IDENTIFICATION OF MICROGLIA AND MACROPHAGES USING ANTIBODIES TO VARIOUS SEQUENCES OF THE IBA-1 PROTEIN

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The Iba-1 protein is traditionally considered a highly selective marker of microglia because of the specific expression of the gene in this particular population of the CNS cells. Alternative splicing creates several isoforms of the Iba-1 protein, which may cause discrepancies in the results of immunohistochomic reactions depending on which epitopes of the immunogen the antibodies selected for the study were developed. In this connection, and with the aim at identifying reliable variants of antibodies to Iba-1 available to researchers in the Russian Federation, we organized with study, seeking to evaluate the results of detecting microglia and macrophages using antibodies to different protein sequences produced by different manufacturers. As material, we used samples of the brain and testis of mature (3–5 months) male Wistar rats (n = 8). Polyclonal and monoclonal (clone JM36-62) antibodies to Iba-1 were used as primary reagents. We found that monoclonal antibodies of the JM36-62 clone enable more selective antigen detection with a better signal-to-background ratio; they can be used as replacements for reagents that are currently not available commercially. Polyclonal antibodies enabled not only immunospecific imaging of microglia and macrophages, but also the identification of cells of the epithelial-spermatogenic layer of the testis. It is assumed that germinal epitelium contains the Iba-1 isoform devoid of an epitope that corresponds to the sequence of the immunogenic antibody clone JM36-62 fragment of the native protein. Functionally, various isoforms of Iba-1 should be investigated further.

Keywords: Iba-1, AIF-1, microglia, macrophages, immunohistochemistry

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ВЫЯВЛЕНИЕ МИКРОГЛИИ И МАКРОФАГОВ С ИСПОЛЬЗОВАНИЕМ АНТИТЕЛ К РАЗЛИЧНЫМ ПОСЛЕДОВАТЕЛЬНОСТЯМ БЕЛКА IBA-1

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Белок Iba-1 традиционно считают высокоселективным маркером микроглии благодаря специфической экспрессии гена именно в этой популяции клеток ЦНС. В результате альтернативного сплайсинга формируется несколько изоформ белка Iba-1, что может служить причиной расхождений результатов иммуногистохомических реакций в зависимости от того, к каким эпитопам иммуногена выработаны выбранные для исследования антитела. В связи с этим, а также с необходимостью определения надежных вариантов антител к Iba-1, доступных для исследователей в Российской Федерации, целью работы было оценить результаты выявления микроглии и макрофагов с использованием антител к различным последовательностям белка, выпускаемых разными производителями. Материалом для исследования служили образцы головного мозга и семенника половозрелых (3–5 месяцев) крыс-самцов Wistar (n = 8). В качестве первичных реагентов использовали поликлональные и моноклональные (клон JM36-62) антитела к Iba-1. Установлено, что моноклональные антитела клона JM36-62 позволяют добиться более высокой селективности выявления антигена при лучшем соотношении сигнал/фон и пригодны для замены реагентов, не доступных в настоящее время к приобретению. Использование поликлональных антител привело не только к иммуноспецифичной визуализации микроглии и макрофагов, но и к выявлению клеток эпителио-сперматогенного слоя семенника. Предполагается, что в клетках эпителио-сперматогенного слоя присутствует изоформа Iba-1, лишенная эпитопа, соответствующего последовательности иммуногенного для антител клона JM36-62 фрагмента нативного белка. Функциональное значение различных изоформ Iba-1 на настоящий момент остается неясным и требует дальнейших исследований.

Ключевые слова: Iba-1, AIF-1, микроглия, макрофаги, иммуногистохимия

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Neuroglia of the central nervous system includes a set of non-neuronal cells that support normal functioning of neurons: nutrients transportation, removal of decay products, myelin formation, signal transmission, etc. [1]. Historically, microglial cells (microglia or microglyocytes) are considered neuroglia, but they have different origin than other glial cells of the central nervous system [2]. Microglia's primary function is development of the brain's immune system [3]. Microglial cells are capable of rapid activation in response to even minor pathological changes in the central nervous system, and in this connection, functional activity of microglia is a widely used indicator of the nervous system's response to infection, trauma, ischemic brain damage, or neurodegeneration [4, 5].

Earlier, antibodies to proteins of class II main histocompatibility complex were used as immunohistochemical markers of microglia. In 1996, scientists isolated the Iba-1 protein (ionized calcium-binding adapter molecule 1) [6], which is considered a highly selective marker of microglia because the *iba1* gene is specifically expressed in this cell population in the brain. Several authors consider this protein to be identical to AIF-1 [7–10]. Currently, Iba-1 is the most common protein in microglia studies. However, adequate interpretation of the results of immunohistochemical reaction is problematic, because the *iba1* gene can undergo alternative splicing, which yields several isoforms of the protein [11]. Since these isoforms spread in slightly different ways [12], study to study, immunohistochemical reactions can yield different results, which depend on the epitopes of the immunogen that triggered production of the antibodies. Alternative splicing and various protein isoforms may be why occasionally antibodies to Iba-1 enable detection of not only microglia and monocyte-macrophage cells, but also some of other origin. For example, a positive reaction to the Iba-1 protein was detected in smooth myocytes [13], breast ductal tumor epithelium [14] hepatocellular carcinoma [15], and in the germinal epithelium of the seminiferous tubules [6, 16]. Thus, the alleged high specificity of the respective antibodies to the Iba-1 protein, considered a microglia marker, is doubtful.

One of the most common antibodies used for microglia labeling are goat antibodies to Iba-1 (Abcam, UK; catalog number: ab5076), which, according to the manufacturer's website, are mentioned in 1145 publications. They are produced against a synthetic peptide that mimics the C-termini of the Iba-1 protein. However, currently, antibodies to Iba-1 are (Huabio, China; catalog: ET-1705-78) are easier to procure in the Russian Federation. The immunogen for these primary reagents is a peptide with a sequence similar to the protein's N-termini. These primary antibodies delivered good results in the context of studies employing tissue samples from various laboratory animals [17–19].

Identification of more reliable variants of antibodies to Iba-1 that can be used for microglia labeling, and determination of their interchangeability are urgent tasks, therefore, this study aimed to evaluate the results of detection of microglia and macrophages that relied on antibodies to different sequences of the Iba-1 protein produced by different manufacturers.

METHODS

Samples of brain and testis tissues of mature (3–5 months old) male Wistar rats weighing 350–400 g (n = 8) were used as material in this study. The animals came from the Rappolovo laboratory animal nursery (Leningrad Region, Russia); they were kept in a vivarium at room temperature under standard conditions, with free access to food and water. Before sampling, the animals were euthanized by an ethyl ether vapors overdose. The sampled material was fixed in zinc-ethanol-formalin and embedded in paraffin in a standard way. Five-micron thick sections were cut from the paraffin blocks on a Leica RM 2125RT rotary microtome, and mounted on adhesive slides HistoBond®+ (Paul Marienfeld; Germany). Brain slices were made at –2.12 mm from bregma.

After deparaffinization (standard technique), we inhibited endogenous peroxidase by incubating the sections in a 3% aqueous solution of hydrogen peroxide for 10 minutes. Before application of the primary antibodies, the slices were treated with normal equine serum (008-000-121; Jackson Immunoresearch, USA) or blocking solution (ab64226; Abcam, UK) at room temperature for 10 minutes. For microglia and macrophages detection, we used antibodies to the calcium-binding protein Iba-1: goat polyclonal antibodies diluted 1:1000 (ab5076; Abcam, UK), and recombinant rabbit monoclonal (clone JM36-62) antibodies diluted 1:1200 (ET-1705-78; Huabio, China). Incubation with primary antibodies took place in a humid box for three days at a temperature of 27 °C.

UltraVision Quanto Detection System HRP (TL-060-QHL; Fisher Scientific, USA), which reacts to mouse and rabbit primary reagents, and a VECTASTAIN Universal Quick HRP kit (PK-8800; Vector Laboratories, USA), which detects primary reagents of mice, rats, and goats, were used as the secondary reagents. In addition, we took secondary reagents from the Cell & Tissue Staining Kit (CTS005 and CTS008; R&D Systems, USA) with monoreactivity to rabbit and goat antibodies, respectively. The incubation modes for secondary reagents were as per the manufacturer's recommendations. For both antibody variants, we performed negative control tests using the appropriate sets of secondary reagents and an HRP detection system. For this purpose, the slices were treated with antibody diluent instead of the primary antibodies solution. Normal rat serum was used to block non-specific binding of anti-mouse secondary antibodies to the closely related rat immunoglobulins.

To visualize the product, we used chromogen 3'3-diaminobenzidine from the DAB+ kit (K3468, Agilent; USA). Once the immunohistochemical reaction was set up, some of the sections were stained with alum hematoxylin or alcyan blue. After dehydration and clearing in orthoxylene (Vecton; Russia), the preparations were put into the Cytoseal 60 medium (23-244257; Richard-Allan Scientific, USA). The resulting preparations were analyzed using a Leica DM750 microscope (Leica; Germany) fit with an ICC50 digital camera (Leica; Germany). To capture images, we used the LAS EZ image capture program (Leica; Germany).

Using various antibodies to Iba-1, we digitally assessed the optical density of the stained product of the immunohistochemical reaction. The data were collected in the striatum area; its processing relied on the Fiji morphometric analysis program (ImageJ). The Region of Interest feature allowed pre-selecting preferred areas (10 × 10 microns). We evaluated the average brightness per unit of area of microglyocytes, blood vessel lumen, and striatum neuropile. The results of the study were given in the optical density units. The resulting data were processed in GraphPad Prism 8 (GraphPad Software; USA) and presented as mean ± SD, with 7 values used to calculate that mean.

We used the UniProt service to cross reference the amino acid sequences of the isoforms of Iba-1 (AIF1 in another classification) [20], and compared sequences P55008-1, P55008-2, and P55008-3.

RESULTS

The preliminary analysis of the preparations confirmed the sections and the tissues of the samples were well-preserved.

A high-intensity immunohistochemical reaction to Iba-1 was observed on brain and testis preparations with a minimum level of background staining, as opposed to rabbit primary reagents (none) and goat primary antibodies (insignificant).

Iba-1-positive cells were found in all the studied rat tissue preparations. In brain, they were typical microgliocytes with a large number of thin branched processes (Fig. 1A–D). Spindle-shaped cells with flattened large processes were seen near gray matter's blood vessels. Monoclonal rabbit antibodies forced the processes of Iba-1-containing cells to grow numerous spike-like outgrowths. Moreover, monoclonal antibody reaction was found in the meninges (oval cells, no processes, intensely colored cytoplasm).

The use of goat polyclonal antibodies to Iba-1, despite the high intensity of the specific reaction, yielded an insignificant background on the preparations: minor staining of the neuropile, and a more pronounced nonspecific background staining of the serum of large blood vessels (Fig. 1A). The intensity of staining of the neuropil and serum of large blood vessels was 0.61 ± 0.03 and 0.75 ± 0.03, respectively. Monoclonal rabbit antibodies did not trigger noticeable background staining: the optical density of the stained chromogen in the neuropile and lumen of blood vessels was 0.57 ± 0.02 and 0.59 ± 0.01, respectively. The average value of the microglyocyte staining intensity for both antibody variants was similar: 0.87 ± 0.09 for polyclonal antibodies and 0.87 ± 0.08 for antibodies of the JM36-62 clone.

The use of both variants of primary antibodies on rat testis tissue samples allowed detecting two types of immunopositive stroma cells of convoluted seminiferous tubules (Fig. 1E–F). In the first case, these are cells found close to the vessels. They have a small, rounded circumnuclear region, and thin non-branching processes. The second type are large oval shape with few or no processes. The latter can be isolated or group (3–4 cells per group).

The third type of cells was detected only with the help of polyclonal goat antibodies to Iba-1. In this case, cytoplasm of cells of the germinal epithelium of the seminiferous (seminiferous epithelium) tubules contained accumulation of the product of immunohistochemical reaction. Morphologically, they are similar to early (rounded) and late (elongated) spermatids (Fig. 1E, double arrow). No Iba-1 immunopositive cells were found in the seminiferous epithelium on any of the studied preparations treated with rabbit primary reagents.

Cell & Tissue Staining Kit, which relies on avidin-biotin amplification, produced immunohistochemical reaction results similar to those registered for VECTASTAIN Universal Quick HRP kit and UltraVision Quanto Detection System HRP.

Comparison of the amino acid sequences of three isoforms of Iba-1/AIF1 (sequences P55008-1, P55008-2, and P55008-3 from the UniProt) has shown that this or that isoform may be lacking such sequences of immunogenic fragments of various antibodies. For example, N-terminus of the immunogenic fragment for monoclonal rabbit antibodies of the JM36-62 clone was found only in P55008-1 (with a sequence of 147 amino acids), and the shorter isoforms P55008-2 and P55008-3 (with sequences of 93 and 132 amino acids, respectively) did not have this fragment. In contrast, the region of C-terminus of the immunogenic fragment for polyclonal goat antibodies Abcam is present in the sequences of P55008-1 and P55008-2 (two isoforms of the three studied).

DISCUSSION

Both primary antibodies used in this work yielded good results of the immunohistochemical reaction, and the ratio of immunospecific signal's intensity to nonspecific background staining was high. Nevertheless, the protocol that involved goat primary reagents produced a higher level of background staining of the brain neuropile and testicular parenchyma compared to the protocol that uses the JM36-62 clone antibodies. Figure 1 shows a seemingly lower background-to-specific signal ratio compared to the case of polyclonal antibodies. An additional quantitative study of the digital images has shown that both variants of antibodies trigger specific immunohistochemical reactions of almost similar intensity. However, the intensity of staining of neuropil and serum from the large blood vessels was indeed higher for preparations stained with polyclonal goat primary reagents. Such reagents are known to impose certain limitations on immunohistochemical studies employing indirect immunolabelling (by Coons) and modifications thereof. Firstly, the most common secondary reagents are mono- or bivalent, i.e., they counter immunoglobulins of one or two animal species (typically — rabbit and mouse), which significantly complicates selection of secondary antibodies and increases the cost of the study. Secondly, non-specific background is more pronounced when goat antibodies are used, while the intensity of reaction increases only slightly because such antibodies react with bovine immunoglobulins, too, and reagents containing bovine serum albumin can contain trace amounts of those [6]. The results of this immunohistochemical study confirm this.

The Iba-1 protein, which consists of 147 amino acid residues and has the molecular weight of 17 kDa [21], is always present both in the body and in the processes of microglial cells. This is why it is widely used in tests designed to evaluate the morphofunctional status of microglia. Interacting with fimbrin, Iba-1 supports crosslinking of actin filaments and regulation of the configuration of plasma membrane [22], so the its level increases with microglial activation [23]. However, this property of Iba-1 also means it is found in other phagocytic cells of the body. Indeed, as shown in numerous papers, antibodies to Iba-1 are often used to identify monocyte-macrophage cells. Thus, resident macrophages of various organs of laboratory animals and humans are immunopositive to Iba-1, including Kupffer cells [18, 24], alveolar macrophages of the lungs and macrophages of the spleen [25, 26], and Langerhans cells [6, 16]. Therefore, this study was expected to yield data pointing ti the presence of Iba-1 in interstitial testicular macrophages and leptomeningial brain cells. In the context of an immunohistochemical reaction involving various antibodies to Iba-1, a much more interesting result has to do with the cells of the germinal epithelium of seminiferous tubules. With goat polyclonal antibodies, the reaction yielded stained products in cells that morphologically are similar to spermatids. Antibodies of the JM36-62 clone did not bind antigens in the germinal epithelium.

Several papers state presence of the Iba-1 protein in spermatids found in the seminiferous tubules [7, 16, 27–29]. However, the results of our study were not consistent therewith for this group of cells with different primary antibodies, which may have been conditioned by properties thereof in the context of detection of epitopes of different specificity, or properties of secondary reagents. Therefore, in order to exclude a false positive reaction from the testicular spermatids, we set up an additional control experiment using Cell & Tissue Staining Kit, identical secondary imaging systems for goat and rabbit antibodies based on avidin-biotin technology, which ensures blocking of endogenous biotin. The results of this reaction were consistent with those registered for the experiments with UltraVision Quanto Detection System HRP (rabbit) and the VECTASTAIN Universal Quick HRP kit (goat). This further emphasizes that the reaction from spermatids is not a

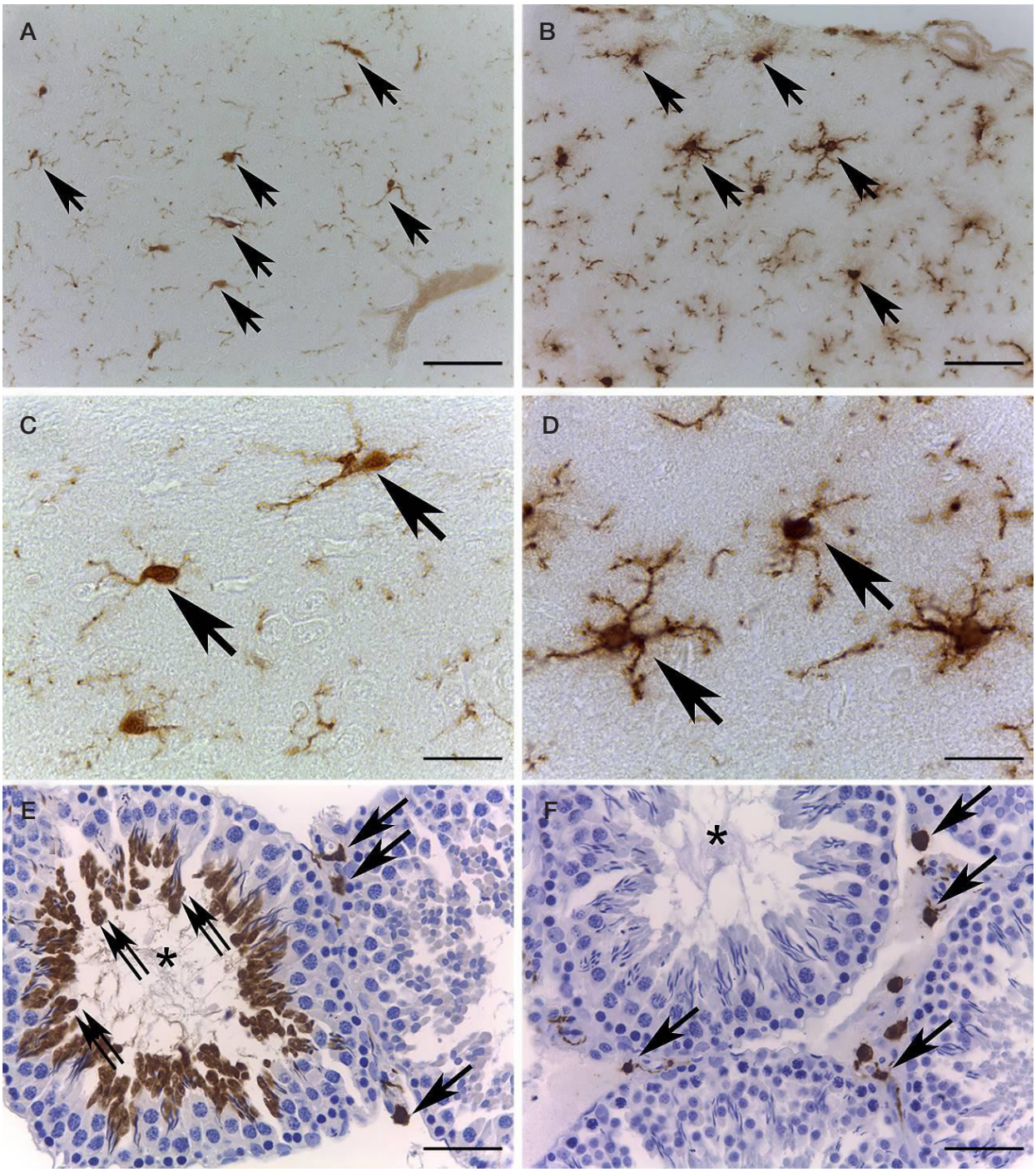

Fig. 1. Iba-1 in rat cells, identified with polyclonal goat (**A**, **C**, **E**), monoclonal rabbit (**B**, **D**, **E**) antibodies. **A–D**. Brain preparations (arrows indicate microglia). **E–F**. Convoluted seminiferous tubules. Arrows point to Iba-1-containing cells in the interstitium, double arrows indicate such in the germinal epithelium, *asterisk* — in the tubule's lumen. Scale: 50 microns (**A**, **B**, **E**, **F**), 20 microns (**C**, **D**)

consequence of non-specific binding of secondary reagents (namely, streptavidin).

This result of the immunohistochemical reaction may come from the differences in antigenic determinants: Iba-1 epitopes in spermatids could have been more like the parotopes of polyclonal, but not monoclonal antibodies. Thus, according to the information provided on the manufacturer's website, a synthetic peptide with the TGPPAKKAISELP sequence localized at the Iba-1 protein's C-terminus was used as an antigen for polyclonal goat antibodies. There is also a note there stating that ab5076 antibodies are capable of detecting at least two isoforms of Iba-1/AIF1. In contrast, the immunogenic peptide for monoclonal rabbit antibodies had the sequence of SQTRDLQGGKAFGL, which corresponds to the N-terminus. A comparison of the sequences of different isoforms of human Iba-1/AIF1 has shown that the sequence of the immunogen for rabbit antibodies corresponds to the protein isoform with a sequence of 147 amino acids, which is accepted as canonical [6]. According to the comparison of the amino acid sequence of protein fragments corresponding to the immunogenic peptide, goat polyclonal antibodies should enable detection of both the canonical isoform and the protein's shorter forms. Thus, our data indirectly indicate that alternative splicing of *iba1* matrix RNAs can exclude from the final transcript the N-terminus detected by monoclonal antibodies of the JM36-62 clone but leave the C-terminus of present isoforms detected

by polyclonal antibodies produced by Abcam. A review of the initial publications that give characteristics of the Iba-1 protein has shown that the authors studied the expression of the gene in various organs [13–16]. Thus, they demonstrated high expression of *iba1* in the testes and spleen, and weak expression in the lungs, kidneys and brain (due to the specific expression of the gene exclusively in macrophages and/or microglia). A large number of other studies covering expression of *iba1* in various tissues [16–18, 24, 25] also suggests that it is not limited to microglia, monocytes, and macrophages. Therefore, it is possible that goat polyclonal antibodies actually detect the Iba-1 protein, in particular, its isoform that is not present in the monocyte-macrophage cells.

CONCLUSIONS

Monoclonal antibodies of the JM36-62 clone enable more selective antigen detection with a better signal/background ratio; they can be used as replacements for a more commonly used reagents. Apparently, higher selectivity of the JM36-62 clone antibodies is the result of the manufacturer choosing a more fitting immunogen. The fact of detection of spermatids with the help of Abcam polyclonal may stem from the presence therein of Iba-1 isoform that is devoid of an epitope corresponding to the sequence of the native protein's N-terminus. Further studies are needed to assess the functional significance of the various isoforms of Iba-1.

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