Combined Immunoperoxidase Analysis for Visualization of Cells of the Blood-Brain Barrier

V. P. Baklaushev, G. M. Yusubalieva, O. I. Gurina, and V. P. Chekhonin

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A method for double immunoperoxidase staining of the blood-brain barrier cell elements was developed using 2mB6 monoclonal antibodies specifically visualizing brain capillary antigen and antibodies to glial fibrillary acidic protein (GFAP). The method is based on consecutive visualization of antigen structures in one section: first using a substrate mixture containing diaminobenzidine (brown coloring) and then a mixture with diaminobenzidine and $CoCl_2$ (blue coloring). This method visualizes cerebral capillary cells and fibrillar astrocytes interacting with them. The method can be used in all variants of double immunoperoxidase studies and for immunodetection of several antigens in immunoblotting analysis.

Key Words: *immunohistochemical analysis; astrocytes; blood-brain barrier*

Several variants of double and triple immunohistochemical staining of cell preparations and tissues by different first and second antibodies in immunofluorescent analysis are known [3,5]. On the other hand, immunoperoxidase analysis with antibodies to antigens of different cell location can be more informative for the solution of some basic and applied problems, for example, in studies of cell elements forming the blood-brain barrier (BBB) [4,9].

We obtained 2mB6 monoclonal antibodies specifically visualizing antigen of brain microvessels (AMVB1). Hybridoma producing these antibodies was created as a result of immunization of mice with preparations of cerebral capillary fractions isolated using a previously described and modified method [6]. We obtained monoclonal antibodies to glial fibrillary acidic protein (GFAP), the main marker of fibrillar astrocytes [2].

Here we developed a method for combined immunoperoxidase staining of BBB components using 2mB6 and GFAP antibodies in the same section.

V. P. Serbsky State Research Center of Social and Forensic Psychiatry, Moscow

MATERIALS AND METHODS

Intact adult male Wistar rats were perfused through the isthmus of the aorta with 4% cold paraformaldehyde in PBS with 1% glutaraldehyde using a Janet syringe by a modified method [1]. The brain was removed and postfixed overnight in the same solution, after which thick sections (40 μ) were made on a freezing microtome. Frozen sections were stored at -20°C in antifreeze (30% ethylene glycol and 30% glycerol in 0.5 M sodium phosphate buffer, pH 7.4).

Double immunoperoxidase visualization of AMVB1 and GFAP was carried out using first monoclonal antibodies (2mB6 and anti-GFAP, respectively), biotinylated anti-mouse antibodies (BA-2000), and Vectastain ABC Kit, Peroxidase, standard (Vector Lab.).

The sections were washed in PBS, endogenous peroxidase activity was then blocked (5 min) with 3% H_2O_2 in methanol, after that the sections were incubated for 1 h with 10% normal equine serum and then 2 h with first monoclonal antibodies (2mB6) diluted 1:10,000 in 1% normal equine serum with

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0.1% Twin-20. After washing in PBS with 0.1% Twin-20 (PBS-T; 3×5 min) the sections were incubated for 1 h with biotinylated equine anti-mouse IgG adsorbed with 2% normal rat serum (1:200 in PBS-T with 1% normal equine serum BA-2000; Vector Lab.). The sections were washed 3 times in PBS-T and then incubated for 1 h with avidin—biotin—peroxidase complex (ABC-Vectastain) as follows: 5 µl reagent A and 5 µl reagent B were added to 1 ml PBS-T with 1% equine serum, after which the sections were washed 3 times in PBS-T. Finally, the sections were stained with 0.025% diaminobenzidine (DAB) and 0.01% H_2O_2 in PBS and then washed in the same buffer.

The staining protocol was completely repeated with the first anti-GFAP antibodies till DAB staining. Peroxidase activity was visualized by staining with the substrate mixture, consisting of 0.025% DAB, 0.01% H_2O_2, and 0.001% $CoCl_2$ in PBS.

RESULTS

The method of double immunoperoxidase staining of brain microcirculatory system and astrocytes involved in BBB functioning with 2mB6 monoclonal antibodies and anti-GFAP antibodies was developed for simultaneous visualization of these structures. This method is a combination of two

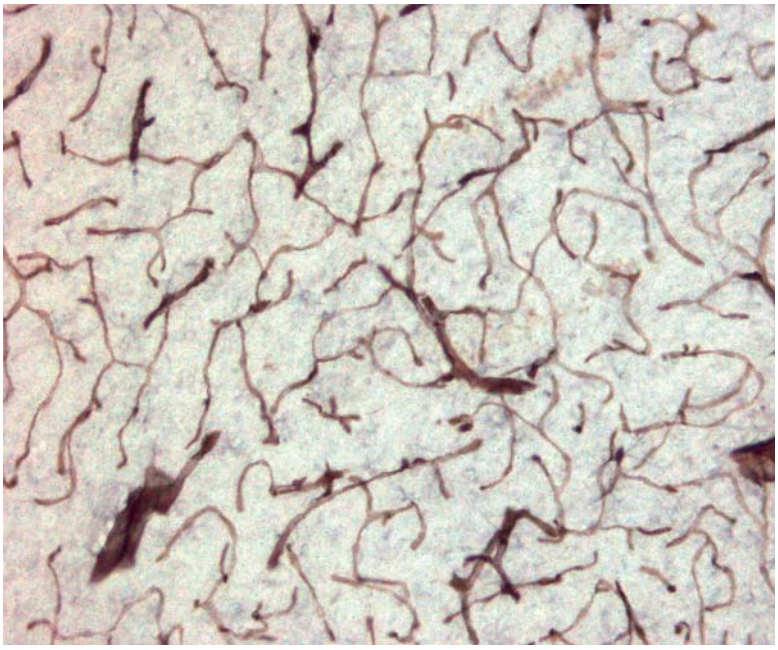

Fig. 1. Visualization of cerebral capillaries with 2mB6 monoclonal antibodies and DAB staining with H_2O_2 (×200).

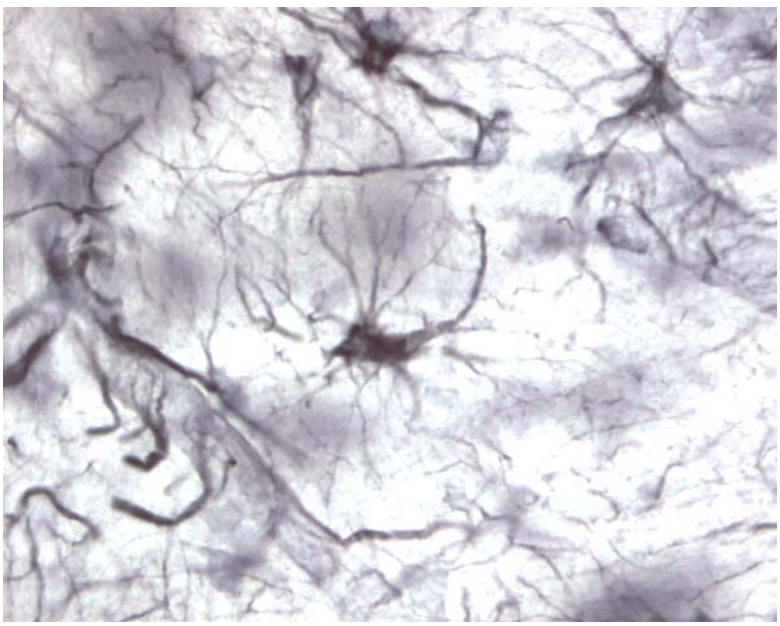

Fig. 2. Visualization of cerebral astrocytes with anti-GFAP-antibodies and DAB development with $CoCl_2$ and H_2O_2 (×1000).

variants of immunoperoxidase staining of one section with chromogens of different colors: DAB and DAB+$CoCl_2$ complex. Cobalt chloride alters the color from brown to blue, allowing clear differentiation between antibodies to two different antigens.

Capillaries were clearly visualized on brain sections after the first staining procedure (brown color; Fig. 1).

Astrocytes were colored blue in immunoperoxidase analysis of GFAP using DAB with $CoCl_2$ (Fig. 2).

The combined immunoperoxidase staining allows analysis of cerebral capillaries and astrocytes on the same section (Figs. 3, 4).

The proposed method clearly shows antigens of different cells (cerebral capillary cells are colored brown) and astrocytes (blue color), which gives spatial picture of cell-cell interactions between structures forming the BBB. This approach is important for *in vitro* BBB modeling and for studies of BBB cell structures in pathological processes characterized by BBB dysfunction.

High-magnification microscopy clearly shows the astrocyte terminal axons entwining the capillaries (Fig. 4), which directly participate in the regulation of selective permeability of BBB for different

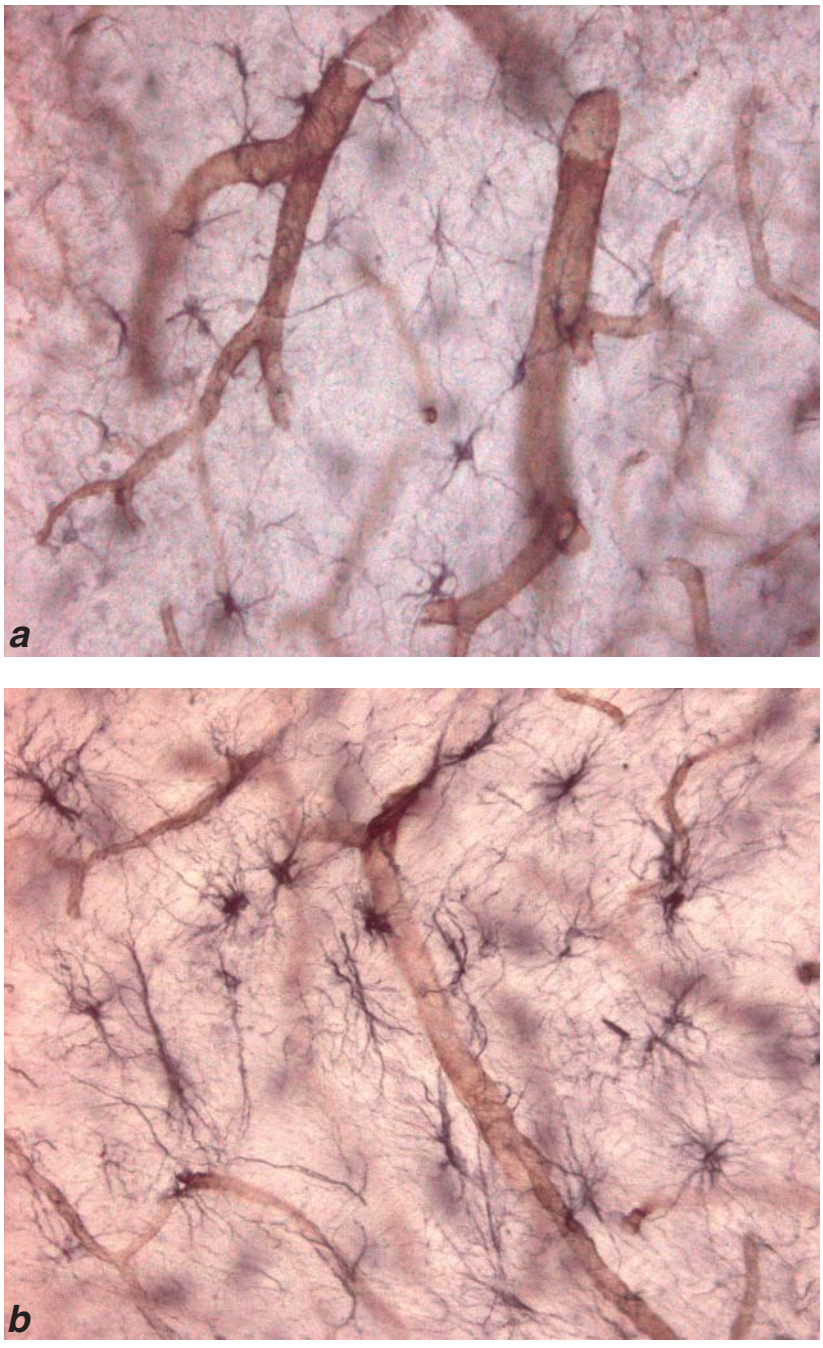

Fig. 3. Double immunoperoxidase staining of cerebral microvessels (*a*) and astrocytes (*b*; ×400).

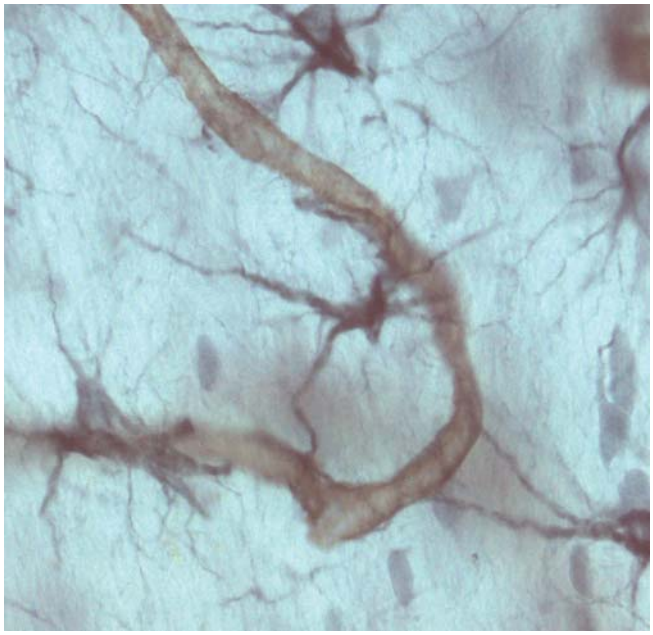

Fig. 4. Visualization of astrocyte-endothelial interactions by double immunoperoxidase staining with anti-GFAP and 2mB6 antibodies (×1000).

substances [4,7] and in the formation and maintenance of the barrier properties of the cerebral epithelium [8].

The study demonstrated the possibility of using our combined immunoperoxidase staining method for simultaneous visualization of cell structures by two first antibodies without special immunofluorescent kits and equipment. There are good grounds to expect that this method will be used for visualization of antigens in immunoblotting analysis with antibodies of different specificity.

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