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Brain Microglia and Microglial Markers

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Recent years have seen a continuing increase in interest in various aspects of the organization and functioning of microglia. However, data on contemporary immunocytochemical methods for detecting microglia are ambiguous and need to be made systematic. Attention in the present review is focused on microglial markers – proteins (Iba-1, CD11b, CD68, HLA-DR, and others) expressed by microgliocytes in normal conditions and on activation evoked by harmful factors. Characterization of markers and immunocytochemical microglial labeling methods is combined with analysis of reports on the origin and structural organization of microgliocytes.

Keywords: microglia, brain, immunocytochemistry, Iba-1, CD11b, CD68, OX-42.

Microgliocytes are resident macrophages in the nervous system and are of mesenchymal origin [21, 36], in contrast to the other cellular elements of nervous tissue, which are from the neuroectoderm. The last few years has seen a continuing increase in interest in various aspects of the organization and functioning of microglia in the CNS. Microglia are traditionally regarded as a key element of the inflammatory process developing in nervous tissue in response to various harmful influences [34, 46]. However, recent studies have shown that the functions of the microglia include a wider range of processes, a significant proportion of which are not directly linked with the development of responses to damage and the initiation of inflammation. Thus, microglia in the intact brain carry out constant monitoring of the state of the synaptic structures of the neuropil and constitute one of the main regulators of synaptic plasticity [68, 70]. It has been suggested that microglial cells are able to undergo directed changes in immunophenotype in regulating the functions of various brain structures and in the process of development of age-related and mental pathology [59, 66]. The mechanisms of these regulatory influences remain largely unclear, partly because of technical difficulties related to the in situ visualization of microglia.

This generates the need to systematize the various accumulated data on microglia and contemporary methods of studying their structural organization. The main focus of the present review is on microglial markers and contemporary immunocytochemical approaches used for detection of microgliocytes and analysis of their structural organization. The characteristics of microglial markers are introduced via information on the origin of the microglia and the terminology used in describing their various types (phenotypes).

The Origin of Microglia. The question of the origin of the microglia was long controversial [2, 46]. Most investigators consistently tend to the view that microgliocytes have a mesenchymal origin, though initially there was no convincing evidence for this. Complex experiments were performed at the end of the 20th century and in subsequent years using transgenic and chimeric animals, and these showed that the immediate precursors of microgliocytes were macrophages formed in mammals at the early stages of intrauterine development in the wall of the yolk sac during the process of embryonic hematopoiesis [33]. On establishment of the circulation, embryonic macrophages enter the vessels of the primitive meninges (meninx primitiva) [17] and migrate into the rudiments of nervous system structures before the

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formation of the intraorgan (intracerebral) vascular bed [8, 36]. On population of the human embryonic telencephalon with microglia, macrophages are concentrated in the rudiments of the vascular plexus [8], which may have a direct relationship with the transepithelial migration of these cells to the CSF-containing space seen in the vascular plexus and in adult animals in experimental studies [48].

During the postembryonic period of ontogeny, replenishment of the microgliocyte population occurs mainly by proliferation of existing microglial cells and their intraorgan precursors [36, 47]. In normal conditions, monocytes do not migrate into nervous tissue or differentiate into microgliocytes. Nonetheless, the potential for populating the brain after artificial depletion of microglia with new cells of bone marrow origin is retained [22]. In pathological processes accompanied by damage to the blood-brain barrier, the differentiation of new microglial cells from monocytes becomes more likely [32], though increases in the microglial population due to division of resident cells can also occur [9].

Contemporary data on the origin of microgliocytes provide evidence that these cells are closely related to other tissue macrophages and other cells of the monocyte series. This relatedness is also confirmed by the general patterns of expression of many markers on both microgliocytes and other tissue macrophages. However, typical microgliocytes (arbitrarily termed resting) are characterized by a complex structural organization different from that of most macrophages. It should be noted that the intact brain contains not only microglia, but also typical macrophages, though these cells are located outside nervous tissue while macrophages are present in the connective tissue of the vascular plexus and meninges, and also constitute a proportion of supraependymal cells and the cellular elements of the fluid filling the CSF spaces [7, 48].

Classification of Microgliocytes (microglial forms). Microgliocytes in the CNS constitute a heterogeneous population. Differences between them have been noted using classical impregnation detection methods. The initial descriptions were of microglia of various forms, discriminated in terms of the number and structural characteristics of the processes. These were unipolar, bipolar, multipolar, and laminar forms, as well as "histiocytic pericytes" [3]. However, this terminology does not fully reflect the functional sate of cells and is poor because of its similarity to the morphological classification of neurons.

The main type of microgliocyte encountered in the mature brain in mammals, including humans, is now designated "ramified" [20] – these are small cells, generally with multiple processes, and very small volumes of perinuclear cytoplasm (Fig. 1).

The primary processes of these cells have numerous branches and different directions; changes in the directions of processes form angles rather than smooth curves (as, for example, in astrocytes) [10]). These cells are characteristic of the gray matter in the intact brain. Microgliocytes in the white matter are smaller and have less branched processes. Located between densely distributed myelin fibers, microgliocytes not infrequently acquire a flattened or fusiform shape [6]. Their processes are less branched (Fig. 2) than those of typical ramified microgliocytes in the gray matter. Subependymal cells whose interependymal processes make contact with the cerebrospinal fluid constitute a special form of microgliocyte [5]. Exposure to harmful factors increases the population of these cells [4].

The reaction of nervous tissue to damage is accompanied by transformation of the phenotype of microgliocytes and the appearance of several new typical and transitional forms. These include rodlike (fusiform), multinucleate, ameboid, giant, and epithelioid microglial cells [12, 24]. Activated microglia are characterized by three immnophenotypes with different sets of markers and, accordingly, roles in inflammatory and regenerative reactions [69]. These are M1 (the proinflammatory phenotype), M2a (the anti-inflammatory phenotype), and M2c (a phenotype characteristic of the stage at which inflammation resolves and tissue remodeling occurs). Changes in the structural organization of the microglia are noted in humans with aging [11], though it remains unclear whether these are always associated with local neurodegenerative processes.

Unfortunately, investigators do not presently have a consistent view as to the terminology for designating the different forms of activated microglial cells. A clearer discrimination of morphological and functional forms of microgliocytes might be obtained by unifying and combining different methods for immunocytochemical labeling and a deeper understanding of the role of the microglia in controlling adaptive and pathological reactions occurring in the CNS.

Microglial Markers. Despite the fact that microglia were discovered about 100 years ago, microgliocytes remained the least studied nervous tissue cells until the last decade of the 20th century. The lack of width of studies of the microglia was due to a number of factors, not the least among which was the lack of reliable and selective methods for identifying this cell population. The main method of detecting microgliocytes until the end of the 1970s consisted of variants of silver nitrate impregnation. Impregnation methods (Rio-Ortega, Penfield, Beletskii, Miyagawa and Aleksandrovskaya), which have been described in detail in a monograph by Beletskii [1], are members of the group of classical neurohistology methods. However, all impregnation methods have common drawbacks, which are the significant duration of the protocols and the amount of work involved, the instability of the solutions used, the low specificity of the reactions, and their poor reproducibility. The development of immunocytochemistry included the opportunity to use antibodies developed against various proteins synthesized by microglial cells, which were termed microglial markers. Immunocytochemical methods are more convenient than classical impregnation methods but have to be adapted to different biological materials and different fixation methods. One important disadvantage of immuno-

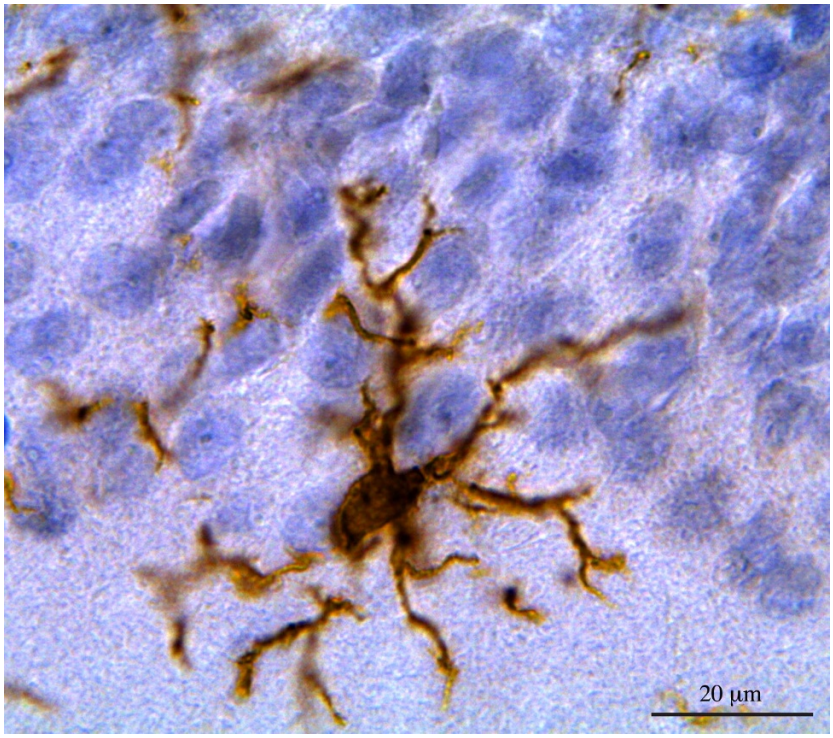

Fig. 1. A microgliocyte in the rat hippocampus. Immunocytochemical reaction for Iba-1. Visualized using diaminobenzidine counterstained with hematoxylin.

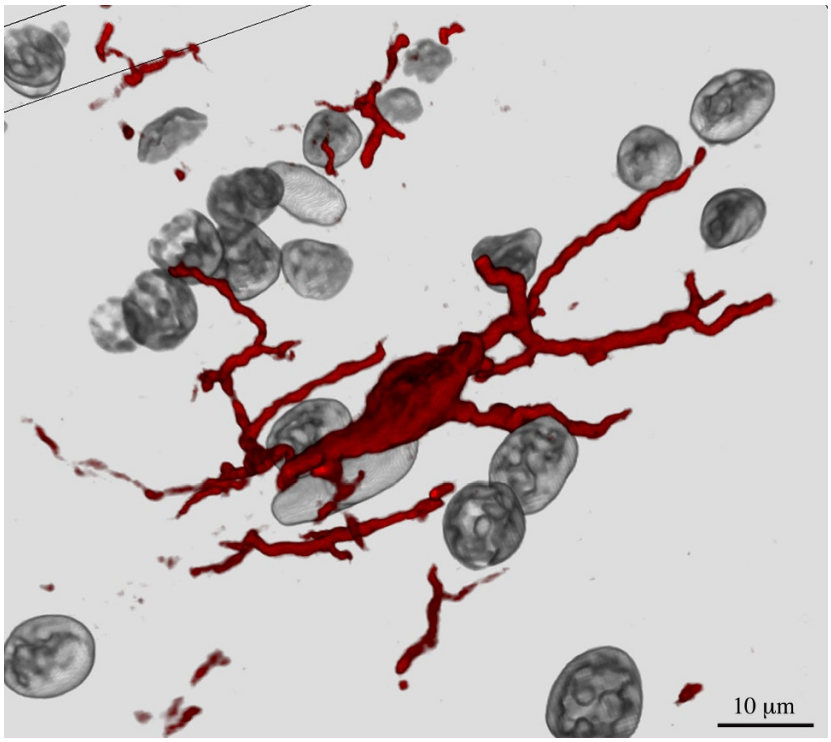

Fig. 2. Three-dimensional reconstruction of a microgliocyte from the subcortical white matter of a rat brain. Immunocytochemical reaction for Iba-1. Fluorescence visualization of immunocytochemical reaction using the fluorochrome Cy3 (red, here dark gray), with nuclei stained with dye SYTOX green (green, here gray). Confocal laser microscopy. Objective: A-Apochromat 63x/1.20 W Korr M27 (water immersion). Z-series size 13.20 μm, number of optical sections 67. Reconstructed using the ZEN 2011 program (Zeiss, Germany).

cytochemical methods is the sometimes rather unpredictable species specificity of the antigens detected. As a result, until recently there was no single immunocytochemical approach giving very clear and selective visualization of the bodies and processes of microgliocytes in laboratory animals and humans using one and the same highly specific marker. The possibility of developing a universal protocol for the immunocytochemical detection of microgliocytes appeared with the discovery of Iba-1 protein by a Japanese group in 1996 [39] and identification of its microglial specificity [41].

Iba-1 Protein and Its Use as a Marker for Microgliocytes. Iba-1 protein (Ionized calcium Binding Adapter molecule 1) is a member of the calcium-binding group of proteins. It has a molecular weight of 17 kDa and consists of 147 amino acid residues forming a compact domain containing two calcium-binding sites rich in hydrophobic amino acids [72]. Iba-1 protein is regarded as analogous to proteins characterized by other authors – AIF-1 (Allograft Inflammation Factor 1), MRF-1 (Microglia Response Factor), and daintain [26, 45]. However, there are doubts regarding the identity of these proteins with Iba-1 [6].

Little is known of the functions of Iba-1. This protein has been shown to take part in reorganizing the cytoskeleton and altering the configuration of the plasmalemma – processes occurring on phagocytosis [54, 55]. Interaction with elements of the cytoskeleton is mediated by the ability of Iba-1 to bind actin molecules [58] forming the corresponding microfilaments.

This new marker has the advantage of the wide interspecies stability of its antigenic epitopes, due to the significant level of conservation of the amino acid sequence of Iba-1 in animals and humans. Thus, comparison of the amino acid sequences of Iba-1 proteins in mice and humans shows them to be 89% identical [72]. The amino acid sequences of the C- and N-terminal fragments of Iba-1 are unique and are not encountered in other proteins [40].

Establishment of the amino acid sequence of Iba-1 protein made it possible to create antibodies to this protein, using synthetic polypeptides corresponding to different parts of the Iba-1 molecule to immunize the animals. Currently available antibodies (for example, rabbit polyclonal antibodies 019-19741, Wako Chemical, and goat polyclonal antibodies ab5076, Abcam) have allowed the successful detection of microgliocytes in the CNS in different mammals and humans [18, 20, 24, 63].

Iba-1 protein is quite uniformly distributed in the cytoplasm and processes of ramified microgliocytes. Studies of the effects of fixation on the retention of the antigenic structure of Iba-1 showed that its antigenic determinants are well preserved both when special immunocytochemical fixatives are used [14] and when the standard, 10% formalin, is used [18]. In the latter case, use of thermal antigen demasking is obligatory; this procedure can be omitted with less "stringent" fixation by increasing the duration of incubation of sections with primary antibodies at elevated temperature or by using highly sensitive detection systems [19]. An obvious advantage of Iba-1 as a selective microglial marker is the fact that it can be used for three-dimensional reconstruction of cells [6] for studies of the complex organization of microgliocyte processes [5].

Monocyte and Macrophage Protein CD68. CD68 is a transmembrane protein with a molecular weight of 110 kDa, present in monocytes and tissue macrophages (including microgliocytes) in humans [30, 37, 60, 62]. In mice, the homolog of CD68 is macrosialin [53]. Both proteins are characterized by high levels of glycosylation and belong to the lysosome-associated membrane protein (LAMP) family [64]. CD68 was initially identified as a protein binding one type of antibody developed against the various epitopes of macrophage antigens, though its function was unclear. It is now recognized that CD68 is involved in the interaction of macrophages with low density lipoproteins and operates as a negative modulator of immune reactions [64]. Several widely used antibody clones have been raised against CD68 – KP1, RMB11, Ki-M6, Y1/82A, Y2/131, and PH-M1 [8, 13, 29, 31]. The most selective of these in relation to tissue macrophages (including microgliocytes) is clone PG-M1 [15, 31], though the antigenic epitope recognized by this clone is species-specific so it cannot be used to study microglia in laboratory animals. In rats, a protein analogous to CD68 is detected using antibodies of clone ED1 [25].

A significant disadvantage of CD68 protein as a microglial marker is the "dotted" nature of the distribution of immunopositive material in the processes of activated and resting microgliocytes, while ameboid microgliocytes are detected quite clearly. These features of the immunohistochemical reaction do not allow the structure of processes to be studied or cells of complex shape to be reconstructed. This would appear to relate to the lysosomal location of this protein. Nonetheless, CD68 and antigen ED1 are quite widely used in neurohistological studies as additional markers for activated macroglia.

CD11b Protein. CD11b protein is part of the type 3 complement receptor (CR3), often designated CD11b/CD18, MO1, or Mac-1 [49, 56], though it is a single protein complex. CR3 is an integrin, while the CD11b molecule is the α subunit of this integrin, which is bound to the β subunit – CD18 [57, 67]. The complement receptor, of which CD11b forms part, is located on the plasma membranes of neutrophil granulocytes and most mononuclear phagocytes and NK cells [23, 44, 67]. Although CD11b is not a highly specific microglial marker, immunocytochemical reactions for this protein are often used as the main or supplementary method for detecting activated microglia. In rats, CD11b is most commonly detected in microgliocytes using monoclonal antibody OX-42 [16, 42, 50, 52]. Despite the fact that OX-42 is the name of a clone reacting with an epitope of CD11b protein, the term "OX-42" is quite often used to refer to the marker itself, creating some difficulty for understanding the functional role of these microglial proteins. A

significant drawback of OX-42 antibody is the fragility of this epitope on embedding in paraffin, so labeling of microgliocytes with CD11b (OX-42) requires use of vibratome [50] or cryostat [16] sections.

Other Microglial Markers. Among other microglial markers, particular note should be made of class II major histocompatibility complex (MHC II) proteins. Microgliocytes, like other tissue macrophages, express these proteins and their expression levels increase on proinflammatory activation of cells and neurodegeneration [71]. In studies of the human brain, activated microglia can be detected using antibodies to HLA-DR antigen [65], which is an MHC II subgroup. In rats, the analogous antigen is termed RT1B and is detected (mostly) using mouse antibodies of clone OX-6. The non-Russian literature contains some references to the use of the microglial marker OX-6 without any indication as to which protein was detected by the immunocytochemical reaction.

Some studies of microglia [38, 51, 61] have used, along with other markers, antigen ED2, so-called on the basis of the antibody clone used to identify it [27, 28]. Of the other proteins expressed by microglial cells, useful information relating to their functional state can be obtained from ferritin, CD115, CD200, inducible NO synthase, F4/80, and Cx3Crl [35, 43, 71, 73].

Conclusions

Thus, microglia are currently detected and their functional state currently analyzed using a variety of immunohistochemical approaches. However, the completeness of the microgliocyte population detected and the quality of the results obtained using different immunohistochemical markers are variable. Some markers (such as HLA-DR, ED2, CD68) are not entirely suitable for analysis of the structural organization of microgliocytes in the intact brain, while others – such as CD200, ferritin, and NO synthase – are insufficiently specific. Furthermore, the majority of antibodies presently available to researchers detect only species-specific epitopes of microglial markers, limiting the potential for comparative interspecies studies.

The variety and functional heterogeneity of microglial markers give rise to sensible questions regarding their adequacy, specificity, and suitability for morphological studies. The analysis of published sources and our own experience presented here leads to the conclusion that of the currently known markers, only two are entirely suitable for structural analysis of microgliocytes in both normal conditions and in the presence of pathological changes to nervous tissue; these are Iba-1 and XD11b (OX-42).

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