:**

The technique incorporates improvements that minimize artifacts such as sporadic staining of normal myelinated fibers. Specimens are fixed for 2-4 days in phosphate buffered formaldehyde (2-3 weeks for human or other large brain) and then cut into slices no more than 3mm thick.

**Solution needed:**

Staining solution- this can be kept for a few months in a suitable bottle. It includes:

* Potassium chlorate 15g
* Water 200ml
* Osmium tetroxide 0.5g(used only once)
* Formalin(37-40%) 0.5ml
* Glacial acetic acid 2.5ml

**Procedure**

1. Transfer slices of tissue (without washing) from the fixative into about 15 time their volume of the staining solution in a screw capped jar. Agitate daily to expose all surfaces of the specimens to the solution. (alternatively place the container on a gently rotating platform)
2. After 7 days, remove the specimens and wash them in running tap water for 24h
3. Either cut frozen sections or process into paraffin before sectioning. Sections should be 20mm or thicker for appreciation of degenerating tracts of fibers in the brain.
4. Dehydrate and clear frozen sections, or dewax paraffin sections and transfer to clean xylene. Coverslip with a resinous mounting medium.

**Result**

Degenerating myelin (early and late products) black

Background: colorless to pale brown. Occasional normal fibers may be black and there may be some fine black granular material on the surface, at the edges of the sections.

In some species Marchi-positive material is normally present

In nerve roots, at the junction between the central and peripheral nervous systems

* **Adams’s OTAN Method for Normal and Degenerating Myelin**

The abbreviation is for **O**smium **T**etroxide and **A**lpha-**N**aphthlamine. The technique is applied to cryostat sections, ideally after calcium-formaldehyde fixation.

 **Solutions needed**

1. Osmium Tertroxide-Potassium Chlorate: this is made up as required and used only once. Osmium tertroxide, 2% stock solution in water, 5ml potassium chlorate(KCLO3), 1% Stock solution in water: 30ml, water: 5ml
2. Saturated-Naphthylamine Solution: dissolve a few crystal of naphthylamine in 40ml of water at 40◦c filter. This solution is used at 37◦c

Procedure

1. Treat the sections with osmium tetroxide-potassium chlorate( solution A) overnight at room temperature, in a tightly closed container.
2. Wash the sections for 10min in water(3 changes with occasional agitation).
3. Treat the sections with saturated-naphthylamine solution B for 20min at 37◦C
4. Wash the sections for 5 min in water( 3 changes with occasional agitations) apply 5 coverslips, using an aqueous mounting medium

 **Result**

Normal myelin is brownish-orange.

Degenerating myelin (late products only) is black. Fat, if present in the tissue is also blackened.