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A Method for the Simultaneous Detection of Mast Cells and Nerve Terminals in the Thymus in Laboratory Mammals

V. V. Gusel'nikova,[2] E. G. Sukhorukova,[1] E. A. Fedorova,[1] A. V. Polevshchikov,[1,2,4] and D. E. Korzhevskii[1,3]

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The aim of the present work was to develop a method for the simultaneous detection of mast cells (MC) and nerve fiber terminals based on methods developed for the histochemical identification of MC with Alcian blue and an immunochemical reaction for synaptophysin. The protocol developed here provides highly selective and reproducible simultaneous detection of MC and nerve endings on paraffin sections of thymus from laboratory mammals. The method can be used both for detecting the spatial interactions of MC and nerve terminals and for independent studies of the innervation of the internal organs of mammals. The optimum fixative was zinc-ethanol-formaldehyde.

Keywords: mast cells, nerve terminals, Alcian blue, synaptophysin.

There is no longer any doubt that the nervous and immune systems function in close cooperation, with mutual influences on each other [2]. One example of this interaction is that between mast cells (MC) and nerve fiber terminals [15, 17]. MC and nerve endings have been shown to be colocated in the myocardium, diaphragm, meninges, gallbladder, ileum, and skin in mammals. Particular attention is currently paid to the role of their interaction in the pathogenesis of various diseases such as irritable bowel syndrome, bronchial asthma, psoriasis, etc., such that the best studied systems are the digestive tract, airways, and skin [10]. Studies of the thymus addressing the colocation of MC and nerve terminals were mainly undertaken in the 1980s and 1990s [8, 13, 14, 16, 18, 19] and used laborious and often poorly reproducible methods and approaches such as impregnation of nerve fibers with metal salts [16], as well as methods using glyoxylic acid [8, 9, 19]. Apart from poor reproducibility, these methods have the disadvantage that they allow detection of nerve fibers but are not suitable for visualization of synaptic contacts. The ability to detect nerve fiber terminals is of fundamental importance in the case of the interaction of MC with nerve fibers. Nerve fibers are identified in immunohistochemical studies using antibodies to defined neuropeptides such as substance P, CGRP, VIP, neuropeptide Y, and others [12–14], or antibodies to enzymes catalyzing noradrenaline and dopamine synthesis, i.e., tyrosine hydroxylase and others [14, 18]. This allows detection of nerve fibers of a particular neurotransmitter type but does not allow large numbers of contacts between MC and nerve endings within a single section to be evaluated.

The aim of the present work was to develop a simple and reproducible method for the simultaneous detection of

[1] Laboratory for the Functional Morphology of the Central and Peripheral Nervous System, Department of General and Special Morphology, Research Institute Experimental Medicine, North-West Branch, Russian Academy of Medical Sciences, St. Petersburg, Russia; e-mail: len48@inbox.ru.
[2] Department of Cytology and Histology, St. Petersburg State University, St. Petersburg, Russia; e-mail: guselnicova.valeriia@yandex.ru.
[3] Department of Basic Problems in Medicine and Medical Technologies, St. Petersburg State University, St. Petersburg, Russia; e-mail: dek2@yandex.ru.
[4] Department of Basic Medicine, Far East Federal University, Vladivostok, Russia; e-mail: alexpol512@yandex.ru.

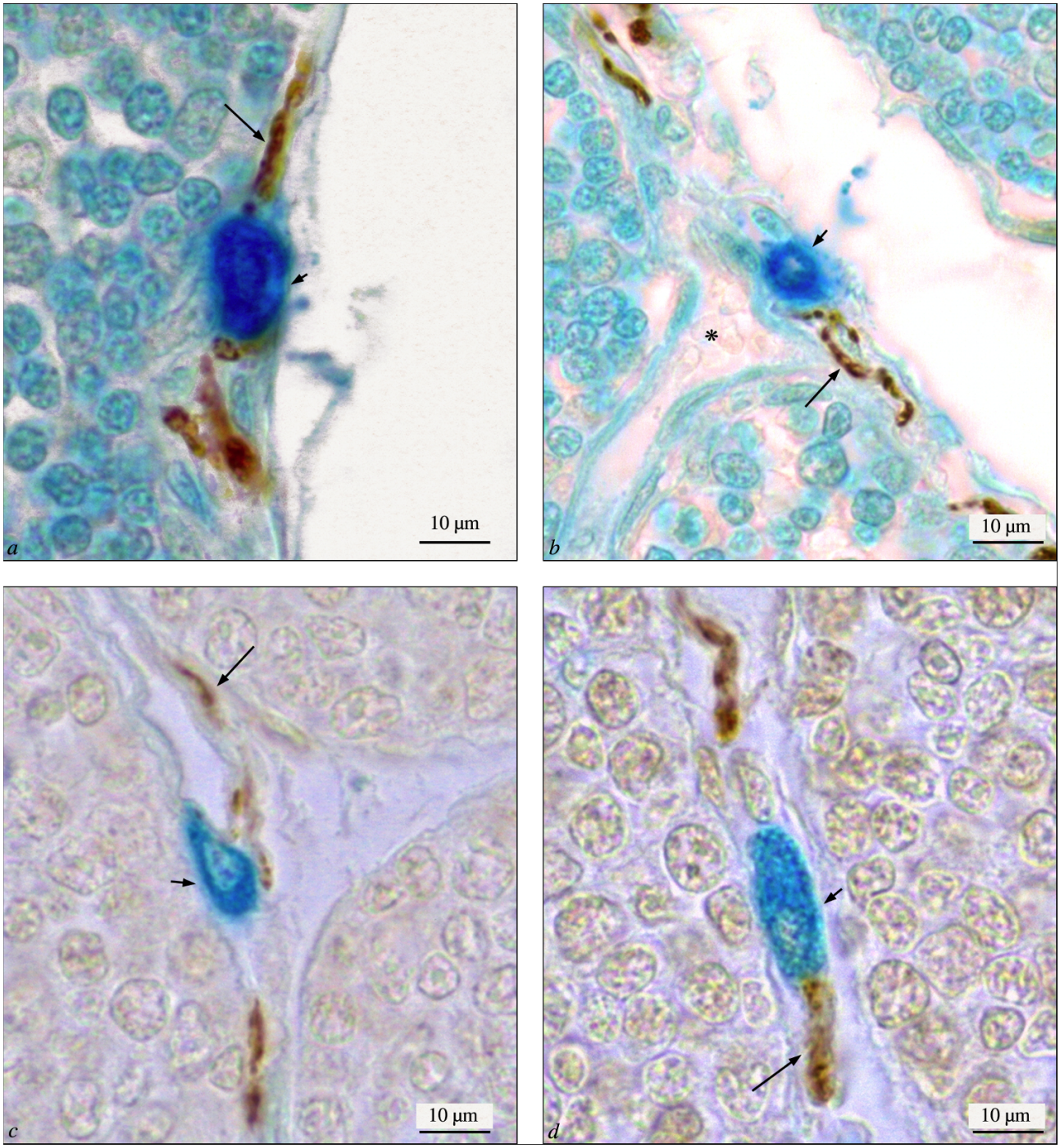

Fig. 1. Mast cells (MC) and synaptophysin-positive terminals (SPPT) of nerve cells in the thymus. *a*, *b*) Subcapsular zone of the rat thymus; *c*–*d*) mouse thymus interlobular connective tissue: long arrows show SPPT, short arrows show MC, and asterisks show vessel lumens. Immunohistochemical reaction for synaptophysin counterstained with Alcian blue: *a*, *b*) progressive staining; *c*, *d*) regressive staining with differentiation.

MC and nerve endings using a combination of methods for histochemical identification of MC with Alcian blue and immunohistochemical detection of synaptophysin (SP).

SP is the main integral protein in synaptic vesicle membranes and is used as a specific marker for synapses. Immunohistochemical staining using mono- and polyclonal antibodies to SP provides a simple and reliable method which is used for evaluation of synaptogenesis, synapse density in the nervous system, and for studying the innervation of the internal organs in mammals [5–7].

Most methods for identifying MC are based on the histochemical detection of the components in their granules. One of these methods, widely used in histological practice, consists of staining with Alcian blue, which, as a cationic stain, forms ionic bonds with polyanionic glycosaminoglycans and thus binds with heparin and chondroitin sulfate in MC granules, staining them blue [3].

The present studies used thymus tissue from adult Wistar rats and C57BL mice. Laboratory animals were kept and sacrificed in compliance with the "Regulations for Studies Using Experimental Animals" (USSR Ministry of Health Decree No. 755 of August 12, 1977). Specimens were fixed in zinc-ethanol-formaldehyde [1] and EFA mix (six parts 96° ethanol, three parts 40% formaldehyde, and one part glacial acetic acid) for 24 h and, after standard histological processing, were embedded in paraffin (NevaReaktiv, Russia; Histomix, BioVitrum, Russia). Sections of thickness 5 μm were attached to slides coated with poly-L-lysine (Menzel, Germany) or a special adhesive [4]. SP was detected by using polyclonal rabbit antibodies (Dako, Denmark; Monosan, Holland); the secondary reagent was HRP conjugate from a Reveal Polyvalent HPR DAB Detection System kit SPD-015 (Spring Bioscience, USA). Reaction product was detected using the chromogen 3,3'-diaminobenzidine from a two-component kit (Spring Bioscience, USA). MC were identified by counterstaining sections with commercial Alcian blue solution (BioVitrum, Russia).

Processing using different regimes of incubation with antibodies to verify the procedure for thermal demasking of antigen and varying Alcian blue staining times identified the following optimum specimen processing protocol:

1) paraffin is removed and sections are rehydrated by standard methods;

2) after washing with distilled water (2–5 min), slides are transferred to 3% hydrogen peroxide for 5–7 min to block endogenous peroxidase;

3) slides are placed in 0.01 M phosphate-buffered saline (PBS) pH 7.4 for 2–3 min;

4) excess fluid around sections is removed with filter paper;

5) the required quantity of blocking solution (Protein block DPB-125, Spring Bioscience, USA) is added to sections and left at room temperature for 10 min. Spreading of reagents during incubation and drying of specimens are prevented by drawing a hydrophobic flowmaster (Liquid Blocker, PAP Pen, Dako Pen) circle around the sections;

6) blocking solution is removed and, without washing, sections are loaded with anti-SP antibodies, spreading the solution evenly over the section by rocking the slide. The slide is placed in a humid chamber and incubated at 27°C for 50 min.

7) preparations are washed with PBS for 5–7 min;

8) excess fluid around sections is removed and the required quantity of HRP conjugate reagent from a Reveal Polyvalent HPT DAB Detection System is added. The slide is placed in a humid chamber and incubated at 27°C for 15 min;

9) reagent is washed from sections and specimens are left in PBS for 10 min;

10) excess PBS is removed and the required quantity of working DAB solution is added to the sections. Histochemical reaction product forms over a period of 1–3 min. This process is monitored under a microscope and the reaction is stopped before appearance of background staining by washing with 3% hydrogen peroxide solution;

11) specimens are washed with distilled water (2–5 min);

12) excess liquid is removed from the slide and the slide is wetted with filter paper around the sections;

13) sections are counterstained with Alcian blue using one of the two methods described, which produce different staining end results.

Progressive staining:

a) the slide is placed in Alcian blue solution and left for 30 min at room temperature;

b) the slide is washed by rocking in two changes of distilled water;

c) specimens are dehydrated, clarified, and embedded in permanent medium (DPX, polystyrene, Canada balsam) by standard methods.

Regressive staining with differentiation with glacial acetic acid (GAC):

a) sections are loaded dropwise with required quantity of Alcian blue solution (such that the stain solution completely covers each section);

b) the slide bearing the sections and stain solution is warmed over a spirit flame (or by some other method) and stained until intense dark blue staining of the sections is obtained;

c) the slide is transferred to distilled water and washed by rocking;

d) excess liquid is removed from the slide and the slide is loaded dropwise with GAC. Slides are washed for a few seconds to remove excess stain (differentiation). As soon as sections become unstained to the naked eye, differentiation is terminated by transferring the slide into distilled water and washed by rocking;

e) specimens are dehydrated, clarified, and embedded in permanent medium (DPX, polystyrene, Canada balsam) by standard methods.

Comparison the results obtained by processing specimens fixed by different methods showed that fixation in zinc-ethanol-formaldehyde gave better preservation of the immunoreactivity of the study antigen and provided for brighter staining of tissue components with Alcian blue than fixation with EFA.

Processing allowed clear detection of SP-positive nerve fiber terminals (SPPT) in specimens. Immunohistochemical detection of nerve terminals produced selectivity and staining intensity which were comparable to results obtained in other studies using anti-SP antibodies [5–7]. SPPT identified by the

dark brown coloration or black coloration produced by intense staining with reagent were seen mainly in the connective tissue of the capsule and septa of the thymus, in the subcapsular zone, and around blood vessels, where they often formed plexuses. MC were clearly detected from the intense turquoise staining of their cytoplasmic granules with Alcian blue. MC were characterized by relatively large sizes (length 10–20 μm, width 6–9 μm), and oval or extended, or, more rarely, round shape, and a large and rather eccentrically located nucleus sometimes coated with granules. MC in the thymus were, like terminals, located within the capsule and septa, subcapsularly, and in perivascular zones. SPPT were often located in the immediate vicinity of MC, creating the impression that there were contacts between them in the thymus.

Staining of specimens with Alcian blue without subsequent differentiation with GAC (progressive staining) improved the orientation of structures in the thymus, not masking the immunohistochemical reaction product. On a general blue-turquoise background, due to Alcian blue-stained nuclei of lymphocytes, endotheliocytes, epitheliocytes, and soft tissue components, MC were visible due to more intense bright blue staining (Fig. 1, *a*, *b*).

The results of differentiation of preparations with GAC (regressive staining) was that all thymus structures were unstained apart from MC and SPPT. The results of the immunohistochemical reaction and staining of the granules of MC were not affected by differentiation with GAC, such that MC contrasted more strongly (as compared with the previous staining version) with the unstained background, making it easier to detect and count them (see Fig. 1, *c*, *d*).

This processing protocol provides highly selective and reproducible simultaneous detection of MC and nerve fiber terminals in sections of thymus from laboratory mammals. The results obtained here are consistent with previous data showing that the thymus contains contacts between MC and nerve fiber terminals [9, 12–14, 16, 19]. The advantages of the method used here (apart from selectivity and reproducibility) include specificity in relation to nerve ending terminals of any neurotransmitter class, while known immunohistochemical methods associated with the use of antibodies to neuropeptides detect nerve fibers of only a given neurotransmitter specificity [12–14]. Botchkarev et al. [11] described a triple staining method based on simultaneous use of antibodies to substance P and CGRP to detect nerve fibers and TRITC- or FITC-labeled avidin to identify MC. This method, although allowing simultaneous detection of substance P- and CGRP-containing nerve fibers and thus extending the range of studies, is laborious – it requires careful selection of fluorochromes with non-overlapping emission spectra and the use of large quantities of multiple reagents. In contrast to methods using the laborious and expensive methods of confocal or electron microscopy, the staining method developed for this study is relatively simple and can be used with paraffin sections and light microscopy. Finally, the method developed here can be used not only for visualizing spatial interactions between MC and SPPT, but also for separate studies of the innervation of various mammalian organs.

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