The Use of Immunohistochemical Method for Detection of Brain Microglia in Paraffin Sections

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 6, pp. 709-712, June, 2010
Original article submitted October 28, 2008

We developed a well reproducible method for detection of brain microglia on paraffin sections on the basis of modern immunohistochemical methods. Polyclonal antibodies to Iba1 antigen obtained after immunization with a synthetic peptide corresponding to 81-93 amino acid fragment of Iba1 antigen selectively interact with microglia cells on paraffin sections of rat brain. Detailed protocol of preparation treatment was drawn up ensuring complete detection of the microglia expressing Iba1 protein.

Key Words: *brain; microglia; immunohistochemistry*

Investigations of brain microglia structure and assessment of its activation after damage and diseases are among the most urgent problems of modern experimental neurobiology. However, microglia investigation *in situ* appears to be a rather complex problem due to lack of simple and well reproducible methods for selective detection of this population. Classical methods of Rio Hortega, Penfield, and Miyagava–Aleksandrovskaya are not quite specific, poorly standardized, and very laborious. Moreover, they cannot be used for specimens embedded in paraffin. Recently, immunohistochemical methods of microglia detection using antibodies to various antigens were reported [3,4], but most of them can detect microglial cells and macrophages only in weakly fixed material on vibratome and cryostat sections.

The aim of this study was to develop a well reproducible method for brain microglia detection in paraffin sections on the basis of contemporary immunohistochemical methods.

MATERIALS AND METHODS

Brains of Wistar rats (n=15) served as the study material. The material was fixed in alcohol-formalin, zinc-formalin, and zinc-ethanol-formaldehyde [1]. Dehydrated specimens were embedded in paraffin using standard method. For identification of phagocytic cells, monoclonal and polyclonal antibodies to CD68 (Dako), CD11b (Chemicon), Iba1 (AbCam) antigens were used. To detect antigen-antibody complexes we used LSAB2, LSAB$^+$, and EnVision$^+$ kits (Dako). The product of immunohistochemical reaction was visualized using DAB$^+$ reagent (Dako). Control procedures necessary for immunohistochemical investigations were performed. Human hippocampal sections were used for additional specificity control. Some sections were poststained with Gill's hematoxylin (BioVitrum) after the reaction.

RESULTS

Among all studied antibodies, only polyclonal antibodies to Iba1 antigen (ab5076, AbCam) obtained after immunization with a synthetic peptide corresponding to 81-93 amino acid fragment of human Iba1 protein allowed performance of immunohistochemical reaction on paraffin sections of rat brain. These antibodies also demonstrated positive reaction to microglia on control preparations (human hippocampal sections). Antibody specificity control was performed using synthetic peptide corresponding to 81-93 amino acid fragment of Iba1/AIF1 protein (AbCam). Zinc-formalin

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0007-4888/10/1496-0768

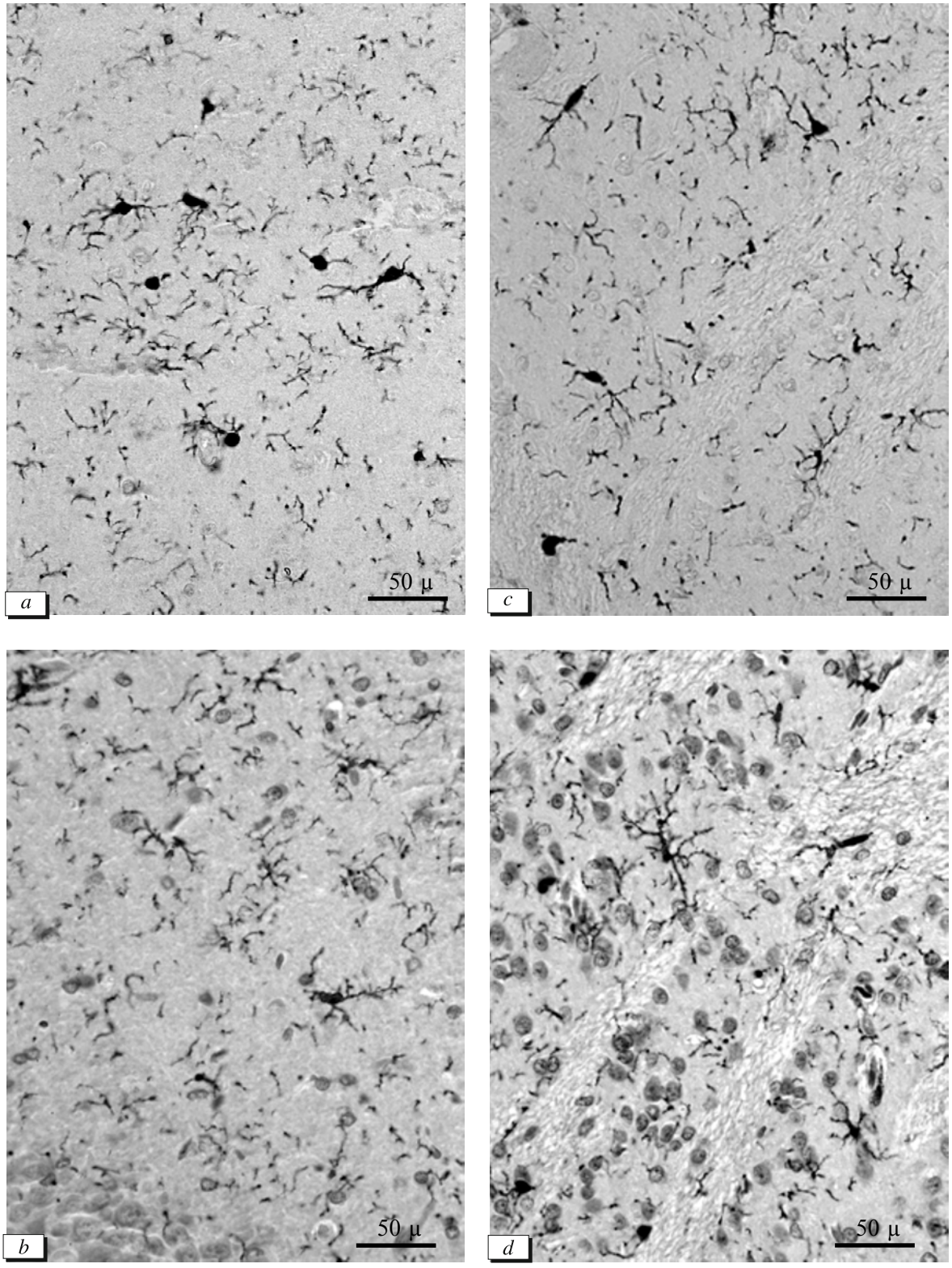

Fig. 1. Microglia of the rat brain. *a*, *b*) fragment of the hippocampus; *c*, *d*) fragment of the striatum. Immunohistochemical reaction to Iba1 protein without (*a*, *c*) and with additional staining with hematoxylin (*b*, *d*). Scale 50 μ.

and zinc-ethanol-formaldehyde appeared to be the best fixatives for microglia detection. In order to insure more complete microglia population, prolonged incubation of the preparation with primary antibodies at higher temperature and streptavidin-biotin amplification system are required. Heat demasking of the

antigen [2] did not substantially intensify the immunohistochemical reaction for Iba1 protein.

Comparison of the results of immunohistochemical reactions performed under different conditions (different temperature and duration of the incubation) resulted in the following sequence of preparation treatment providing the most complete detection microglial cells expressing Iba1 protein.

1. Remove paraffin and re-hydrate the sections using standard procedures.

2. Place the slide with sections into a vial with 3% hydrogen peroxide for 5-10 min for inhibition of endogenous peroxide.

3. Wash out peroxide with distilled water and place the slide into 0.01 M phosphate-saline buffer (PBS), pH 7.4, for 5-10 min.

4. Carefully sponge the slide around the specimen (dry field have to appear) and encircle the sections with a hydrophobic pen (*e.g.* DakoPen). Cover the specimen with sufficient amount of 5% blocking BSA solution (fraction V according to Kohn) in PBS and leave it for 10 min at room temperature.

5. Remove the excess of blocking solution (do not wash the slide) and apply sufficient amount of primary antibodies to Iba1 (dilution 1:200). Place the slide into a wet chamber (for this purpose any plastic box with a lid and wet filter paper and glass sticks on the bottom can be used) and place into a thermostat for 80 min at 40°C.

6. Wash out antibodies with PBS and place the slide in this buffer for 5-10 min. Carefully sponge the slide around the specimen, apply sufficient amount of secondary antibodies (Link reagent from LSAB$^+$ kit), and leave the slide for 35 min at room temperature.

7. Wash out antibodies with PBS and place the slide in this buffer for 5-10 min.

8. Carefully sponge the slide around the sections, apply sufficient amount of streptavidin-peroxidase conjugate (Str/HRP reagent from LSAB$^+$ kit) on the specimen, and leave it for 20 min at room temperature.

9. Wash out antibodies with PBS and place the slide in this buffer for 5-10 min.

10. Carefully sponge the slide around the specimen and apply sufficient amount of working solution of chromogen DAB$^+$ on the specimen. This is the stage of appearance of stained product of chemical reaction. This process needs to be controlled under a microscope to stop the reaction before background staining.

11. Wash out the chromogen solution and wash the preparations in three portions of distilled water 3-5 min each.

12. Additionally stain the sections with Jill hematoxylin solution for 0.5 min and wash out the dye with distilled water and blue the specimen with alkaline or tap water.

13. Dehydrate the sections in 96% and absolute ethyl alcohol (instead of absolute ethyl alcohol 99% isopropanol can be used), clear in xylene using standard procedures, and embed in balm, polystyrol, permount, DPX, or another similar medium.

The results of immunohistochemical reaction performed according to proposed protocol are presented (Fig. 1). Iba1-positive nerve cells are clearly identified. The reaction product exhibits cytoplasmic localization and is located in the perinuclear cytoplasm and in cell processes. The reaction intensity in microglia can differ sometimes, which can be explained by different functional state of cells (Iba1 protein is necessary for processes of phagocytosis and migration of microglial cells [5]). Additional staining with hematoxylin facilitates identification of anatomical structures and makes it possible to assess simultaneously the state of other cells of the nervous tissue.

Thus, immunohistochemical detection of Iba1 antigen according to the proposed protocol can be used for detection of microglia on paraffin sections of the brain.

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