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Expression of la Antigen on Perivascular and Microglial Cells after Sublethal and Lethal Motor Neuron Injury

WOLFGANG J. STREIT, MANUEL B. GRAEBER, AND GEORG W. KREUTZBERG

Department of Neuromorphology, Max Planck Institute for Psychiatry, D-8033 Martinsried, Federal Republic of Germany

The expression of immune-associated (MHC class II) antigen was studied immunohistochemically over several months in the rat facial nucleus after nerve transection and after intraneural injection of toxic ricin. Cells expressing Ia antigen were of a perivascular type and parenchymal ramified microglia. In the first few weeks after nerve lesions we observed a gradual increase in the number of Ia-immunoreactive cells starting with an initial appearance of Ia-positive perivascular cells which were succeeded by increasing numbers of Ia-positive ramified microglia. In long-term animals Ia expression was almost exclusively found in microglia. We propose (a) the existence of a population of immunocompetent perivascular cells normally present in adult rat brain that can be stimulated to express Ia antigen, and (b) the existence of a subpopulation of ramified microglia that arises through transformation of Ia-positive perivascular cells in the adult under pathological conditions.

INTRODUCTION

Previous studies from our laboratory have shown that CNS microglial cells, which become activated locally during neuronal regeneration and degeneration, demonstrate an upregulation of a number of intracellular and surface molecules (reviewed in (33)). One aspect of the microglial activation process concerns the elevated expression of antigens of the major histocompatibility complex (MIIIC). After axotomy of the facial nerve there is markedly enhanced appearance of MHC class I antigens predominantly on perineuronal microglial cells (34), as detected with monoclonal antibody OX-18 (8). In addition, within the first week after axotomy some scattered cells in the regenerating facial nucleus reveal a positive reaction with monoclonal antibodies directed against MHC class II (Ia) molecules. Both, the uniform expression of class I antigens by microglia, as well as the scattered appearance of some Ia-positive cells in the sublethally injured and regenerating facial nucleus may indicate an elevated state of immune responsiveness of certain cellular elements of nervous tissue. However, since nerve transection represents a sublethal and sterile lesion that is not accompanied by an infiltration of blood-borne cells, the accentuated expression of MHC molecules may also suggest a yet unknown function of MHC antigens related to nerve regeneration.

A number of studies have reported elevated expression of MHC antigens under conditions of lethal neuronal injury and during autoimmune conditions (1, 4, 15, 21, 36, 38, 39). In addition, various other neuropathological conditions, such as amyotrophic lateral sclerosis, Parkinson's and Alzheimer's disease, and viral infections, are accompanied by MHC antigen expression on glia, but not on neurons (19, 23, 25, 35). Surprisingly, neuronal expression of MHC class I antigens has recently been suggested to occur in facial motor neurons following axotomy (20), as well as in neurons terminating within the CNS (28).

In addition to the axotomy paradigm, we have been working with an experimental model of lethal neuronal injury employing injections of the toxic lectin from Ricinus communis (RCA60) into the facial nerve, which results in the rapid and selective demise of facial motor neurons (32). As part of the local glial reaction to neuronal death there is massive proliferation of microglia and the formation of microglia-derived brain macrophages. Similar to the axotomy paradigm, there are no detectable inflammatory infiltrates of blood-borne cells in the degenerating facial nucleus. In the present study we sought to further investigate the time course of occurrence of Ia-positive cells in the axotomized facial nucleus and to identify the cell type(s) responsible for MHC class II expression. In addition, we wanted to compare qualitatively the temporal and spatial distribution of Ia-positive cells after sublethal (axotomy) and lethal (ricin injection) neuronal injury.

MATERIALS AND METHODS

Surgery

Young adult Wistar rats weighing approximately 200 g were used for all experiments. Animals were divided
into three groups: the first group underwent a unilateral axotomy by transecting the facial nerve near its exit from the stylomastoid foramen; the second group received a unilateral intraneural injection of 0.5 μl of R(CA)60 (Sigma, L-8508) at 1 mg/ml, as described previously (32); and in the third group, animals received bilateral lesions consisting of nerve transection on one side and ricin injection on the other.

**Tissue Collection and Fixation**

Following survival periods of 3, 4, 6, 7, 11, 15, 21, 27, 34, 41, 44, 106, and 128 days, three rats per time point were decapitated under ether anesthesia, and the brains were rapidly removed. Cryostat sections cut at 20 μm were mounted on gelatinized slides and allowed to dry at room temperature for 30 min. Fixation was carried out consecutively in 3.7% phosphate-buffered formaldehyde (5 min), 50% acetone (2 min), 100% acetone (2 min), 50% acetone (2 min), followed by two 5-min washes in 0.1 M Tris-buffered saline (TBS), pH 7.2, and TBS containing 1% bovine serum albumin.

For the electron microscopic demonstration of OX-18 immunoreactivity, animals were deeply anesthetized with 30% chloral hydrate and perfused with 4% freshly containing 1% bovine serum albumin.

**Staining Procedures**

Mouse ascites monoclonal antibodies (mAb) OX-3, OX-6, OX-17, and OX-18 directed against various determinants of rat MHC class II and class I (OX-18) antigens (34) were purchased from Serotec (Camon, Wiesbaden, FRG). The fixed sections were incubated for 45 min at room temperature using the following optimal antibody dilutions: OX-3 (1:100), OX-6 (1:500), OX-17 (1:100), and OX-18 (1:2000). Specific antibody binding sites were visualized using the avidin-biotin method (Vector Laboratories, Burlingame, CA), and 3,3′-diaminobenzidine as a peroxidase substrate. In order to minimize nonspecific background staining, the biotinylated secondary antibody was absorbed with normal rat serum from our Wistar strain prior to use.

**RESULTS**

**Microglia Respond to Neuronal Injury**

In bilaterally operated animals motor neurons were preserved on the axotomized side, but disappeared on the side of ricin application (Figs. 1A and 1B). Typically, axotomized neurons were surrounded by increased numbers of perineuronal microglial cells, while in the ricin-treated nucleus almost all neurons were degenerated and had been replaced by an extensive accumulation of glial cells consisting largely of phagocytic microglia. Examination of the microglial reaction to bilateral lesions using monoclonal antibody OX-18 showed perineuronal microglial processes expressing immunoreactivity as late as 2 months after axotomy and diffuse immunostaining associated with cell processes of microglia-derived brain macrophages throughout the ricin-injected nucleus (Figs. 1C and 1D). Immunocytochemistry at the ultrastructural level demonstrated OX-18 reaction product to be localized on the plasma membrane of perineuronal microglial cells (Fig. 1E). Reaction product was undetectable on other glial cells or neurons. In sections of unoperated facial nuclei and in other regions of normal brain, immunoreactivity with OX-18 was confined to endothelial cells.

**Ia Immunoreactivity in Normal Brain Tissue**

The choroid plexus of the fourth ventricle consistently showed identical staining patterns with mAbs OX-17 and OX-6. Immunoreactivity was not seen on cuboidal cells of the choroidal epithelium, but was localized along fenestrated capillaries and thus likely represented staining of endothelial cells. As shown in Fig. 2A, the stain was discontinuous in that not the entire capillary tree was decorated and only intermittent endothelial cells showed positivity. Unlike mAbs OX-17 and OX-6, staining with mAb OX-18 revealed considerably less immunoreactivity with very few scattered cells being labeled (not shown). Numerous Ia-positive cells were seen in the meninges, and the pattern of staining observed here was identical to that described by Vass et al. (38).

The brain parenchyma including the unoperated facial nucleus was largely devoid of Ia immunoreactivity. In some areas of white matter, notably in the molecular layer of the cerebellum, the cerebellar peduncles, and the spinal trigeminal tract, Ia-positive microglial cells could be identified (Fig. 2C). These resting microglia were labeled with mAb OX-17 and OX-6, but not with OX-3. Ia-immunoreactive microglia were also found in the cerebellar gray matter, although this was an uncommon finding. Generally, the occurrence of Ia-expressing resting microglia was limited to white matter. In addition, other Ia-positive cells could be observed consistently in sections of normal brain including the unoperated facial
FIG. 1. Cresyl echt violet-stained sections of axotomized (A) and ricin-treated facial nuclei (B) 11 days after operation. A large proportion of glial cells are found in perineuronal positions after nerve transection, whereas after ricin injection neurons have degenerated and been replaced by glial cells. Sections are from a bilaterally operated animal. X68. (C, D) Adjacent sections to A and B, respectively, stained with mAb OX-18. Perineuronal microglia express MHC class I antigen after axotomy. Diffuse immunostaining associated with processes of microglia-derived macrophages is seen in the degenerated nucleus. X126, Normarski optics. (E) The electron micrograph shows a microglial cell (M) located beside a facial motor neuron (N). OX-18 reaction product is found associated with the microglial plasma membrane (arrows). X18,225.

These cells were seen to occur singly, and seemed to be randomly distributed throughout the brain regions examined. They were often observed in close association with small vessels (Fig. 2B), but were not endothelial cells, as the endothelial surface of vessels was always negative for Ia antigen. Instead they seemed to be of the perivascular type, possibly representing a cell population related to pericytes.
FIG. 2. Localization of Ia antigen in normal brain. (A) Choroid plexus of the fourth ventricle stained with mAb OX-6. Note intermittent staining of capillaries. ×192, Normarski optics. (B) Section of midbrain stained with mAb OX-6. A small blood vessel with three perivascular cells positive for Ia antigen is shown. Endothelial cells (arrows) are negative. ×240. (C) Section of inferior cerebellar peduncle stained with mAb OX-17 demonstrates immunoreactivity on ramified microglia. ×152. Inset shows Ia-positive microglial cell in gray matter of the cerebel-lum. ×240, OX-17.
EXPRESSION OF Ia ANTIGEN

FIG. 3. OX-17 immunoreactivity in the axotomized facial nucleus 6 days after operation (A, C, D). An increase in the number of Ia-positive cells (A) can be detected compared to the contralateral unoperated side (B). The cells do not have microglial morphology, but are of the perivascular type. x77. (C) Higher magnification of Ia-positive perivascular cells. (D) In the white matter just outside the facial nucleus ramified Ia-positive microglia (arrows) can be detected in addition to perivascular cells. Dashed line indicates boundary of facial nucleus. x191.

Time Course of Appearance and Identification of Ia-Expressing Cells in the Regenerating and Degenerating Facial Nucleus

During the first week after axotomy there was a noticeable increase in the number of Ia antigen-expressing cells in the axotomized facial nucleus compared to the unoperated side (Figs. 3A and 3B). In almost every instance the close association of these cells with small blood vessels could be confirmed. The cells were often elongated and their cytoplasmic processes were in alignment with the course of the vessels (Fig. 3C). Perineuronal microglia, a most prominent histologic feature during the early stages of the retrograde reaction, were rarely found to be Ia immunoreactive in any of the preparations examined at this time. Some scattered microglia expressing Ia antigens on their ramified processes were, however, quite apparent in regions of white matter surrounding the facial nucleus (Fig. 3D). Occasional ramified microglia demonstrated Ia expression also in the root of the axotomized nerve, but there was no obvious increase in immunostaining compared to the contralateral unoperated side. In contrast, the nerve root of the degenerating facial nucleus after ricin application showed an early increase in Ia immunoreactivity 3 days after surgery. In bilaterally operated animals microglial cells in the genu n. facialis revealed intense immunostaining on the ricin-treated side compared to that on the axotomized side (Figs. 4C and 4D). During these early time points considerably more Ia-positive cells could also be identified in the degenerating than in the axotomized nucleus (Figs. 4A and 4B).

Over a period of 4–5 weeks after nerve lesioning a gradual and continuous increase in the number of Ia-immunoreactive cells was observed in both experimental situations (Fig. 5; Table 1). During this time period there seemed to be a slight preponderance of Ia-positive cells in the degenerating compared to the regenerating facial nucleus. While this quantitative difference was not striking, in qualitative terms considerable differences were noted between ricin-treated and axotomized nuclei after identical postoperative survival periods. The number of Ia-expressing microglial cells increased on the ri-
FIG. 4. Comparison of OX-6 immunoreactivity after axotomy (A, C) and ricin injection (B, D). Note increased number of Ia-positive perivascular cells in the nucleus after 4 days in B compared to A. X77. X 90. The nerve root after ricin (D) contains numerous microglial cells strongly positive for Ia antigen, while on the axotomized side (C) only very faint Ia-immunoreactivity can be seen 3 days after operation. X98.

cin side at the expense of rapidly disappearing perivascular cells, and by the end of the fourth week almost the entire population of Ia-positive cells consisted of ramified microglia (Figs. 5E and 5F). In contrast, after axotomy perivascular cells were conspicuous until the end of week 4, at which time approximately equal proportions of perivascular and microglial cells were present (Figs. 5A and 5B). In some of these preparations we were able to observe Ia-expressing cells with a transitional morphology intermediate between perivascular and microglial cells suggesting differentiation of perivascular cells into microglia (Figs. 5C and 5D). As survival times after axotomy exceeded 1 month, more microglial cells kept appearing while Ia-positive perivascular cells were decreasing (Fig. 6). Whereas after 3 months hardly any Ia-positive cells could be detected in the ricin-treated nucleus, Ia-positive cells persisted on the axotomized side in the form of ramified microglia until day 128. Quite frequently perineuronally located microglial cells were observed during these late stages.

Ultrastructural Identification of Ia-Positive Cells

Throughout all preparations including normal brain we observed perivascular cells expressing Ia antigen. With the aid of ultrastructural immunocytochemistry we could clearly exclude the possibility that these cells were endothelial in nature, because reaction product was absent from the luminal surface of endothelial cells and Ia-immunoreactive cells were always seen to be completely surrounded by a basal lamina (Fig. 7) and were thus consistent in their morphology with the definition of a pericyte (27). In view of the overall low density of Ia-positive perivascular cells in normal brain, however, this cell population could not possibly include all pericytes and is thus likely to represent either a subpopulation of pericytes or a separate population of pericycle-like cells. We suggest using the term “perivascular cell” until the identity of this cell population is elucidated. In preparations from animals with long postoperative survival times perineuronally located Ia-positive microglial cells could be identified.

DISCUSSION

Our results demonstrate a gradual and long-lasting increase in the number of Ia-immunoreactive cells during regeneration and degeneration of facial motor neurons.
FIG. 5. OX-6 immunoreactivity 27 days after axotomy (A–D) and ricin injection (E, F). (A) Low power view shows large numbers of Ia-positive cells occupying not only the area of the facial nucleus proper, but also surrounding regions of the brain stem. ×30. (B) Higher magnification of the axotomized nucleus reveals a mixed population of Ia-positive cells consisting of perivascular and microglial cells. ×100. C and D demonstrate Ia-positive cells in close vicinity of small blood vessels that could represent transitional forms between perivascular and microglial cells. ×240. (E) Panoramic view of degenerated nucleus demonstrates Ia-positive cells in the nucleus (n7) and the nerve root (r7). ×24. (F) Four weeks after ricin injection almost the entire population of Ia-immunoreactive cells consists of ramified microglia. ×240.
Cell types expressing Ia antigen include perivascular and microglial cells. Regarding the time course we found that the appearance and disappearance of Ia-positive cells happens faster during ricin-induced degeneration than after axotomy. In both experimental situations Ia immunoreactivity first becomes apparent on perivascular cells located within the basal lamina, but with longer postoperative survival periods there is a tendency for Ia-positive microglia to appear. This shift in morphology and location of Ia-positive cells is complete at least 2 weeks earlier after ricin treatment than after transection. Thus, if Ia expression is considered to indicate immunological activation of microglia, it can be concluded that microglial immune competence is achieved sooner with increased severity of the lesion. This concept is supported by earlier findings, namely, that during ricin-induced degeneration local microglia demonstrate significantly enhanced mitotic activity compared to simple nerve crush lesions and transform into brain macrophages (32). It remains to be shown what, if any, neuronal and/or glial factors are released in an injury-dependent manner to regulate microglial activation.

We could not confirm the findings of Maehlen et al. (20) showing immunoreactivity for MHC class I molecules on facial motor neurons. Our previous studies with mAb OX-18 and another antibody against class I molecules which showed no reactivity (34), as well as the present study employing ultrastructural immunocytochemistry, have consistently failed to demonstrate immunoreactivity on structures other than the microglial and endothelial cell surfaces. Possibly, OX-18 staining of neurons is caused either by differences in fixation or by nonspecific binding of the secondary biotinylated antibody which, in our experience, needs to be preabsorbed with normal serum from the strain of experimental animals used in order to achieve the lowest background staining. The weak staining results obtained with mAb OX-3 in comparison with those obtained with mAbs OX-17 and OX-6 are likely to be caused by OX-3 having a binding affinity toward a polymorphic determinant of Ia antigen. Unlike monomorphic determinants which are found in all individuals of a species, polymorphic determinants are found only in some (17).

Hickey and Kimura (16) have recently reported about perivascular microglia that are bone marrow derived and function as antigen presenting cells in the rat CNS. Since the present results offer further support for the immune competence of perivascular cells and microglia, a clarifying remark about the terminology employed seems appropriate at this point. The term “perivascular microglia” is used to describe the anatomic location of some parenchymal microglia lying outside the basal lamina of blood vessels (5, 27, 31), while the term “perivascular cell” refers to a pericyte-like cell enclosed within the basal lamina and thus located outside the brain parenchyma (12). On the basis of their morphology and Ia immunoreactivity, the cells described by Hickey and Kimura are most likely identical with the perivascular cells described here. However, we would like to extend their observations by suggesting that the Ia-positive microglia we observed after late postoperative intervals when perivascular cells were decreasing arise through morphological transformation of Ia-positive perivascular cells. This is based mainly on our observation that concomitant to the disappearance of Ia-positive perivascular cells there is an increase in Ia-positive microglia up to a point where almost all perivascular cells have disappeared and microglia predominate. Further support for such a transformational process comes from the studies of Hager (13) showing that in traumatized brain tissue perivascular cells can break through the basement membrane and become microglia. In addition, Bartlett (3) reported that the adult rodent brain contains large numbers of hemopoietic stem cells which may be a source of microglia.

### Table 1

Summary of Results: Ia-Positive Cells in the Regenerating and Degenerating Facial Nucleus

| Weeks after injury | Axotomy | Ricin injection | Unoperated control |
|-------------------|---------|-----------------|-------------------|
| 1                 | Perivascular cells (+) | Perivascular cells (++) | Perivascular cells (±) |
|                   | Microglial cells (±)   | Microglial cells (±)   | Microglial cells (±)   |
| 2                 | Perivascular cells (++) | Perivascular cells (++) | Perivascular cells (±) |
|                   | Microglial cells (±)   | Microglial cells (±)   | Microglial cells (±)   |
| 3                 | Perivascular cells (++) | Perivascular cells (++) | Perivascular cells (±) |
|                   | Microglial cells (±)   | Microglial cells (±)   | Microglial cells (±)   |
| 4                 | Perivascular cells (++) | Perivascular cells (++) | Perivascular cells (±) |
|                   | Microglial cells (±)   | Microglial cells (±)   | Microglial cells (±)   |
| 6                 | Perivascular cells (+) | Perivascular cells (±) | Perivascular cells (±) |
|                   | Microglial cells (±)   | Microglial cells (±)   | Microglial cells (±)   |
| 15                | Perivascular cells (±) | Perivascular cells (±) | Perivascular cells (±) |
|                   | Microglial cells (±)   | Microglial cells (±)   | Microglial cells (±)   |

*Note. *, sparse (0–5 cells/section); +, few (5–10 cells/section); ++, intermediate (10–20 cells/section); +++, many (>20 cells/section).*
FIG. 6. (A) OX-6 immunoreactivity 44 days after axotomy is largely confined to microglia, and only few immunoreactive perivascular cells can be found. (B) One hundred twenty-eight days after transection Ia-positive cells are ramified microglia. Note perineuronal microglia in A and B (arrows). x200.

Clearly, the subpopulation of microglia that may arise from perivascular cells represents only a minority of the total microglial population, because very few Ia-positive microglia can be identified in the facial nucleus during the first week after lesioning when microglial proliferation peaks (18). However, during that early period microglia are strongly positive for MHC class I antigens (34). Therefore, we propose that the total microglial population is heterogeneous with regard to their ability to express different MHC antigens, as well as with regard to their recent origin. While the majority of microglia are already present in the normal brain, under pathological conditions perivascular cells may become stimulated to form an auxiliary, albeit small, source of antigen presenting cells in addition to the existing population. This concept of microglial heterogeneity could
help to explain the long-standing and continuing debate about the origin of microglia (cf. (22)).

The prolonged presence of Ia-positive cells in our experimental paradigms necessarily implies the prolonged presence of a stimulant, as this seems to be a minimal requirement for maintaining Ia expression (37). The most potent stimulatory substance known is interferon-γ (IFN-γ) which has been shown to induce Ia expression on microglia in vivo (30). However, we have so far been unable to detect IFN-γ either immuno-cytochemically or by enzyme-linked immunosorbent assay in regenerating or degenerating facial nuclei. Probably other, yet unknown, factors may be involved in regulating Ia expression under these conditions. The fact that inflammatory infiltrates consisting of T lymphocytes are absent in our models further supports the involvement of alternative stimulatory factors. Thus, if the peripheral immune system is not involved one may postulate a nonimmunological function of Ia antigens during glial reactions. It has been demonstrated that MHC class II antigens can mediate cell adhesion by interacting directly with the CD4 antigen of lymphocytes (6). The authors have suggested that this interaction may be important for cellular communication among lymphocytes, so that, by the same token, the presence of both class II and CD4 antigens on CNS microglia (26) might facilitate similar interactions among this cell type. This thought seems especially attractive in view of the recent demonstration that microglial cells also express antigens typical for B lymphocytes (24).

Contrary to some reports (20, 29), but confirming those of others (14, 38), our findings have demonstrated Ia immunoreactivity on perivascular cells and microglia in normal brain. A peculiar characteristic of Ia-positive
microglia is their preferential localization in normal white matter. The reason for this is presently unknown, but it is an intriguing finding that could account for the susceptibility of white matter to demyelination in multiple sclerosis. Woodroofe et al. (39) have pointed out the possibility that Ia-positive microglia in white matter surrounding inflammatory lesions function as antigen presenting cells thereby extending the lesion. Another glial cell type found predominantly in white matter is the fibrous astrocyte. Since fibrous astrocytes rapidly appear in the gray matter of the facial nucleus after nerve transection (10), as well as after ricin intoxication (32), it is apparent that the microenvironment of regenerating and degenerating gray matter favors changes in the structure and biochemistry of glial cells. Similarly, during acute EAE in the rat GFAP-positive fibrous astrocytes are prominent in gray matter (2), as well as Ia-positive perivascular cells and microglia (38). On the basis of these observations one could speculate that the occurrence of GFAP-positive fibrous astrocytes and Ia-positive microglia in gray matter are regulated simultaneously or mutually through chemical changes in the microenvironment of injured central nervous tissue. For example, the release of interleukin-1 from microglia (9) or interleukin-3 from astrocytes (7) during the early stages after injury could be such a chemical change, since the interleukins have been shown to act as growth factors for microglia and astrocytes.

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