Immunohistochemical analysis of microglial changes in the experimental acute hepatic encephalopathy

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Hepatic encephalopathy (HE) is a syndrome of impaired brain function in patients with advanced liver failure and it manifests in form of psychometric tests alterations up to decreased consciousness and coma. The current knowledge about HE mainly focused on the theory of ammonia neurotoxicity and neuroinflammation. Microglia being resident innate immune cells of the brain when activated are responsible for the neuroinflammatory reactions.

The aim – immunohistochemical study of the microglial changes in different rat brain regions in conditions of experimental acute HE (AHE).

Materials and methods. We used acetaminophen induced liver failure model in Wistar rats. Four from 10 animals that survived up to 24 h after acetaminophen injection constituted “compensated group”; 6 animals which died within 24 h – “decompensated group”. Microglial reactive changes were analysed by the evaluation of the relative area (S rel., %) of CD68 expression in the brain cells not associated with meninges and vessels, as well as the changing in shape and number of these cells.

Results. Acetaminophen-induced AHE in rats was characterized by the regional- and time-dependent dynamic increase in CD68 expression level in the rat brain in form of significant (relatively to control) increase of CD68 S rel. in brain cells and the number of such cells. The highest and statistically significant changes observed at the 24th hour of experiment were, respectively: subcortical white matter – 0.24 (0.20; 0.26) and 11.00 (8.00; 13.00); thalamus – 0.13 (0.90; 0.18) and 6.00 (3.00; 7.00); caudate/putamen – 0.13 (0.12; 0.18) and 7.00 (4.00; 11.00) – all indicators were statistically significant compared to control. In the survived animals, indicators were, respectively: subcortical white matter – 0.24 (0.16; 0.26) and 10.00 (8.00; 12.00); caudate/putamen – 0.12 (0.10; 0.15) and 6.00 (4.00; 10.00) – the differences were significant compared to control.

Conclusions. The highest and significant indicators were revealed at 24 h (compared to earlier time points) of the experiment in the white matter, thalamus and caudate/putamen. This fact reflects time-dependent dynamic boosting of reactive changes in microglia and presumably may indicate the regions of the most active neuroinflammatory response within the brain parenchyma in the conditions of AHE. The appearing of a small percentage of cells with amoeboid transformation among CD68+ cells may mean partial functional insufficiency of such cells due to probable suppressive impact of ammonia or other influencing factors, as well as insignificance of the material that needs to be phagocytosed under established conditions.
Immunohistochemical analysis of microglial changes in experimental acute liver failure

T. V. Shuliatnikova

Hepatic encephalopathy (HE) is a syndrome of impaired brain function in patients with advanced liver failure [1]. This complex state presents in a wide spectrum of neuropsychiatric manifestations from alterations of psychometric tests up to progressive spatiotemporal disorientation, decreased consciousness and coma [2]. The pathogenesis of HE is complicated and specific disease mechanisms remain to be determined, however the current knowledge about HE development mainly focused on the theory of ammonia neurotoxicity as a result of substantial liver dysfunction. Besides this concept, the role of systemic inflammation and neuroinflammatory response of the brain was emphasized [1]. Microglia being resident macrophages of the central nervous system when activated by systemic pro-inflammatory cytokines are responsible for the brain neuroinflammatory reactions [3]. Neurotoxic forms of activated microglia release a range of pro-inflammatory and cytotoxic mediators including IL-1β, IL-1α, TNF-α, NO, prostanooids all inducing damaging effect on the neighboring tissue elements and affect brain homeostasis [3]. The most prominent neuropathological sign of acute hepatic encephalopathy (AHE) is the development of severe brain edema mostly caused by cytotoxic decompensated astrocyte swelling. Though the fine pathophysiological links of the brain edematous changes in AHE are still not entirely clarified, it was proposed that pro-inflammatory cytokines released by the part of activated microglia might be directly related to these mechanisms [4].

Different animal and cell culture studies have shown the controversial influence of acute or chronic hyperammonemia on microglial and astroglial reactivity mostly indicating microglial activation associated with increased expression of proinflammatory IL-6 and TNF-α cytokines [5–7]. However, the precise primary cellular source of noted cytokines synthesis, secretion and consequential causal factors inducing inceasement of them are still inconsistent [8]. Considering the above, as well as the fact of small amount of in vivo studies of microglial reactivity in AHE conditions, it would be useful to study reactive response of this neuroglial pool in animal model of acute liver failure (ALF). The concept of microglial activation still has not clear definitions, however as a functional sign of the latter it was widely considered acceptable to use CD68 marker, that indicates highly activated phagocytic microglia/macrophages [9]. Moreover, given the recently identified wide
context-dependent and region-dependent morpho-functional heterogeneity of the microglial population in the brain [10,11], a more detailed research of the microglial reactivity in different brain structures would make a great contribution in the better understanding of the cerebral dysfunction mechanisms in the conditions of ALF.

Acetaminophen is the most widely used antipyretic and/or analgesic drug and its overdosing is the primary cause of ALF in many countries [12]. Due to the fact, that such overdosing also causes ALF in rodents this model can be used for analysing mechanisms of acute hepatic encephalopathy similar to that in humans [13,14].

Aim

Immunohistochemical study of the microglial changes in different rat brain regions in the conditions of experimental acute hepatic encephalopathy.

Materials and methods

For experimental purpose Wistar rats, 200–300 g, were used. All procedures were conducted according to the European convention for the protection of vertebrate animals (Strasbourg, 18 March 1986; ETS No. 123) and the Directive 2010/63/EU. Rats were divided into control group (n = 5) and ALF-group (n = 10). For induction of AHE type “A” ("Acute liver failure" – according to the American Association for the Study of Liver Disease updated guidelines), we used acetaminophen (paracetamol, N-acetyl-p-aminophenol [APAP]) induced liver failure (AILF) model [13,14]. The detailed description of all steps and characteristics of the experimental model can be found in our previous paper [15]. After intraperitoneal (i.p.) acetaminophen injection, rats were examined for signs of changed major physiological parameters, lethargy, loss of reflexes. Six rats were euthanised up to 24 h after the acetaminophen injection. Euthanasia was achieved by an i.p. administration of sodium thiopental euthanasia due to the above severe clinical symptoms. Four animals that survived up to 24 h after the procedure were designated to group “AILF-A” – compensated ALF; 6 animals which died within 24 h after injection constituted the group “AILF-B” – decompensated ALF. In the control group “AILF-C”, all animals survived up to 24 h. In 24 h after ALF-procedure, all survived and control animals were euthanised by i.p. injection of sodium thiopental solution.

The material of the brain and liver tissue was processed according to standard procedures with formation of paraffin blocks. For general histopathological analysis hematoxylin-eosin stained sections were used. Immunohistochemical (IHC) study involved detection of immunopositive labels using mouse monoclonal anti-CD68 antibody (clone PG-M1, RTU, Dako, Denmark) and UltraVision Quanto detection system (ThermoScientific, USA) at magnification ×200 in the standardized field of view (SFV) of the Scope A1 microscope (Carl Zeiss, Germany). Morphometric calculations were done using Videotest-Morphology 5.2.0.158 program. The characteristics of CD68 expression were evaluated as the relative area (S rel., %) of immunopositive labelling to SFV. In addition, numbers of immunopositive cells and the percentage of positive cells with changed morphological type to amoeboid type were assessed. Latter ones show more round form due to more abundant cytoplasm, with shortened, thickened processes in relation to rod-shaped microglia (inset on the Fig. 4). To identify specific features of glial reactivity such regions were selected as prefrontal cortex, underlying white matter, thalamus, hippocampus and caudate nucleus/putamen region.

Digital data were statistically processed by Statistica® for Windows 13.0 (StatSoft Inc., license № JP-ZB04I382130ARCHN10-J) with evaluating median, lower and upper quartiles. For comparison between groups Mann–Whitney and Kruskal–Wallis tests were used. The results were considered significant at 95 % (P < 0.05).

Results

In our study all experimental animals showed the clinical signs of growing acute brain disfunction finished by comatose state. Pathohistological changes in their liver tissue have evidenced its widespread toxic damage. Thus, at the period up to 24 h, all AILF-rats had pathohistological signs of centrolobular necrosis, focal hemorrhages, as well as severe balloon dystrophy of hepatocytes. The degree of these changes had dynamic increase with the time after injection. The IHC study of the brains showed that in control animals, the expression of the CD68 in the brain tissue was unequally expressed, region-specific and manifested at a low level in all studied parameters. Control animals demonstrated the highest indicators of the CD68+S rel. (%) and the numbers of CD68+ cells in the subcortical white matter in relation to other regions. Moreover, white matter was characterized by the most significant indicators (in comparison to other regions) percentage of CD68+ cells with morphologic transformations into amoeboid phenotype. Cortical, hippocampal, thalamic and caudate/putamen regions of control rats demonstrated relatively smaller numerical indicators of the studied parameters. The percentage of round-shaped amoeboid immunopositive cells in all noted regions was represented at the minimum level (Table 1).

In the brain tissue of survived rats with compensated AILF (AILF-A) group in relation to control group, by 24 h of the experiment the moderate, regionally heterogeneous elevation in the indicators of microglial reactivity was found. The highest and statistically significant parameters of CD68+S rel. (%) and the number of CD68+-cells were characteristic for caudate/putamen and subcortical white matter compared to other studied areas. The cortex, thalamus and hippocampus also showed increase of S rel. (%) and numbers of CD68+ cells, however they did not statistically differ from the control values. The same statistical insignificance of differences applied to moderate increase in the number of cells changing their morphology to amoeboid shape in subcortical white matter of survived rats (Table 1).

The brain microglial reactive changes in non-survived animals with decompensated AILF (AILF-B) demonstrated statistically significant regional-dependent increase in two studied parameters. The highest rates of the indicators of the CD68+S rel. (%) and numbers of CD68+-cells were
Table 1. The indicators of microglial reactivity in different brain regions in animals from different experimental groups

<table>
<thead>
<tr>
<th>AILF-A</th>
<th>AILF-B</th>
<th>AILF-C</th>
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<tr>
<td>Cortex</td>
<td>Cortex</td>
<td>Cortex</td>
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<tr>
<td>S rel. (%) in SFV</td>
<td>CD68(^+) cells number in SFV</td>
<td>% of CD68(^+) cells in SFV with amoeboid morphology</td>
</tr>
<tr>
<td>0.13 (0.10; 0.16)</td>
<td>5.00 (4.00; 7.00)</td>
<td>3.00 (1.00; 5.00)</td>
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<tr>
<th>Subcortical white matter</th>
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<td>S rel. (%) in SFV</td>
<td>CD68(^+) cells number in SFV</td>
<td>% of CD68(^+) cells in SFV with amoeboid morphology</td>
</tr>
<tr>
<td>0.24 (0.16; 0.26)*</td>
<td>13.00 (8.00; 12.00)*</td>
<td>6.00 (3.00; 11.00)</td>
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<tr>
<th>Hippocampus</th>
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<tr>
<td>S rel. (%) in SFV</td>
<td>CD68(^+) cells number in SFV</td>
<td>% of CD68(^+) cells in SFV with amoeboid morphology</td>
</tr>
<tr>
<td>0.14 (0.11; 0.17)</td>
<td>7.00 (4.00; 9.00)</td>
<td>4.00 (1.00; 8.00)</td>
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<th>Thalamus</th>
<th>Thalamus</th>
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<tr>
<td>S rel. (%) in SFV</td>
<td>CD68(^+) cells number in SFV</td>
<td>% of CD68(^+) cells in SFV with amoeboid morphology</td>
</tr>
<tr>
<td>0.10 (0.07; 0.13)</td>
<td>5.00 (2.00; 7.00)</td>
<td>2.00 (1.00; 4.00)</td>
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<th>Caudate/putamen</th>
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<tr>
<td>S rel. (%) in SFV</td>
<td>CD68(^+) cells number in SFV</td>
<td>% of CD68(^+) cells in SFV with amoeboid morphology</td>
</tr>
<tr>
<td>0.12 (0.10; 0.15)*</td>
<td>6.00 (4.00; 10.00)*</td>
<td>3.00 (2.00; 5.00)</td>
</tr>
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Data are presented as median (Me) with lower and upper quartiles (Q1; Q3); *: significant differences in indicators of the same brain region compared to the control animals (P < 0.05); AILF-A: compensated AILF; AILF-B: decompensated AILF; AILF-C: control group.

Fig. 1. Dynamics of the relative area of CD68\(^+\) expression (in the microscope SFV, %) in the subcortical white matter of AILF-B rats in the period after injection.

Fig. 2. Dynamics of the relative area of CD68\(^+\) expression (in the microscope SFV, %) in the thalamus of AILF-B rats in the period after injection.

Fig. 3. Dynamics of the relative area of CD68\(^+\) expression (in the microscope SFV, %) in the caudate/putamen of AILF-B rats in the period after injection.

Fig. 4. CD68\(^+\) expression in the caudate/putamen of the non-survived rat (AILF-B group) 24 h after the injection. The inset shows the magnified image of an amoeboid cell (anti-CD68, Dako, Denmark). ×200.
noted in (descending order): subcortical white matter, caudate/putamen and thalamus. In the hippocampus and cortex increase of noted indicators was not statistically different relatively to the control. The slight increase in the percentage of cells with changed morphology in such regions as caudate/putamen, thalamus and subcortical white matter of AILF-B rats was also not statistically different relatively to the indicators of control animals (P > 0.05) (Table 1).

Thus, the data from 3 experimental groups clearly demonstrated the substantial, reliable increase (compared to control values) in indicators of CD68\(^+\) S rel. (%), as well as the number of CD68\(^+\) cells in the subcortical white matter, caudate/putamen and thalamus of non-survived AILF-B-animals and for subcortical white matter and caudate/putamen of survived AILF-A-rats.

In the AILF-B group depending on the time after acetaminophen injection, when decompensation of animal state occurred, the numerical rates of microglial reactive changes were different. The maximal level of the CD68\(^+\) S rel. (%) was found in caudate/putamen, thalamus and subcortical white matter at 24 h after the initiating of the experiment (Fig. 1–4).

**Discussion**

Besides the old issues on microglia origin, the novel problem of heterogeneity of microglial populations within different or even the same adult brain region has arisen recently [10]. The already established concept of M1-like/ M2-like microglial polarization also has been revised and now is the subject of great controversy [11]. However, this subdivision still exists and is actively used by current neuroscience researchers due to the lack of the evidence-based tools for evaluating the precise microglial effect on tissue media under certain conditions within the tissue in *in vivo* experiments or during the study of postmortem human material. In the brain tissue CD68\(^+\) cells can be generally represented by microglia and perivascular macrophages, as well as hematogenous phagocytes entering the brain as a result of various brain-systemic challenges [10]. Therefore, in our study we excluded positive cellular immunolabelling related to the structures of blood brain barrier (BBB).

The current knowledge on the microglial role in the impairment of the astrocytic water metabolism under action of ammonia neurotoxicity remains unclear to a large extent. Studies of microglial reactive using different models of ammonia intoxication, including primary cell cultures and acute or chronic HE animal models, have shown controversial results. Zemtsova I. et al. has experimentally demonstrated the direct activation of cultured rat microglia by ammonia in form of increased expression of Iba-1 (ionized calcium binding adaptor molecule 1) – protein involved in reorganization of the cytoskeleton, required for morphology changing, cell migration and phagocytosis. Although this was accompanied by activated cellular migration, there was simultaneous inhibition of phagocytic microglial activity. Despite noted “activation” signs cultured microglia did not increase proinflammatory cytokines mRNA expression, whereas ROS production was induced [3]. On the other hand, Rao et al. using primary microglial cultures has shown an increase in the levels of proinflammatory cytokines, oxy-radicals and nitric oxide after ammonia exposure, and conditioned media derived from ammonia-treated microglia when added to cultured astrocytes led to significant astrocyte swelling [5]. Thrane R. V. et al. in the slower azoxymethane-induced HE demonstrated that microglia did not become activated prior to the onset of neurological dysfunction at the terminal stage of HE and coincides with BBB opening. In the same study using a rapid acute hyperammonemic AHE model induced by ammonia load authors did not show any signs of microglial activation, despite animals developed high levels of plasma ammonia and severe neurological impairment. Consequently, authors suggested that microglial activation does not contribute to the early neurological dysfunction during either HE and AHE [7].

Our study revealed partially similar characteristics of microglial reactivity but in the conditions of AILF-induced AHE, although, this did not apply to all indicators. In AILF-animals a small percentage of the brain CD68\(^+\) cells, as in control rats, was presented as round-shaped (amoeboid) forms which indicated activation of their phagocytic activity at that moment. Moreover, the increase in number of CD68\(^+\) cells in specified brain regions, as well as the relative area of the marker expression (with respect to control) indirectly evidence to inducing of migration activity of these phagocytes within the brain tissue, while not excluding invading parenchyma through the brain barriers. As noted above, in both lethal and survived animals, morphological transformations of cells towards the amoeboid form did not have significant differences compared to control for the same regions. Altogether, noted features presumably may indicate the early structural reactive changes of microglia and simultaneous lack of their functional activity under action of ammonia and other neurotoxins elevated due to acute liver failure. However, this does not exclude the relative insignificance of the amount of tissue material that needs to be phagocytosed under established experimental conditions. Elevation of S rel. CD68\(^+\) and number of such cells appeared to have a region-dependent specificity: indicators were significantly higher in the subcortical white matter, caudate/putamen of the both groups and in thalamus of non-survived rats, compared to control. This fact allows to conclude that indicated regions are possibly ones where neuroinflammatory response unfolds most intensely in the conditions of AILF. Dynamic evaluating of noted parameters in the uncompensated and euthanized AILF-B-rats helped to recognize that changing of microglial reactivity developed in time-dependent manner. The highest indicators of the CD68\(^+\) S rel. (%) and numbers of cells were found in the white matter, thalamus and caudate/putamen at 24 h after injection (if compared to all earlier time points) (Fig. 1–3). The revealed difference in higher, statistically significant values of microglial reactivity in non-survived rats may indicate predominance of neurotoxic effects of CD68\(^+\) cells, as well as represent phagocytosis activation as a subsequent mechanism of tissue cleansing after the irreversible damaging impact of neurotoxins. These features can be comparable to our previous study on astroglial reactivity in the conditions of experimental AILF, where the most severe edematous...
and destructive changes in astrocytes were characteristic for decompensated cases of AILF at 24 h after injection [15]. This fact can indirectly evidence the close interaction between astro- and microglia in the conditions of AHE, although this still does not give a clear answer to the question: "Which one of this pair has a primary influence on the other and what is this effect?" Clarifying these questions requires more comprehensive technological approaches and complex study.

Conclusions

1. In the conditions of AILF in the rat brain there is a dynamic, region-specific increase in microglial reactive changes.
2. The highest indicators of microglial reactive changes were revealed at 24 h after initiation of AILF in the subcortical white matter, thalamus and caudate/putamen which presumably may indicate the regions of the most active neuroinflammatory response within the brain tissue in acute hepatic encephalopathy.
3. The small percentage of cells with amoeboid shifting among CD68+cells may mean partial functional insufficiency of these cells due to probable suppressive impact of ammonia and other influencing factors or relative insignificance of the amount of tissue material that needs to be phagocytised under established conditions.

Prospects for further research. Considering the central role of neuroglia in adaptation and responses of the brain to various systemic and intracerebral challenges, it is necessary to study the interaction between different glial pools in the conditions of somatogenic toxic encephalopathies, including hepatic one, not only in experimental models, but also using postmortem human material.

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Conflicts of interest: author has no conflict of interest to declare.

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