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A Method of Enhanced Immunofluorescence by Amplification of the Immunoperoxidase Reaction

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The aim of the present work was to develop a simple and effective means of converting the immunoperoxidase reaction into an immunofluorescence reaction. Use of fluorochrome-conjugated antibodies to horseradish peroxidase significantly increased the intensity of the immunofluorescence reaction as compared with the indirect Coons method, which was confirmed by quantitative evaluation of fluorescence intensity. The conversion method developed here does not require the protocol to be expanded by a large number of steps or any reagent substitutions, which facilitates the transition from the immunoperoxidase reaction to the immunofluorescent reaction.

Keywords: immunoperoxidase reaction, immunofluorescence reaction, conversion, amplification.

Immunocytochemical reactions for fluorescence visualization of test markers have some particular characteristics as compared with the widely employed immunohistochemical methods (using an enzyme label) used for standard light microscopy. The main one of these is the lower sensitivity of immunofluorescence reactions than the avidin-biotin polymers and multimeric amplification systems used with the immunoperoxidase system, which are in wide use [3]. This property is responsible for the main difficulties encountered by investigators trying to transfer from immunoperoxidase reactions to immunofluorescence reactions. Thus, while immunocytochemical reactions using peroxidase labels and one of the standard immunohistochemical kits yield good-quality preparations with clearly discriminable immunopositive structures, reactions using the same primary antibodies with the appropriate secondary antibodies bound to fluorochrome do not give detectable fluorescence, the most likely cause of failure being the low sensitivity of the immunofluorescence reaction. There are several methods for increasing reaction sensitivity during the processing of preparations for fluorescent and confocal microscopy. For example, positive effects can in some cases be obtained by prolonged thermal antigen demasking, though introduction of this procedure into the protocol requires special buffer solutions, which significantly increases the total processing time and can also have adverse effects on preparation quality, leading to detachment of sections from slides and changes in the hematoxylin counterstaining properties of the tissues. When the concentrations of study antigen in cells is high, good results can be obtained by increasing the duration of incubation of sections in primary antibodies to several days [3]. Increases in the sensitivity of immunofluorescence reactions can also be obtained with streptavidin amplification using a conjugate of streptavidin with a fluorochrome. However, the reaction sensitivity of this method is below that of the analogous avidin-biotin amplification method used in combination with peroxidase label for light microscopy. Furthermore, use of fluorochrome-conjugated streptavidin leads to an increase in the number of steps in processing preparations and requires inclusion of an additional stage blocking endogenous avidin and biotin binding loci [4].

The most effective way of progressing from immunoperoxidase reactions to immunofluorescence reactions is provided by tyramide amplification of the peroxidase reaction [13]. This method provides significant increases in reaction sensitivity, though there are significant disadvantages – perifocal diffusion of the tyramide-fluorochrome complex from

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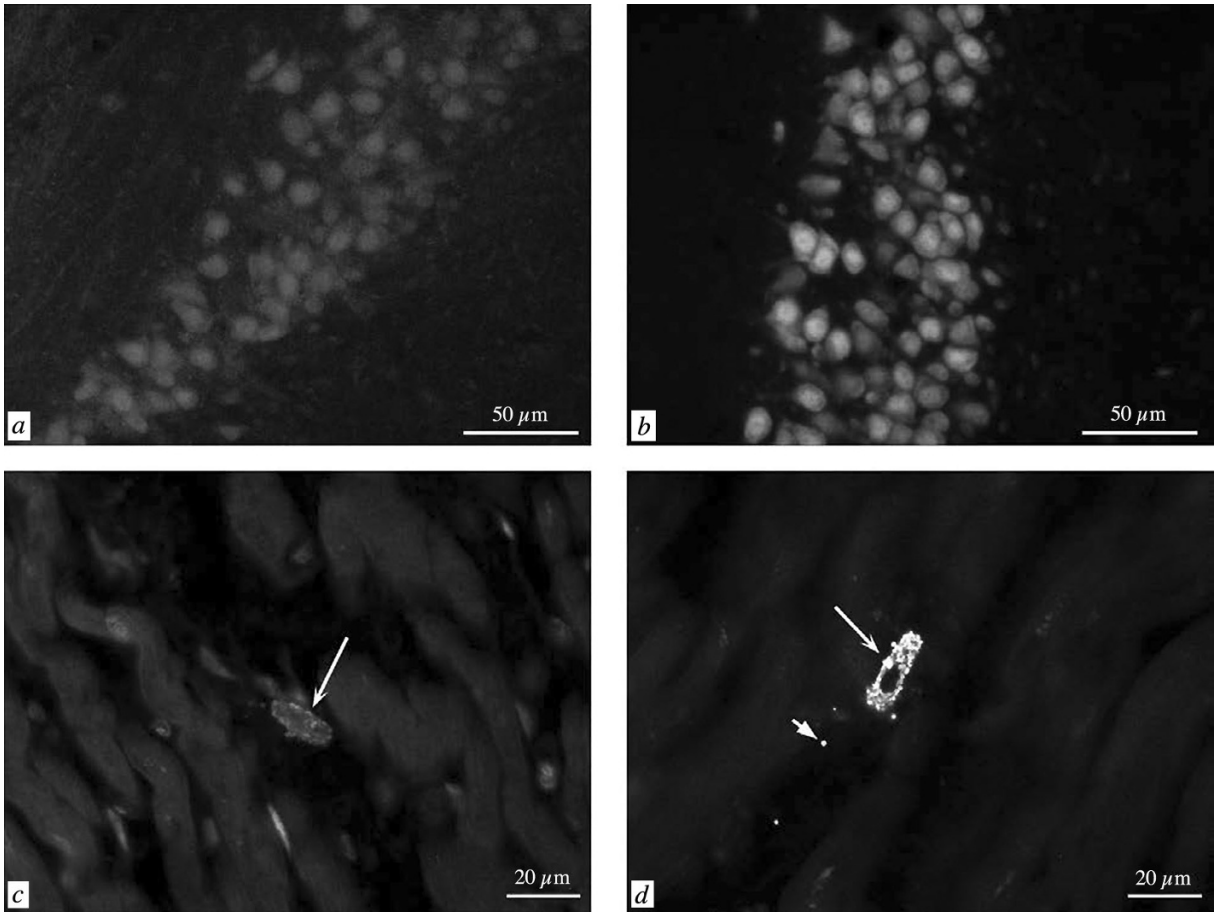

Fig. 1. Use of different methods of processing preparations for immunofluorescence reaction for NeuN and mast cell tryptase. *a*, *b*) Rat hippocampal field CA3, immunocytochemical reaction for neuronal nuclear marker NeuN. N PLAN 40×/0.65 objective; *c*, *d*) human ventricular myocardial, immunocytochemical reaction for human mast cell tryptase. N PLAN 63×/0.80 objective; *a*, *c*) secondary reagent – tetramethylrhodamine isocyanate-conjugated antibodies; *b*, *d*) secondary reagent – fluorochrome Cy3-conjugated antibody to horseradish peroxidase. Long arrows show tryptase-immunopositive mast cells; the short arrow shows a tryptase-immunopositive granule located outside the mast cell cytoplasm.

the antigen location. This drawback significantly limits the use of this method, such that it can be used only in studies of relatively well separated discrete structures [4]. Thus, there is a need to develop a simpler methodological approach which, on the one hand, could be used for conversion of immunoperoxidase reactions to immunofluorescence reactions without displacing the label from the location of the antigen and, on the other, is able to provide significant amplification of the signal and an increase in the intensity of the immunofluorescence reaction, providing a simple transition from an immunoperoxidase reaction to an immunofluorescence reaction.

The aim of the present work was to develop a simple and reliable means of converting an immunoperoxidase reaction to an immunofluorescence antigen detection reaction giving results comparable in quality to results obtained using immunoperoxidase reactions but not requiring addition of a large number of additional steps into the protocol or substitution of reagents.

Study materials consisted of brain sections from adult male Wistar rats ($n = 5$) and specimens of ventricular myocardium ($n = 4$) from humans (men and women) aged 23–93 years who had died from various diseases, from the collection of the Department of General and Special Morphology, Institute of Experimental Medicine. Brain samples were fixed in zinc-ethanol-formaldehyde [5], while myocardial samples were fixed in 10% formalin solution. After fixation and standard histological processing, samples were embedded in paraffin using standard methods. Sections of thickness 5 μm were attached to slides with an adhesive coating (Menzel, Germany). After deparaffination, rehydration, and washing with distilled water, sections were processed following protocols developed previously for specific primary antibodies. Our studies used two versions of the primary antibodies to two different markers – mast cell (MC) tryptase and neuronal nuclear antigen NeuN. These markers were selected for several reasons, the main of which was their high specificity for specific cell types, such that the corresponding antibodies

are widely used in scientific and clinical diagnostic investigations [8, 10, 11]. Thus, tryptase is a specific serine protease found in human MC granules and is used as a major marker for identifying these cells [12]. NeuN protein is located in the nuclei and perinuclear cytoplasm of most CNS neurons in mammals, but is not seen in gliocytes, with the result that NeuN is widely used as a universal neuron-specific marker [9]. Another reason influencing selection of markers for the present studies was the existence of well developed protocols for primary antibodies to tryptase and NeuN, allowing high-quality properties to be made for immunohistochemical reactions for light microscopy [1, 2, 6, 7]. According to these protocols for antibodies to NeuN (mouse monoclonals, clone A60) (Chemicon, USA, diluted 1:400) and antibodies to human tryptase (mouse monoclonals, clone AA1, manufacturer's dilution, Dako, Denmark), sections are incubated with primary antibodies in a moist chamber for 60 min at 40°C without preliminary thermal antigen demasking. Secondary reagents in both cases are provided by the EnVision+ System Labelled Polymer-HRP Anti-Mouse kit (Dako, Denmark) or MACH2 Universal HRP Polymer Kit for Mouse or Rabbit (Biocare Medical, USA), which include secondary antibodies conjugated with horseradish peroxidase. Immunohistochemical reaction product is visualized using 3,3'-diaminobenzidine (Dako, Denmark).

While addressing the task of transferring from the immunoperoxidase reaction to the immunofluorescence reaction, we initially conducted an analysis of all possible variants of manipulations providing fluorescence microscopy results of quality comparable with results obtained with the immunoperoxidase reaction. This analysis led us to verify the simplest approach giving a successful transition from the immunoperoxidase reaction to an immunofluorescence reaction with minimal substitution of reagents. This approach was based on the use of antibodies against horseradish peroxidase (HRP) conjugated with fluorochrome. These antibodies must also be used after processing sections with secondary antibodies conjugated with HRP, and it is logical to suggest that this means can be effective in addressing the challenge of amplifying the immunoperoxidase reaction signal as compared with the direct Coons method, where the fluorochrome is conjugated directly with the secondary antibodies.

During development of protocols for fluorescence microscopy, we introduced changes into the procedure for processing preparations with primary antibodies to NeuN: an additional brief (25 min) thermal demasking stage in modified citrate buffer S1700 (Dako, Denmark) was introduced and the duration of incubation of sections with primary antibodies was increased (with a reduction in the incubation temperature). As a result, immunofluorescence reactions using anti-NeuN antibodies were run by incubating sections with primary antibodies in a moist chamber for 72 h at 27°C after preliminary thermal antigen demasking. The regime for processing sections using primary antibodies to MC tryptase was not altered from the corresponding protocol (see [2] for more detail) used for the immunoperoxidase reaction.

When incubation of sections with primary antibodies and washing in phosphate-buffered saline were complete, preparations were loaded with secondary antibodies. For each of the primary antibodies used, two reactions with different secondary antibodies were run in parallel to compare immunofluorescence reaction intensities obtained using the indirect Coons method on the one hand and the method using antibodies to HRP on the other. The first variant of using secondary antibodies consisted of processing sections with rabbit anti-mouse antibodies conjugated with tetramethylrhodamine isocyanate (TRITC, diluted 1:20, Dako, Denmark) for 90 min at 27°C followed by washing in distilled water and embedding in Fluorescence Mounting Medium (Dako, Denmark). In the second variant, washing sections to remove primary antibodies was followed by loading with the required quantity of MACH2 reagent from the Universal HRP Polymer Kit for Mouse or Rabbit (Biocare Medical, USA) and incubating in a moist chamber for 40 min at 27°C. After incubation and washing of sections in phosphate-buffered saline, slides were loaded with goat anti-HRP antibody conjugated with the fluorochrome Cy3 (Cy3-conjugated AffiniPure Goat Anti-Horseradish Peroxidase, Jackson ImmunoResearch, USA; dilution 1:100) and kept at 27°C for 30 min. Sections were then washed in distilled water and embedded in Fluorescence Mounting Medium (Dako, Denmark).

Preparations were examined under a Leica DM2500 fluorescence microscope (Germany) fitted with a filter system for fluorescence at 340–560 nm. An excitation filter BP = 515–560 nm (third – N2.1) and N PLAN 40×/0.65 and N PLAN 63×/0.80 objectives were used. Photographs were taken using a Leica DFC420 camera (Germany) with identical lighting conditions and camera settings. Images were processed using computer program Leica Application Suite V3 (Leica Microsystems, Switzerland/Germany). Fluorescence intensity (FI) in the red channel was measured using computer program Image J (Image Processing and Analysis in Java, USA). Comparison of FI was run in the clearest points of each photomicrograph. FI values corresponding to pixel brightness in the digital image were expressed in arbitrary units (U).

Comparison of the results obtained using different variants of sample processing showed that FI observed using antibodies to HRP was significantly greater than the FI of analogous structures seen using TRITC-conjugated secondary antibodies. Thus, analysis of rat brain preparations processed with anti-NeuN antibodies (Fig. 1, *a*, *b*) showed that use of (secondary) antibodies conjugated with TRITC gave very low FI in nuclei and the perinuclear cytoplasm of neurons – 36 and 25 U, respectively (see Fig. 1, *a*). The margins of neuron nuclei were not clearly visible, such that it was not always possible to identify nucleus shape. Despite the fact that the nucleus and perinuclear cytoplasm had different FI, visual discrimination of these two cell compart-

ments was often impossible because of the low overall level of fluorescence (see Fig. 1, *a*).

Conversion using anti-HRP antibodies (see Fig. 1, *b*) gave FI for hippocampal neuron nuclei (150 U) which was more than four times greater than values recorded using the indirect Coons method. In this case, analysis of hippocampal field CA3 neurons gave clearly discriminable nuclear margins, while nucleolar locations could be identified in some neurons (dark areas). The perinuclear space was characterized by a smaller FI than the nucleus, though overall this parameter was quite high in this case (107 U), allowing visual discrimination of the two structural compartments of neurons – the nucleus and the perinuclear cytoplasm (Fig. 1, *b*).

Analogous results were obtained using antibodies to MC tryptase (see Fig. 1, *c*, *d*). Analysis of human myocardial preparations showed that when secondary antibodies conjugated with TRITC were used, FI for MC was low (39 U) and was virtually no different from the intensity of background fluorescence in labeled tissue (see Fig. 1, *c*). This made it very difficult to identify MC, which in this situation could be seen only using a high microscope enlargement (at least a ×63 objective). Only those MC located in large intermuscular connective tissue layers or around blood vessels could be identified, while MC in fine intermuscular layers were essentially indistinguishable because of the strong background fluorescence of the surrounding muscle tissue and collagen fibers. The boundaries of granules located in the MC cytoplasm were not identified, with the result that the cytoplasm appeared homogeneous. Granules outside MC could very rarely be identified, again because of their low fluorescence intensity (see Fig. 1, *c*).

Conversion using antibodies to HRP gave high FI for MC granules (159 U) with the essentially complete absence of fluorescence from muscle tissue (see Fig. 1, *d*). This provided for easy identification of MC at low microscope magnification. At high magnification (×40 and ×63 objectives), the margins of tryptase-positive granules were visible in the MC cytoplasm despite their dese packing. Granules outside MC were well discriminated (see Fig. 1, *d*, short arrow).

Thus, the results obtained here led to the development of a simple and reliable method for converting immunoperoxidase reactions into immunofluorescence reactions based on the use of fluorochrome-conjugated anti-HRP antibodies. This method provides an easy transition from immunoperoxidase reactions to immunofluorescence reactions by introducing one additional step at the final stage of processing preparations, which yielded antigen detection results of quality comparable to that the immunoperoxidase reaction. FI in this case was significantly greater than obtained using the indirect Coons method. In contrast to the tyramide amplification method, the conversion of the immunoperoxidase reaction described here avoids displacement (diffusion) of label from the antigen site, and can thus be used to identify small structures located close together (e.g., cytoplasmic granules). The conversion method described here requires minimal replacement of reagents and can be used without making significant changes to the protocol developed for the immunoperoxidase reaction.

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