PRIMARY CORTICAL GLIAL REACTION VERSUS SECONDARY THALAMIC GLIAL RESPONSE IN THE EXCITOTOXICALLY INJURED YOUNG BRAIN: MICROGLIAL/MACROPHAGE RESPONSE AND MAJOR HISTOCOMPATIBILITY COMPLEX I AND II EXPRESSION

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Abstract—The excitatory amino acid analog, N-methyl-D-aspartate, was injected intracortically into nine-day-old rats. Resulting axon-sparing lesions in the developing sensorimotor cortex, which secondarily affect thalamic neurons that become deprived of cortical targets, provide an experimental model for the study of the glial response in distantly affected areas. The microglial/macrophage response was studied using tomato lectin histochemistry and major histocompatibility complex I and II immunocytochemistry. Blood–brain barrier integrity was evaluated. In the cortical lesion site, where blood–brain barrier breakdown occurs, the rapid microglial response was restricted to the degenerating area. Microglial changes were first seen at 4 h post-injection, peaking at days 3–5. Reactive microglia changed morphology, increased tomato lectin binding and expressed major histocompatibility complex I. Additionally, some cells expressed major histocompatibility complex II. In the secondarily affected thalamus, the microglial response was not as pronounced as in the cortex, was first seen at 10 h post-injection and peaked at days 3–5. Reactive microglia showed a bushy morphology, were intensely lectin positive and expressed major histocompatibility complex I.

The exceptional response of the nine-day-old brain to cortical lesions makes this model an interesting tool for studying the implications of microglial major histocompatibility factor expression in still enigmatic processes such as wound healing and plasticity. © 1998 IBRO. Published by Elsevier Science Ltd.

Key words: development, glia, postnatal, retrograde changes, tomato lectin.

Neural damage is accompanied by a gliosis phenomenon involving a marked astroglial reaction and a rapid microglial response.14–34,35 As described by Del Rio Hortega more than 75 years ago,12,13 microglial cells usually react to injury in a graded manner. Becoming apparent by morphological, immunophenotypical and functional changes, microglial cell activation can imply proliferation and migration, and it may even involve active phagocytosis when neuronal death or axonal degeneration occurs.50,64 In an attempt to differentiate between these different activation states, several authors have focused on the study of the microglial up-regulation or de novo expression of immunomolecules such as major histocompatibility complexes (MHCs).19,53,61,87 Although these reports show that MHC expression on activated microglial cells occurs after neuronal and axonal degeneration, the complete significance of this MHC expression on microglial cells is still unclear, and it may involve functions other than antigen presentation.

In this sense, the study of microglial MHC expression during development as well as following injury in the immature brain may give some insights into the role of the expression of these immunomolecules in the brain. The immunological immaturity of the early postnatal brain, reflected by the incomplete myelination10 and the lack of a fully developed blood–brain barrier (BBB),7,32 is an important characteristic of the immature brain. Moreover, Kolb et al. have shown that animals who received cortical lesions during the early second postnatal week of age show no behavior deficit in several tasks. This behavior outcome is correlated with extraordinary recovery of the cortical damaged area and no abnormalities in corticocortical or subcorticocortical (including

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Abbreviations: BBB, blood–brain barrier; FCS, fetal calf serum; MHC, major histocompatibility complex; NMDA, N-methyl-D-aspartate; TBS, Tris-buffered saline; VB, ventrobasal complex.
thalamocortical connections, clearly implying that the post-injury environment in the developing brain differs from the adult brain. Therefore, modulation of the microglial response may occur in a different way.

Taking advantage of the particular brain response to injury at this developmental stage, the aim of the present work was to analyse the developmental microglial MHC expression associated with distinct grades of neural damage: excitotoxic cell death induced by the intracortical injection of N-methyl-D-aspartate (NMDA) and secondary affectation of distant thalamic relay neurons that lose their cortical targets.

**EXPERIMENTAL PROCEDURES**

**N-Methyl-D-aspartate injections**

Long-Evans black-hooded nine-day old rats (day of birth equals day 0; Harlan Sprague-Dawley, Inc.) of both sexes were used. Under ether anesthesia, each pup was placed in a stereotaxic frame adapted for newborns (Kopf) and the skull was opened using a surgical blade. Thirty-seven nanomoles of NMDA (Sigma, M-3262) diluted in 0.15 μL of saline solution (0.9% NaCl, pH 7.4) were injected into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral from bregma and at 0.5 mm of depth) by using a 0.5-μL Hamilton microsyringe (needle gauge 25). After suture, the pups were placed in an incubator and maintained at 36°C for 2 h before being returned to their mothers. The incubator was used to maintain normothermia because NMDA-induced lesions in the immature brain are highly dependent on body temperature. In control animals, the same procedure was followed, except they received an injection of 0.15 μL of saline solution (0.9% NaCl). Four NMDA-injected animals and two saline-injected control animals were used for each of the eight post-injection survival times. All efforts were made to minimize animal suffering in all procedures.

**Fixation and histology**

At 4 h, 10 h, one, three, five, seven, 14 and 30 days after NMDA injection, rats were anesthetized by ether inhalation and perfused intracardially for 10 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed immediately, immersed in the same fixative for 4 h and either embedded in paraffin or quickly frozen with dry CO₂ after being cryoprotected in a 30% sucrose solution in 0.1 M phosphate buffer. Paraffin coronal sections (10 μm thick) or frozen coronal sections (30–40 μm thick) were obtained. Alternate cryostat sections were mounted on gelatin-coated slides or stored free-floating in De Olmos solution in 100% methanol, free-floating sections were rinsed in 0.05 M Tris-buffered saline (TBST containing 1% Triton X-100 and incubated for 2 h at room temperature with the biotinylated lectin obtained from Lycopersicon esculentum (tomato; Sigma, L-9389) diluted to 6 μg/ml in TBS+1% Triton X-100. After incubation, sections were washed in TBS and incubated for 1 h at room temperature with avidin peroxidase (Sigma, A-3151) in a 1:70 dilution in TBS. Following several rinses in TBS, the peroxidase reaction product was visualized using 50 μg of 3,3’-diaminobenzidine with 33 μl of hydrogen peroxide in 100 ml Tris buffer. Finally, sections were mounted on gelatin-coated slides, dehydrated, cleared in xylene and coverslipped in DPX. Control of tomato lectin specificity was carried out by preincubating the sections for 30 min in a 0.1 M solution of N-acetyllactosamine (Sigma, A-7791), a sugar to which the lectin binds, and then incubating the sections in the lectin solution with the same sugar.

**Tomato lectin histochemistry**

Tomato lectin histochemistry for the specific visualization of microglial cells has been described previously. Briefly, after endogenous peroxidase blocking with 2% hydrogen peroxide in 100% methanol, free-floating sections were rinsed in 0.05 M Tris-buffered saline (TBST containing 1% Triton X-100 and incubated for 2 h at room temperature with the biotinylated lectin obtained from Lycopersicon esculentum (tomato; Sigma, L-9389) diluted to 6 μg/ml in TBS+1% Triton X-100. After incubation, sections were washed in TBS and incubated for 1 h at room temperature with avidin peroxidase (Sigma, A-3151) in a 1:70 dilution in TBS. Following several rinses in TBS, the peroxidase reaction product was visualized using 50 μg of 3,3’-diaminobenzidine with 33 μl of hydrogen peroxide in 100 ml Tris buffer.

**Immunocytochemistry for major histocompatibility factor classes I and II and serum proteins**

After endogenous peroxidase blocking with 2% hydrogen peroxide in 100% methanol, cryostat sections for MHC class I and II immunocytochemistry were treated with 10% fetal calf serum (FCS) in TBS (pH 7.4) for 30 min, and incubated overnight at 4°C with either OX6 (primary antibody (Serotec, MCA-46G) or mouse anti-rat OX18 primary antibody (Serotec, MCA-51), diluted to 1:200 or 1:600, respectively, in TBS containing 10% FCS and 1% Triton X-100. After washing with TBS, the sections were incubated at room temperature for 1 h with the secondary antibody biotinylated anti-mouse (Amersham, RPN-1001) in a 1:200 dilution. Sections were then rinsed in TBS and incubated for 1 h at room temperature with streptavidin–peroxidase (Dakopatts, P-364) at a 1:400 dilution in 10% FCS in TBS. After rinsing again in TBS, the peroxidase reaction product was visualized by incubating the sections in 100 ml of Tris buffer containing 50 mg 3,3’-diaminobenzidine and 33 μl hydrogen peroxide.

A similar procedure was carried out for the immunocytochemical detection of serum proteins on paraffin sections as a measure of BBB permeability. For this purpose, we used rabbit anti-rat serum proteins (Dakopatts, Z-0178) diluted to 1:1500 as primary antibody and biotinylated anti-rabbit (Amersham, RPN-1001) in a 1:200 dilution. Sections were then rinsed in TBS and incubated for 1 h at room temperature with streptavidin–peroxidase (Dakopatts, P-364) at a 1:400 dilution in 10% FCS in TBS. After rinsing again in TBS, the peroxidase reaction product was visualized by incubating the sections in 100 ml of Tris buffer containing 50 mg 3,3’-diaminobenzidine and 33 μl hydrogen peroxide.

**Quantitative analysis**

Histological sections processed for tomato lectin histochemistry and for the demonstration of MHC class I and class II were quantified as described previously. Sections were digitized by a video camera mounted on a Leitz microscope and interfaced to a Macintosh computer. National Institute of Health Image software (version 1.52) was used to quantify the histochemical and immunocytochemical staining. The quantification measure, referred to as the “reactivity grade”, was defined as the ratio between values of a specific area in the cerebral hemisphere ipsilateral to the NMDA injection versus the same area in the contralateral control hemisphere. This ratio method compensates for the variability of staining between sections. The value of this ratio represents: (i) the intensity of marker binding (tomato lectin, MHC I or MHC II) by measuring...
the density of staining, and (ii) the density of labeled cells in
that area, measured as the percentage of the delimited area
above a staining threshold (threshold is defined as the
density of staining in the contralateral control area).
The formula used to calculate the “reactivity grade” is
summarized as follows:

\[
\text{REACTIVITY GRADE} = \frac{\text{MEAN STAINING DENSITY}_{\text{ipsilat.}}}{\text{MEAN STAINING DENSITY}_{\text{Contralat.}, \text{(Threshold)}}} \times \frac{\text{AREA ABOVE THRESHOLD}_{\text{ipsilat.}}}{\text{AREA ABOVE THRESHOLD}_{\text{Contralat.}}} \times \frac{\text{Factor considering a possible increase in marker binding}}{\text{Factor considering possible hyperplasia and/or hypertrophy of labeled cells}}
\]

The excitotoxic lesion induces de novo expression of
MHC molecules in the ipsilateral hemisphere, but the
control hemisphere remains negative. Accordingly, for
MHC class I and class II processed sections, the factor
considering the areas above threshold was error inducing
and therefore was omitted from the formula.

A “reactivity grade” was obtained for each area of study
(i.e. neocortex and thalamus), each marker (tomato lectin,
MHC I and MHC II) and for each animal (mean of two
sections). Four animals for every NMDA-injected group
were analysed. The same measurements were done in saline-
injected animals. Measured areas included 0.38 mm² area
of the neocortex at the level of the injection site, and 1.45 mm²
area of the caudal thalamus, including the ventrobasal
complex (VB), reticular nuclei and the internal capsule.

Statistical analysis was performed using Statview 4.0 soft-
ware. ANOVA with Fisher’s PLSD post hoc comparisons
were used.

RESULTS

Injection of 37 nmol of NMDA into the right
sensorimotor cortex of nine-day-old rats caused a
primary lesion involving a neuronal loss and glial
response across the entire thickness of the cortex at
the injection site. Additionally, a glial response was
observed in the VB of the thalamus. This thalamic
affectation was likely due to secondary changes
caused by the loss of target cortical neurons. No
apparent neuronal degeneration or glial response
was seen in the contralateral hemisphere at any
survival time, therefore it was used as an additional
control.

Tomato lectin allowed the visualization of lesion-
induced activated microglial cells, as well as ameboid
and ramified microglia in non-damaged areas and in
control brains (Fig. 1D). Moreover, blood vessels
and ependymal cells were also stained using this
histochemical technique (Fig. 1D–F). These findings
are in agreement with previous studies using tomato
lectin histochemistry for the visualization of micro-
glial cells in the normal and developing rat brain,\(^4\) in
hypomyelinated mutant mice,\(^81\) and in the injured
early postnatal and adult rat brain.\(^1, 56\)

Saline controls

In gray matter areas of saline-injected control
animals with survival times ranging from 4 h to seven
days, tomato lectin-positive microglial cells were
evenly distributed and showed a primary ramified
morphology. As a normal feature of development,
microglial cells in white matter tracts such as the
corpus callosum and the internal capsule appeared
ameboid in shape, showed increased lectin binding
and expressed MHC class I, as described pre-
viously.\(^5\) The adult pattern of ramified microglial
cells was first observed in 14-day-old control animals.
In the cortex of saline-injected animals, a few reactive
microglial cells showing mild MHC I expression were
observed in the needle track until three days post-
injection. No microglial response was observed in
the thalamus of saline controls, and no MHC II
expression was observed in any control animal.

In saline-injected animals, serum protein extra-
vasation was observed surrounding the needle track
until five days post-injection. Moreover, in normal
and saline control animals, serum protein immuno-
reactivity was associated with ependymal cells, pial
elements and choroid plexuses. Generally, sections of
animals with shorter survival times showed intense
background neuropil staining, which progressively
decreased with age.

Neocortical injection site

As observed in Hematoxylin–Eosin-stained sec-
tions, and in comparison to the control contralateral
side, where no abnormal changes were observed, the
injection site had already lost its laminar cortical
cytoarchitecture at 4 h post-injection (Fig. 1A, B). At
this time-point, neuropil vacuolation, increased eosin-
ophilia of neuronal cells and signs of an incipient
glial response were commonly observed across all
cortical laminae at the level of the injection site (Fig.
1C). By 10 h post-injection, besides necrotic neuronal
cells, some apoptotic-like figures, characterized by
darker cell bodies, could be observed. Even though
necrotic and apoptotic-like figures were still seen at
one and three days post-injection, massive neuronal
loss was obvious (Fig. 2C). At these time-points, we
observed an increase in the number of non-neuronal
cells, including macrophages and peripheral blood
cells, mainly neutrophils. Scarring of cortical tissue
became apparent by five days post-injection and
cortical thickness diminished from then on.

Changes in microglial morphology and distri-
bution were observed by tomato lectin histochemistry
at 4 h post-injection when compared to the control
hemisphere (Fig. 1D, E). Morphological changes
involved retraction of cell processes and the appear-
ance of a beaded morphology (Fig. 1F), mainly in
microglial cells located at the edge of the lesion area.
In some cases, elongated cells following a radial
orientation seemed to emerge from the corpus callo-
sum and migrate towards the lesion site (Fig. 1E).
Already at this time post-injection, immunoreactivity for serum proteins, used to identify BBB disruption, showed neuropil staining and strongly positive neuronal cell bodies scattered in the degenerating area and the surrounding tissue (data not shown). However, the accumulation of serum proteins became more evident at 10 h and one day post-injection, when the strongest immunoreactivity was observed (Fig. 4A–C).

Microglial reactivity was more evident at 10 h post-injection, when reactive cells were found exclusively in areas undergoing neuronal degeneration (Fig. 2A). Reactive microglial cells showed an ameboid or pseudopodic morphology with a further increase in lectin binding. Moreover, round peripheral blood cells (tomato lectin and MHC class I positive) were also present at the lesion site. From one day post-injection, pseudopodic reactive microglia/macrophages typically accumulated at lesion margins, while ameboid cells and smaller blood infiltrated cells were observed characteristically at the center of the lesion (Fig. 2B, D, G, H).
Fig. 2. Micrographs showing the cortical degenerating area at 10 h (A), one day (B) and three days (C–J) post-injection. (A, B, D, G, H) Tomato lectin (G and H are high magnifications of D). (C) Hematoxylin–Eosin. (E, I) MHC I. (F, J) MHC II. D–F were obtained from parallel sections. Magnifications: ×73 (A, B); ×46 (C–F); ×291 (G–J).
this time-point, a subpopulation of reactive microglia/macrophages (regardless of their morphology) also expressed MHC class I antigen (Fig. 2E, I). Additionally, round or pseudopodic cells in the lesion site and few ramified cells in the periphery expressed MHC class II (Fig. 2F, J).

At three days post-injection, and as shown by quantitative analysis (Fig. 5), the microglial/macrophage reactivity started to peak, and a massive increase in the number of tomato lectin-positive cells was evident (Fig. 2D). At five days post-injection, the morphology of reactive microglia/macrophages was more uniform and accumulation of round tomato lectin- and MHC I-positive cells around blood vessels was seen frequently (Fig. 3A, B); some of these cells were also MHC II positive (Fig. 3C). At this time-point, microglia/macrophages were densely packed and a lectin-positive and strongly MHC I-positive glial scar started to form, accounting for the maximum of MHC I immunoreactivity shown in our quantification analysis (Figs 5, 9). By seven days, tomato lectin binding had decreased (Fig. 3D), although MHC class I and II expression was maintained (Fig. 3E, F). MHC-positive cells were usually round or pseudopodic in shape, and tended to accumulate in clusters around blood vessels. MHC labeling was still present at 14 and 30 days post-injection, the last survival times examined (Fig. 3H, I). At survival times longer than day 7, tomato lectin binding was down to control levels (Fig. 3G), although the glial scar was mildly positive. From three days post-injection, when the peak of microglial/macrophage response took place, serum protein immunoreactivity was strictly limited to the lesioned cortical site and started to diminish, being faint by day 14 (Fig. 4D–G).

Secondarily affected thalamus

Although Hematoxylin–Eosin-stained sections showed no significant morphological changes in thalamic neurons, a characteristic microglial response was observed by tomato lectin histochemistry (Fig. 6, Fig. 7). Moreover, no immunoreactivity for serum proteins was detected in the thalamus of any animal.

Thalamic reactive microglial cells were typically seen at the border between the VB and reticular nuclei (Fig. 6B, D, F). At more caudal levels of some animals, evident microglial reaction was specifically located in the centrolateral nucleus. Additionally, and in comparison to the contralateral side and to saline-injected controls, an increase of ameboid microglial cells, characteristically MHC I positive, was observed in the ipsilateral corpus callosum and internal capsule of lesion animals (Fig. 6A, B, D, F).

In these areas, cells with the appearance of activated microglia were first evident by 10 h post-injection (Fig. 6B), but MHC class I expression was not seen until one day post-lesion (Fig. 6C). Apart from a very faint labeling of ramified microglial cells at the 10 h time-point, the thalamus appeared to be negative for MHC class II.

At three and five days, when the microglial reactivity was at its peak, as shown qualitatively (Figs 6E, F, 7E, F) and quantitatively (Figs 8, 9), reactive microglial cells characteristically presented a bushy-like morphology, with enlarged cell bodies and short pseudopodic processes (Fig. 7A, B, D, F); however, microglial response in the thalamus never reached the intensity of the cortical response (Fig. 9). Some of these reactive microglial cells showed MHC I expression from days 1 to 5, this being maximum at three days post-injection (Fig. 7C, E). Neither round lectin-positive cells or blood infiltrates were observed at any time. Few reactive microglial cells still remained in the VB seven days post-lesion (Fig. 6G, H), when no evidence of microglial reaction was observed in the centrolateral nucleus.

Although not studied specifically in the present report, neuronal death in the sensorimotor cortex also causes Wallerian degeneration of cortical descending efferents. Microglial reactivity associated with cortical efferents was first seen at one day post-injection mainly in the white matter tract area, but also in pontine gray nuclei in some animals. This response disappeared by five days. Few MHC I-positive cells were only observed at one day post-injection, and no MHC II expression was seen at any time.

DISCUSSION

The present study revealed a rapid microglial response in the cortex and thalamus following an NMDA-induced focal lesion in the immature sensorimotor cortex. Microglial reactivity, seen by 4–10 h post-injection, was characterized by changes in cell morphology, an obvious increase in lectin binding and MHC class I expression. Moreover, some ameboid or round tomato lectin-positive cells in the cortical lesion site also expressed MHC class II antigen. Characteristics of the microglial response in primary and secondary injured areas, as well as possible induction factors for MHC expression, are discussed below.
Microglial MHC expression to developmental injury

Fig. 3.
Fig. 4. Digitized images of the neocortical damaged areas in sections processed for the detection of serum proteins at 4 h (A), 10 h (B), one day (C), three days (D), five days (E), seven days (F) and 14 days post-injection (G). Color scale oscillates from violet (lack of staining) to red (maximal intensity of staining). Note that the highest and widest staining is found at 10 h and one day post-injection, decreasing and becoming more restricted at later survival times. Magnification: × 19 (A–G).
In previous work, we described the microglial response to an excitotoxic injury in the six-day-old rat brain, where neuronal degeneration and extensive microglial reaction in the neocortex, hippocampus, rostral thalamus and some dorsal thalamic nuclei was observed. In contrast, and in order to study the glial response associated with different grades of neuronal affectation in the immature brain, we have induced a focal axon-sparing lesion in the nine-day-old sensorimotor cortex. Cortical lesions at this age are followed by remodeling of adjacent cortical areas and recovery of cortical connectivity, including thalamocortical projections. The same experimental lesion model was used for the study of the astroglial response and metallothionein expression.

**Sensorimotor cortex connections in development**

Both primary and secondary somatosensory cortex have well-described reciprocal projections with the ventral posterior medial and the ventral posterior lateral thalamic nuclei, which constitute the VB. Several studies have reported VB degeneration following different kinds of somatosensory cortical injury in the adult rat brain. Moreover, substantial thalamic atrophy is seen after cortical aspiration lesions made at birth. These lesions are accompanied by formation of abnormal cortical connections and behavioral loss. However, the response to cortical injury of the rat brain during the second postnatal week of age seems to have a different outcome, which may be explained by its particular developmental stage and may determine a specific pattern of glial response.

Recent findings suggest that somatosensory cortex barrel fields, as well as the periphery-related patterns in thalamocortical projections from the VB, are already established one day after birth. Subsequently, during the first 10 days of life, the rat neocortex increases three-fold in thickness, develops its characteristic laminar distribution, and the establishment of thalamocortical, corticothalamic and corticocortical connections takes place. Elimination of exuberant cortical efferents and mature arborization of corticothalamic projections is not achieved until the end of the second postnatal week, even though anterograde labeling studies demonstrate that axons arising from laminae V and VI of the nine-day-old sensorimotor cortex send collaterals to the reticular nuclei and, by then, terminate in the VB and the posterior nucleus, constituting one of the major inputs of thalamic relay neurons.

In view of these neuroanatomical studies, we have used the nine-day-old rat brain, where thalamocortical connections are already well established, corticothalamic projections have achieved their targets but not their adult pattern, and cortical efferents have...
Fig. 6. Contralateral control (A) and ipsilateral thalamus (B–H) at 10 h (A, B), one day (C, D), three days (E, F) and seven days post-injection (G, H). (A, B, D, F, H) Tomato lectin. (C, E, G) MHC I. Note the presence of microglial response in the VB and the internal capsule (IC), but not in the reticular nuclei (Rt). Magnification: × 46 (A–H).
Microglial MHC expression to developmental injury

reached the brainstem and spinal cord, even though myelination is not yet completed.\textsuperscript{33,39} These developmental characteristics of the nine- to 10-day-old rat brain may explain recent results showing that, after frontal cortical aspiration lesions at this age, the remaining cortex develops a greater dendritic arbor than normal adults, while no obvious abnormality in cortical and subcorticocortical connections was apparent, and significant behavior sparing was evident.\textsuperscript{46,48} These results, taken together with other significant features of the 10-day-old neocortex, such as the continuing growth of neuronal dendritic trees, the trophic actions of developing astrocytes and the high expression of neurotrophin-3 by microglial cells,\textsuperscript{15} point to this developmental stage as the most suitable for studying the putative role of glial cells in enigmatic events associated with developmental plasticity.

Cortical microglial response

The microglial response after injury to the developing neocortex has been studied previously following aspiration lesions,\textsuperscript{60} stab wounds,\textsuperscript{39} or excitotoxic and ischemic–hypoxic insults.\textsuperscript{1,31,80} In these studies, as in our excitotoxic lesion study, microglial

Fig. 7. High-magnification micrographs of microglial cells in the contralateral control (A) and ipsilateral (B-E) VB of the thalamus. (A, B, D, F) Tomato lectin. (C, E) MHC I. Note the changes in cell morphology when compared to the contralateral thalamus microglia (A). Microglial response at one and three days post-injection is accompanied by MHC I expression (C, E). Magnification: $\times 182$ (A–F).
activation first appeared within hours after the insult, suggesting that microglial activation primarily occurs prior to neuronal degeneration. According to our results, this early activation state is followed by a more pronounced activation, at 10 h post-lesion, involving drastic morphological changes and MHC class I antigen expression. Several factors, such as extravasated cytokines or signals from affected neurons, could trigger this microglial activation. Moreover, according to our observations, at one day post-lesion, when neuronal debris is present, microglial/macrophages become phagocytic, show MHC I expression and de novo expression of MHC class II antigens. Moreover, at this time-point and at three days post-injection, several tomato lectin-positive cells also expressed the intermediate filament vimentin and the metal binding protein metallothionein. Although specific morphological distinction between these different activation stages of microglial cells is difficult, it is likely that different grades of activation co-exist in the NMDA injection site.

Although a similar pattern of microglial response has been described in the excitotoxically injured adult brain, our developmental lesions showed a faster resolution of cellular debris, an earlier peak of microglial/macrophage response and significantly less scarring than what has been reported for the adult brain. In this sense, one of the striking characteristics of the microglial response observed in the cortical injection site is the increase in the number of tomato lectin-positive cells already seen three days after the lesion. Despite the high proliferating capability of microglial cells in the young brain, leading to a massive cell number increase between postnatal days 5 and 20, both irradiation and monocyte labeling studies support the hypothesis that the majority of brain macrophages seen after an injury where the BBB is disrupted originate from blood monocytes. However, the three-day delay between BBB opening and massive monocyte infiltration suggests that an early activation of resident microglial cells may be important to trigger the recruitment of blood-borne monocytes.

The fate of brain macrophages after repair still remains an interesting and controversial topic. Although no conclusions can be drawn from our findings, three possible mechanisms of elimination have been suggested: (i) phagocytic cell degeneration in situ, supported by the observations that two-thirds of ameboid microglial cells present in the corpus callosum degenerate in the course of development and are engulfed by local companion cells; (ii) subsequent transformation into microglial-like cells, also demonstrated using bloodstream injection of carbon particles; and (iii) re-entrance to the blood circulation, suggested by ultrastructural observations following laser beam lesions. The accumulation around blood vessels of round tomato lectin- and MHC I-positive cells seen in our five-day survival group may support this last hypothesis, although it is impossible to conclude whether accumulated cells are leaving or entering the brain parenchyma.

**Thalamic microglial response**

In general, the thalamic microglial response was less intense, but only mildly protracted when compared to the cortical lesion site. These differences in microglial activation probably reflect distinct patterns of neuronal and axonal injury. The fact that the intracortical NMDA injection at this age causes mild affection of thalamic relay neurons, in addition to the likely Wallerian degeneration of corticothalamic terminals, may outline a thalamic microglial response.
Microglial MHC expression to developmental injury

Figure 9. Summary graphs showing microglial "reactivity grades" in the cortical injection site and the secondarily affected thalamus at all survival points in a time-scale. Note that a "reactivity grade" of 1 implies equivalence to the control hemisphere. Even though the temporal pattern does not differ between areas, the microglial reactivity in the thalamus appears to be weaker and lacking MHC II expression.

characterized by bushy-like morphologies in the absence of round phagocytic cells. This lack of massive thalamic neuronal degeneration, contrasting with studies of cortical lesions in newborn and adult animals, \(^{44,65,72,75}\) correlates with previous studies reporting considerable behavioral recovery after cortical lesions at postnatal day 9, \(^{48}\) and might be explained by the progressive maturation changes occurring in the VB during the second postnatal week in the rat. \(^{11}\) In addition to this putative thalamic plasticity, another factor that may influence the microglial cell reaction relates to the integrity or disruption of the BBB. Although some studies suggest that the tightening of the cortical BBB occurs between postnatal days 4 and 8, \(^{32}\) blood vessel proliferation and sprouting are thought to be maximal between postnatal days 8 and 9, \(^{7,16,70}\) suggesting a residual BBB leaking until postnatal day 10, mainly as a consequence of new vessel formation. Whatever the maturation state of the BBB in the postnatal day 9 brain, we have shown that the cortical excitotoxic injection caused no BBB leakage in the thalamus. Thus, thalamic microglial response is likely to occur both in the absence of abnormal levels of serum proteins or blood cell infiltration.

Therefore, several of these events may modulate the thalamic microglial response characterized by morphological changes to "bushy-like" cells with short, thick processes, MHC class I up-regulation, but no apparent increase in the number of reactive cells. According to in vitro studies, several molecules seem capable of triggering a microglial response, \(^{25,58,68,69}\) although the presence and effect of these molecules in the secondarily affected thalamus remain speculative. Microglial cells are extremely sensitive to changes in their surrounding microenvironment, being capable of responding to extracellular changes that may occur in the target-deprived thalamic nuclei, such as changes in potassium, \(^{62}\) ATP concentration, \(^{62}\) or the presence of calcitonin gene-related peptide. \(^{67}\) Moreover, recent observations showing that cortical ischemic damage can lead to changes in c-fos, jun B or heat-shock protein-70 gene expression in thalamic regions during the first 24 h after infarction \(^{35,71}\) suggest a role for these stress proteins in regulating the expression of putative glial activation signals.

Our observations contrast with studies reporting retrograde changes in the adult thalamus accompanied by a microglial response characterized by the presence of phagocytic cells and MHC class II antigen expression, following different types of cortical injury, such as ischemia, \(^{62}\) excitotoxicity, \(^{5,36,38}\) cryogenic damage \(^{69}\) or aspiration lesions. \(^{86}\) These differences between the adult and the immature brain would possibly imply that microglial activation signals differ, mainly due to the developmental stage and the grade of thalamic affectation. The drastic retrograde degeneration in the thalamus seen after newborn and adult cortical lesions, contrasting with the milder changes observed after lesions at postnatal day 9, may account for the presence of phagocytic cells and the expression of MHC class II only in the degenerating adult thalamus.

Microglial major histocompatibility complex expression

Classically, MHCs have been considered as tools for antigen presentation to T lymphocytes in different tissues: class I MHC proteins present antigen that has been synthesized within the antigen-presenting cell itself, and class II MHC proteins present ingested antigens such as secreted products from infected, transformed or damaged cells. The observation that several stimuli could induce MHC expression on neural cells has lead to extensive study of MHC class I and/or MHC class II expression under distinct types of axonal, terminal or cell body degeneration. \(^{5,8,19,20,63,79,87}\) As MHC expression is restricted to endothelial cells and areas devoid of BBB in the adult brain, \(^{66}\) these studies agree in that microglial
expression of MHC class I antigen is a sensitive marker for variations in the brain microenvironment, including changes caused by motoneuron regeneration,77 proximity to a degenerating area19 or transient cerebral overpressure.41 Furthermore, developmental events such as tissue remodeling in the postnatal corpus callosum also provide the signals for MHC class I induction on ameboid microglial cells (see Results section). Accordingly, small changes in the extracellular milieu, such as variations in acid/base status, increased extracellular concentrations of glutamate or potassium ions,43 and extravasation of serum constituents,73 may provide a sufficient stimulus for MHC class I expression. Thus, this increased MHC class I antigen expression would indicate a general state of microglial metabolic activation rather than their involvement in true antigen-presenting function, especially in thalamic regions, where neither extravasation of serum proteins or blood-borne cells were observed. In addition, after the insult, some cortical site microglial cells/macrophages, mainly showing a round or pseudopod form, also expressed MHC class II antigens. Neither the normal immature brain or the secondarily affected thalamus showed consistent expression of these immunomolecules. This is in agreement with previous developmental studies reporting MHC class II expression only by phagocytic cells seen after motoneuron degeneration, ischemic-hypoxic injury in neonatal rats27 or in the degenerating optic tract when it achieves myelination,57 suggesting that the process of phagocytosis and MHC class II expression are linked.

In an attempt to elucidate the possible MHC-inducing signals, in vitro studies have shown the ability of several molecules, such as interferon-γ, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, interleukin-1β and interleukin-3 to induce MHC expression on microglia.21,30,52 Moreover, ciliary neurotrophic factor, tumor necrosis factor-α, lipopolysaccharide and interferon-γ have been shown to induce the microglial expression of MHC class I and/or MHC class II when injected, either intraperitoneally or intravenicularly, into the developing rat.55,64,65 The presence of these factors has not yet been demonstrated in the injured developing brain, but these putative inducing molecules may be produced locally by reactive astrocytes and microglial cells, or extravasated through the leaking BBB and secreted by infiltrating blood cells in the cortical injection site.

Moreover, as expression of MHC II on microglia is selective and involves a small population of cells, this molecule is likely to be related to the functional state of some activated microglia. As damaged tissue offers several potential sources of antigen that can be recognized as “foreign”, a putative function of MHC II expression could include an elevated state of immune alertness or processes other than antigen presentation, like non-immunological roles such as cell interaction and adhesion.51,77

CONCLUSIONS

We conclude that excitotoxic lesions in the developing sensorimotor cortex are accompanied by an expression of MHC class I and class II on reactive microglia/macrophages, which peaks at days 3–5 post-lesion. BBB disruption in the injection site precedes the expression of these immunomolecules. In contrast, secondary affectation of thalamic relay neurons that lose their cortical targets causes a microglial response characterized by expression of MHC class I, but not MHC class II. These events in the immature thalamus occur in the absence of BBB leakage. These distinct patterns of microglial response and MHC expression may be determined by different activation signals, depending on the severity of neuronal injury, tissue damage and BBB integrity.

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