Lectin histochemistry and ultrastructure of microglial response to monosodium glutamate-mediated neurotoxicity in the arcuate nucleus

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Summary. In this study we describe the most relevant morphological features of the microglial reaction that takes place in the arcuate nucleus (AN) after neurotoxic injury induced by a single subcutaneous injection of monosodium glutamate (MSG) in neonatal rats. The time course of the reaction was evaluated by lectin-histochemistry. Microglial/macrophagic cells were labelled with the lectin obtained from Lycopersicon esculentum and with B4 isolecitin from Griffonia simplicifolia. The microglial response was also studied by ultrastructural observations. The histochemical study revealed the presence of few reactive microglial cells at 6 h post-injection. These cells were intensely stained and had a globular morphology but contained no neuronal debris inside them when observed under the electron microscope. At 12 h post-injection, the number of microglial cells had increased and, at the same time, intense phagocytic activity was observed ultrastructurally. The microglial reaction peaked at 24 and 36 h post-injection, when the number of microglial/macrophagic cells was maximum, although the ultrastructural observations showed that at 36 h the amount of debris ingested by macrophages was decreased with respect to animals sacrificed at 24 h. Finally, at 4 days after neurotoxic injection the number and morphology of microglial cells were similar to those observed in the control rats. The ultrastructural study also revealed the existence of microglial cell mitosis in the territory of the AN together with a strong increase in the number of supraependymal cells resembling macrophages in the third ventricle during the lesion. Our data demonstrate that activated microglial cells initially extend throughout the damaged territory, but from 24-36 h onwards they are especially patent in the ventrolateral portions of the AN.

Key words: Microglia, Neurotoxicity, Lycopersicon esculentum lectin, Griffonia simplicifolia lectin, Arcuate nucleus

Introduction

In recent years several studies have focused on the glial response following brain damage. One of the earliest known cellular changes which occurs in the microenvironment surrounding injured neurons is a microglial reaction (see Streit, 1996). This reaction varies depending on the type of insult, the brain area affected, the severity of the injury and even the animal species studied (Araujo and Wandosell, 1996; Streit, 1996; Popovich et al., 1997).

The microglial response after neuronal injury induced by the administration of different excitatory neurotoxins has received particular attention. In most cases, the neurotoxic agent employed has been an analogue of glutamate such as kainic acid (Murabe et al., 1981; Akiyama et al., 1988; Andersson et al., 1991; Marty et al., 1991; Kaur and Ling, 1992; Pasinetti et al., 1992; Finsen et al., 1993; Jorgensen et al., 1993; Mitchell et al., 1993; Morgan et al., 1993), ibotenic acid (Coffey et al., 1990), domoic acid (Appel et al., 1997) or quinolinic acid (Schwarz et al., 1983). In other cases, substances (agonist or antagonist) that also interact with glutamatergic receptors have been used, such as NMDA (Acarin et al., 1996), AMPA (Araujo and Wandosell, 1996) or MK-801 (Olney et al., 1989; Fink et al., 1996). Moreover, since the work of Olney (1969), it is well established that administration of monosodium glutamate (MSG) to neonates of different species of rodents causes selective destruction of a large part of the neurons of the hypothalamic arcuate nucleus (AN). Although the neurotoxic effects of MSG have been studied in considerable depth (for references, see Meister et al., 1989; Pastor et al., 1990), little attention has been devoted to the participation of the glia after this type of experimental brain damage (Olney, 1971; Lemk-
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Johnston et al., 1976) and, hence, further studies are required to better define the characteristics of glial responses consecutive to the neurotoxic lesion induced by MSG.

In the present work we studied the microglial reaction occurring secondary to the neuronal necrosis induced in the rat AN by a single subcutaneous injection of MSG (4mg/g b.w.) administered on the 4th postnatal day. Our chief aim was to determine the spatial and temporal changes that occur in microglial cells using histochemical and ultrastructural techniques. Histochmical characterisation of microglial cells by lectins is a suitable and reproducible method. For this purpose, two lectins are of special interest: the lectin from Lycopersicon esculentum (LEA) and the B4 isoelectin from Griffonia simplicifolia (GSA I-B4) (see Acarin et al., 1994; Hewicker-Trautwein et al., 1996). A further aim of the study was to compare the ability of these lectins to demonstrate resting and activated microglia.

Materials and methods

Experimental animals

A total of 60 neonatal male Sprague-Dawley rats was used in this study. From weaning, animals were housed under standard stable conditions and were caged by litters; temperature was 20±2 °C and relative humidity 50±5%. Forty rats received a single subcutaneous injection of an aqueous solution of monosodium glutamate (MSG; Sigma, G-1626) at a dose of 4 mg/g b.w. on day 4 of life (experimental rats). Twenty control rats were injected with a comparable volume of saline. Experimental groups of 8 rats and control groups of 4 animals were sacrificed 6, 12, 24 and 36 h, and 4 days after injection. One half of the rats of each group were used in histochemical procedures and the other half in the ultrastructural studies.

Lectin histochemistry

Animals were sacrificed by decapitation and brains were immediately removed. Blocks containing the arcuate nucleus (AN) were immersed for 72 h in Bouin's solution and then dehydrated and embedded in paraffin. Serial coronal sections (10 μm thick) mounted on glass slides were dewaxed and rehydrated. Endogenous peroxidase was blocked for 10 min with 2% hydrogen peroxide in absolute methanol. Sections were rinsed twice for 10 minutes in TPBS (40 mM Tris, 3.5 mM KH₂PO₄, 8.4 mM Na₂HPO₄, 120 mM NaCl, 0.02% NaN₃, at pH 7.8), once for 10 minutes in TPBS+1% Triton X-100 (Merck, 8603) and incubated for 24 h at room temperature with the lectin diluted in TPBS+1% Triton X-100. Biotinylated lectins obtained from Lycopersicon esculentum (LEA, Sigma L-9389, at a dilution of 20 μg/ml) and B4 isoelectin from Griffonia simplicifolia (GSA I-B4, Sigma L-2140, at a dilution of 5 μg/ml) were used. After rinsing in TPBS (three times for 10 minutes each), sections were incubated for 1 h at room temperature with extravidin peroxidase (Sigma, E-2886) in a 1:100 dilution in TPBS. Sections were rinsed in Tris-buffered saline (TBS) for 15 minutes, and the peroxidase reaction product was visualised using 40 mg of 3,3'-diaminobenzidine (Sigma, D-5637) and 68 mg of imidazole (Sigma, I-0250) in 100 ml TBS for 5 minutes at room temperature, and a final incubation in the same solution plus 50 μl of 33% H₂O₂ for 3 minutes. A final counterstaining was carried out using Weigert's haematoxylin. Sections were then dehydrated, cleared in xylene and coverslipped with DePeX.

Ultrastructural study

Under intraperitoneally-administered sodium thiopental anaesthesia (30 mg/kg b.w.), animals were perfused through the left ventricle with 4% paraformaldehyde and 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, at 4 °C, following a wash of the vascular tree with 150 mM NaCl solution. After perfusion, the hypothalamus was removed and blocks containing the AN were kept in the fixative solution over 12 h and then post-fixed in 1% osmium tetroxide in the same phosphate buffer for 90 min at 4 °C. They were then dehydrated in acetone, contrasted with 2% uranyl acetate and embedded in Durcupan (Fluka). Ultrathin sections were taken on a LKB Ultratome III 8800 ultramicrotome and studied with a Philips EM-201 electron microscope.

Results

Lectin histochemistry

In the AN of control rats sacrificed at 6 h, both lectins used in this study (LEA and GSA I-B4) labelled few microglial cells (Fig. 1A,B, respectively). These featured a cell body of some 12 μm in diameter out of which emerged prolongations that in turn branched into smaller and shorter fibres, which corresponded to the quiescent or ramified microglia of the nervous system (Fig. 1C). Both lectins also labelled the walls of blood vessels (Fig. 1A,B, arrows). No modifications in the microglial cells were seen in any of the animals of the control groups.

After MSG treatment, microglial cells acquired the characteristics of activated cells; both their globular

Fig. 1. A (LEA) and B (GSA). Resting microglial cells in coronal sections of the AN of control rats. The blood vessels are also lectin-labelled (arrows). C (GSA). Higher magnification of resting microglia in the AN of a control rat shows the characteristic ramified morphology. D (LEA). Inferior ventrolateral portion of the AN 36 h after MSG administration. Both types of activated microglial cells are observed: globular (arrows) and pseudopodic o ameboid (arrowheads). Scale bars: 100 μm (A and B), 20 μm (C), and 40 μm (D).
forms, which were rounded and without prolongations (Fig. 1D, arrows), and their pseudopodic or ameboid forms, which had short thick branches (Fig. 1D, arrowheads).

The administration of MSG caused the destruction of most neurons in the AN. The lesion was very prominent at 6 h and was characterised by the presence of numerous pyknotic nuclei that stained intensely with Weigert’s hematoxylin (Fig. 2 A,B). These nuclei alternated with scarce globular microglial cells that were shown both by LEA (Fig. 2A) and GSA (Fig. 2B); with the latter lectin they appeared to be more abundant.

At 12 h after MSG injection (Fig. 2 C,D), microglial cells showing the morphological features of activated microglia (both globular and pseudopodic or ameboid cells) were seen among the pyknotic nuclei. These microglial cells were spread throughout the AN and did not tend to cluster in any of its portions. Staining with LEA (Fig. 2C) at this time revealed more positive elements than the reaction with GSA (Fig. 2D).

The labelling of microglia with lectins was especially patent at 24 h after treatment with MSG and was mainly localised in the periventricular and inferior ventrolateral portions of the AN (Fig. 3A, LEA; Fig. 3B, GSA), where it was possible to visualise the two different morphological types of activated microglia. Staining was more intense when it was performed with LEA, which also labelled the ependyma of the third ventricle (Fig. 3A). Likewise, some microglial cells were seen in the lateral territories of the median eminence (Fig. 3A,B).

In rats sacrificed at 36 h after MSG injection, the reaction was very similar to that found at 24 h. Large numbers of LEA-labelled (Fig. 3C) and GSA-labelled (Fig. 3D) microglial cells were observed in the inferior ventrolateral and periventricular portions of the AN.

In the AN of animals that had survived for 4 days after MSG injection, scanty small microglial cells were observed dispersed in the ventrolateral portion of the AN, where there was a substantially reduced population of neurons as the result of MSG treatment (Fig. 3 E,F). The microglial cells were very similar to the resting microglia observed in control animals.

Ultrastructural observations

In rats sacrificed at 6 h after MSG administration, activated globular microglial cells were observed (Fig. 4A). These were scarce throughout the territory of the AN and did not yet contain debris from degenerated neurons. At 12 h after MSG injection the macrophagic cells displayed large amounts of neuronal debris in their cytoplasm (Fig. 4B) and hence were considerably increased in size. At 24 h after treatment with the neurotoxic agent the amount of ingested material was maximum and frequently included whole degenerated neurons (Fig. 4C). At 12 and 24 h of survival, a common finding was the presence of apparently binucleate microglial cells at the site of the lesion (Fig. 4 B,C). At 36 h after MSG administration, the activated microglial cells contained less neuronal debris in their cytoplasm (Fig. 4D). Finally, at 4 days after treatment with MSG the microglial cells of the AN were scarce and did not display the ultrastructural features of activated cells (images not shown).

Our study also revealed that following the lesion induced by MSG the number of macrophagic cells in the infundibular recess of the third ventricle was increased (Fig. 4E). During this period, especially between 24 and 36 h post-lesion, macrophagic cell mitosis was observed inside the lesioned territory, more frequently in the ventral portions of the AN, where they were most abundant (Fig. 4F).

Discussion

The present work analyzes the pattern of microglial response in the AN of the hypothalamus after neuronal degeneration induced by a subcutaneous injection of MSG (4 mg/g b.w.) into four-day-old rats. The neurotoxic effect of this substance in young rodents has been documented for 3 decades (Olney, 1969, 1971) and has been used to study the role of the AN in neuroendocrine mechanisms (Olney, 1969; Burde et al., 1971; Redding et al., 1971; Nemeroff et al., 1971, 1976; Meister et al., 1989; Pastor et al., 1990). It should be noted that MSG continues to be used as an additive in the food industry and in this sense some authors have reported that it may cause alterations in humans (see Samuels and Samuels, 1993; Olney, 1994).

Here, we characterised the time course as well as the localisation of this microglial reaction using lectin histochemistry (LEA and GSA) and electron microscopy. LEA and GSA have been widely used owing to their specific binding properties to the membrane of resident and reactive microglial cells (Kaur et al., 1990; Marty et al., 1991; Acarin et al., 1994; Hewicker-Trautwein et al., 1996).

Our experimental model is characterised by the simplicity of its use (a single subcutaneous injection of MSG). This contrasts with the administration of other neurotoxic agents through the intracerebral (Marty et al., 1991; Acarin et al., 1996), intraventricular (Murabe et al., 1981, 1982) or epidural (Akiyama et al., 1988; Kaur and Ling, 1992) routes. Moreover, the use of rats in the post-natal period permits the study of the microglial response to neurotoxicity.
reaction in the immature hypothalamus, when the blood-brain barrier is still not completely developed (Xu et al., 1993).

The most important characteristics of the microglial reaction in the AN after MSG administration are:

1) The reaction is established rapidly and is circumscribed within the lesioned territory. Both lectins and electron microscopy reveal the presence of activated microglial cells 6 h after injection. At this time, microglia are not abundant and at the site of the lesion the ramified forms observed in control animals are not found. The microglial cells found at 6 h after MSG injection may correspond to resident microglia, which are activated and change their morphology, thus representing the first response to the lesion. In the ultrastructural study we observed that although neuronal degeneration was generalised at 6 h, phagocytosis of cell debris had not yet begun.

2) The intensity of the response increases at 12, 24 and 36 h, as evidenced by the number of cells labelled with both lectins. From 24 h, the dorsal territories of the AN begin to recover; this is reflected by the disappearance of activated forms in those portions and the appearance of forms resembling the ramified ones. The microglial reaction attains its maximum expression at 24 and 36 h in the ventral territories.

Using the electron microscope, it is possible to assess phagocytic activity on the basis of neuronal debris contained in the macrophagic cells present at the site of the lesion. In this sense, it is seen that the amount of debris is greater at 12 and 24 h than at 36 h; i.e., at 36 h after MSG injection, macrophages are very abundant but phagocytic activity has begun to decline.

3) The microglial reaction disappears rapidly. Four days after administration of the neurotoxic agent the numbers of microglial cells decreases dramatically. At the same time their morphology is changed and readopts the appearance of the ramified forms observed in the control animals. In the ultrastructural study, the phagocytosis of neuronal debris was seen to have concluded (images not shown). These observations are consistent with those of Olney (1971), who reported that at 4 days post-lesion the degenerated elements are well debrided.

Summarising our results, we found that after lesion of the AN by a subcutaneous injection of MSG the microglial reaction begins after about 6 h. It reaches a maximum around 24 h and begins to decline at around 36 h. After 4 days it has completely disappeared. Thus, under the conditions used here the microglial response does not seem to contribute to the neuronal degeneration (as had been suggested in other studies in vitro; see Giulian and Baker, 1986; Colton and Gilbert, 1987; Giulian et al., 1993) since this begins after 15 min and peaks at 90 min according to Mestres and Rascher (1983). Additionally, the microglial response precedes the astrocyte reaction (analysed using GFAP immunoreactivity) that takes place in the arcuate nucleus between 4 and 60 days after MSG-induced lesion (Blázquez et al., 1997).

Comparing our observations to the time course of the microglial response reported by others, it seems clear that under our conditions the reaction debuts, reaches a maximum, and disappears more rapidly than in the experiments carried out by Murabe et al. (1981) or Marty et al. (1991), who used kainic acid in their lesions, or those of Fix et al. (1996), who used MK-801, or Acarin et al. (1996), who administered NMDA intraventricularly to rats of 6 days of age. In these previous studies the reaction usually lasted for at least 7 or 14 days. We believe that this apparent discrepancy can be attributed to the extent of the lesions, which in our case were smaller, since the response persists for at least as long as debris remains to be cleared. That is, in the same way that the transition to the phagocytic state is determined by the presence of neuronal debris as a result of degeneration, it may be assumed that once the debris has been cleared the microglial cells revert to their normal resting state (Streit, 1996). Our data are also consistent with the findings of Olney et al. (1971), who report that beyond 2-4 days after treatment, with the exception of the observed reduction in the neuronal population, few remnants of the lesion persist.

In recent studies on the microglial reaction following cytotoxic injury considerable attention has been paid to the origin of the large number of microglial-macrophagic cells that usually accumulate at the site of the lesion (Streit and Kreutzberg, 1988; Marty et al., 1991; Acarin et al., 1996). It is generally admitted that the cells come from both the activation/proliferation of endogenous microglia and from the infiltration of recruited blood monocytes. In view of the closeness of the AN to the third ventricle, our experimental model permits the study of the arrival of cells through the cerebrospinal fluid. As is known, in the ventricles of the brain, and more specifically in the third ventricle, there is a system of resident macrophage cells (Bleier et al., 1975; Bleier and Albrecht, 1980) that could be mobilised towards the site of the lesion under circumstances of neuronal necrosis. In this sense, Mestres and Rascher (1983) detected a strong increase in the number of supraependymal cells in the third ventricle concomitant with the progress of the MSG-induced lesion. Our study also reveals that the number of supraependymal cells with macrophagic characteristics is increased strongly during the hours following the injection of MSG. To reach the territory of the AN, the site of the lesioned neurons, these supra-
Fig. 4. A. Electron micrograph of a globular microglial cell 6 h after MSG administration. Neuronal debris is not yet present in the cytoplasm. B. Electron micrograph of a binucleate macrophage 12 h post-injection. Its cytoplasm contains a large amount of ingested debris. C. Binucleate phagocyte in the arcuate nucleus 24 h following MSG injury. The nucleus of the engulfed neuron displays an advanced stage of pyknosis. Other residual structures are incorporated in the cytoplasm of the microglial cell. D. 36 h after the injection of MSG the amount of phagocytic debris decreases in the macrophages of the AN. E. Supraependymal cell appended to the ventricular lining of the third ventricle 24 h after MSG administration. F. A mitotic microglial cell in the ventrolateral portion of the AN 36 h post-lesion. Membranous residues are prominent in the cytoplasm. Scale bars: 2 μm (A,C,D,F) and 4 μm (B,E).
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ependymal cells must cross the ependyma by trans-ependymal diapedesis, as suggested in a previous work carried out at our laboratory (Amat-Peral et al., 1994).

A second mechanism that could be invoked to account for the rapid increase in macrophage numbers at the site of the lesion is the proliferation of endogenous microglia. In this sense, the present study demonstrates the existence of microglial cell mitosis in the affected territory, especially between 24 and 36 h after the injection of MSG. Although both mechanisms apparently help to explain the increase in the number of phagocytic cells, it is not possible to determine the extent to which both of them do this.

The histochemical images obtained in the present work show that both lectins are good labels for locating the microglial reaction. The differences between them are insignificant in the control animals and up to 12 h post-lesion. The differences in labelling observed at 6 and 12 h could be related to discrete variations among the animals of the same group as regards the extent of the territory affected. When the microglial response peaks (between 24 and 36 h), the intensity of labelling seems to be stronger with LEA than with GSA. Thus, in our experience, LEA would be the label of choice for studying the microglial reaction occurring in the AN after lesions induced with MSG.

Our work also demonstrates that among the different models proposed to study microglial function after injury in vivo, the use of MSG in young animals is of great interest. In the hypothalamic AN the microglial response is rapid but occurs after neuronal destruction and completely disappears a few days later, once the debris has been cleared and before the astroglial reaction is initiated.

Acknowledgements. Supported by DGICYT grant number PB94-1410.

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Accepted July 14, 1998