

Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal Rat.

N. Benjelloun, Sylvain Renolleau, Alfonso Represa, Yehezkel Ben-Ari, Christiane Charriaut-Marlangue

► **To cite this version:**

N. Benjelloun, Sylvain Renolleau, Alfonso Represa, Yehezkel Ben-Ari, Christiane Charriaut-Marlangue. Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal Rat.. Stroke, American Heart Association, 1999, 30 (9), pp.1916-23; discussion 1923-4. inserm-00486285

HAL Id: inserm-00486285

<https://www.hal.inserm.fr/inserm-00486285>

Submitted on 25 May 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Stroke

American Stroke
AssociationSM

A Division of American
Heart Association



JOURNAL OF THE AMERICAN HEART ASSOCIATION

Inflammatory Responses in the Cerebral Cortex After Ischemia in the P7 Neonatal Rat • Editorial Comment

N. Benjelloun, S. Renolleau, A. Represa, Y. Ben-Ari, C. Charriaut-Marlangue and Giora Z. Feuerstein

Stroke 1999;30;1916-1924

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 72514
Copyright © 1999 American Heart Association. All rights reserved. Print ISSN: 0039-2499. Online
ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://stroke.ahajournals.org/cgi/content/full/30/9/1916>

Subscriptions: Information about subscribing to Stroke is online at
<http://stroke.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
<http://www.lww.com/reprints>

Inflammatory Responses in the Cerebral Cortex After Ischemia in the P7 Neonatal Rat

N. Benjelloun; S. Renolleau, MD; A. Represa, PhD; Y. Ben-Ari, PhD; C. Charriaut-Marlangue, PhD

Background and Purpose—The contribution of inflammatory response to the pathogenesis of ischemic lesions in the neonate is still uncertain. This study described the chronological sequence of inflammatory changes that follow cerebral ischemia with reperfusion in the neonatal P7 rat.

Methods—P7 rats underwent left middle cerebral artery electrocoagulation associated with 1-hour left common carotid artery occlusion. The spatiotemporal pattern of cellular responses was characterized immunocytochemically with the use of antibodies against rat endogenous immunoglobulins to visualize the area of the breakdown of the blood-brain barrier. Infiltration of neutrophils and T lymphocytes was demonstrated by antibodies against myeloperoxidase and a pan-T cell marker, respectively. Antibodies ED1 and OX-42 were applied to identify microglial cells and macrophages. The response of astrocytes was shown with antibodies against glial fibrillary acidic protein. Cell survival was assessed by Bcl-2 expression. Cell death was demonstrated by DNA fragmentation with the use of the terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) assay and Bax immunodetection.

Results—Endogenous immunoglobulin extravasation through the blood-brain barrier occurred at 2 hours of recirculation and persisted until 1 month after ischemia. Neutrophil infiltration began at 24 hours and peaked at 72 to 96 hours (30 ± 3.4 neutrophils per 0.3 mm^2 ; $P < 0.0001$), then disappeared at 14 days after ischemia. T cells were observed between 24 and 96 hours of reperfusion. Resident microglia-macrophages exhibited morphological remnants and expressed the cell death inhibitor Bcl-2 at 24 hours of recirculation. They became numerous within the next 48 hours and peaked at 7 days after ischemia. Phenotypic changes of resident astrocytes were apparent at 24 hours, and they proliferated between 48 hours and 7 days after ischemia. Progressively inflammatory cells showed DNA fragmentation and the cell death activator Bax expression. Cell elimination continued until there was a complete disappearance of the frontoparietal cortex.

Conclusions—These data demonstrate that perinatal ischemia with reperfusion triggers acute inflammatory responses with granulocytic cell infiltration, which may be involved in accelerating the destructive processes. (*Stroke*. 1999;30:1916-1924.)

Key Words: brain injuries ■ ischemia ■ leukocytes ■ newborn ■ reperfusion ■ rats

Perinatal ischemic brain injury may lead to the death of neonates and important neurological impairment manifested as cerebral palsy and mental retardation in the surviving infants.¹⁻³ This is due to the fact that the basal ganglia as well as certain regions of neocortex are relatively vulnerable to injury in the neonatal period.⁴ Perinatal asphyxia, with its attendant bradycardia and hypotension, is an ischemic condition that is followed by reperfusion on resuscitation.

Reperfusion after an ischemic episode elicits in adult brains the appearance of deleterious blood leukocytes, potentially a source of toxic free radicals and cytokines causing inflammatory reactions and leading to a delayed death of neurons and the expansion of cerebral damage (for reviews, see References 5 and 6). In contrast, the physiological events that follow reperfusion in the neonate have not been largely examined. With the use of the classic model of Rice et al⁷ in

See Editorial Comment, page 1923

7-day-old rats, neuronal death was found to be associated with an activation of both microglia and astrocytes, while granulocytic cells were not involved.⁸⁻¹⁰ It is presently unclear whether those effects result from the combination of both processes (ischemia and hypoxia) or from ischemic insult alone. To investigate the mechanisms of reperfusion injury, we recently developed a model of stroke by transient unilateral focal ischemia in P7 rats by permanent left middle cerebral artery (MCA) occlusion with 1-hour occlusion of the left common carotid artery. Ischemia with reperfusion in the anastomoses via the carotid artery produced a well-delineated cortical infarct at 48 hours of reperfusion¹¹ and neuronal death with features characteristic of apoptosis.^{11,12}

This study was designed to investigate inflammatory responses in this model between 2 hours and 3 months of

Received February 4, 1999; final revision received April 29, 1999; accepted June 2, 1999.

From the Université René Descartes, Paris, France.

Correspondence to C. Charriaut-Marlangue, INSERM U29, 123 bd de Port-Royal, 75014 Paris, France. E-mail cm@u29.cochin.inserm.fr

© 1999 American Heart Association, Inc.

Stroke is available at <http://www.strokeaha.org>

reperfusion. Our data strongly suggest that inflammatory cells may be involved in promoting secondary cell death in neonatal ischemia with reperfusion.

Materials and Methods

Animals and Neonatal Ischemia

Experiments involving animals were approved by the French Ethical Science Committee (statement No. 04223). Experiments were performed in 7-day-old Wistar rats (weight, 15 to 23 g) of both sexes, as previously described.¹¹ Rat pups were anesthetized with an injection of chloral hydrate (300 mg/kg IP). After 15 minutes, rats were placed on their back, and a median incision was made in the neck to expose the left common carotid artery. Rats were then placed on the right side, and an oblique skin incision was made between the ear and the eye. After excision of the temporal muscle, the cranial bone was removed from the frontal suture to a level below the zygomatic arch. Then the left MCA, exposed just after its apparition over the rhinal fissure, was coagulated at the inferior cerebral vein level. After this procedure, a clip was placed to occlude the left common carotid artery and was removed after 1 hour. Carotid blood flow restoration was verified with the aid of a microscope. Both neck and cranial skin incisions were then closed. The surgical procedure lasted approximately 20 minutes. During surgery, body temperature was maintained at 37°C to 38°C. Rat pups were placed in an incubator maintained at 37°C until they woke up, and then they were transferred to their mother for long-time survival.

Tissue Preparation

Each of the following survival times after reperfusion studied was represented by a group of 5 animals: 6, 24, 72, and 96 hours; 7 and 14 days; and 1 and 3 months. At each point, animals were perfused transcardially with warm heparinized saline followed by a phosphate-buffered solution (0.1 mol/L, pH 7.4) containing 4% paraformaldehyde. Brains were immediately removed and postfixed for 1 hour in the same fixative solution at room temperature. Then they were placed in a phosphate-buffered solution containing 10% sucrose for 2 days at 4°C. The brains were frozen in isopentane at -40°C. They were then stored at -80°C until used. For control, 2 sham age-matched rats were killed and processed in the same way. Coronal cryostat sections (10 μm thick) were collected on gelatin-coated slides. Cresyl violet was used to identify the site of the lesion and to evaluate degenerating cells.

Immunocytochemistry

Antisera

A mouse monoclonal antibody against glial fibrillary acidic protein (GFAP) (1:200, Boehringer Mannheim) was used to visualize astrocytes. Rat microglia and brain macrophages were immunostained by the mouse monoclonal antibody OX-42 (1:500, Serotec) and ED1 (1:500, Serotec). OX-42 recognizes the complement C3bi receptor expressed by monocytes/macrophages and activated ramified and amoeboid microglia. ED1 antibodies label most rat macrophages, peripheral blood monocytes, and activated microglia.¹³ The area of leakage of the blood-brain barrier (BBB) was identified by immunodetection of rat endogenous immunoglobulins in brain parenchyma (1:200, Dako). Rabbit antibodies against myeloperoxidase (MPO) (1:200, Sigma) were used to visualize polymorphonuclear leukocytes (PMNL). Mouse monoclonal antibodies against rat T lymphocyte (Pan-T, 1:200, Serotec) were used to identify infiltrating T lymphocytes. Bcl-2 antibodies were purchased from Boehringer Mannheim and Bax (P-19) antibodies from Santa Cruz (Tebu). They recognized p26 and p21 proteins to identify key regulators of apoptosis in surviving and dying cells, respectively.

Immunostaining Procedure

Sections were incubated overnight at 4°C in the first antibody at the dilution indicated above in PBS 0.1 mol/L containing 2% BSA, 0.3% Triton X-100. The secondary (anti-rabbit or anti-mouse IgG) biotinylated antibodies (1:200 to 1:400 dilution, Dako) were visualized by

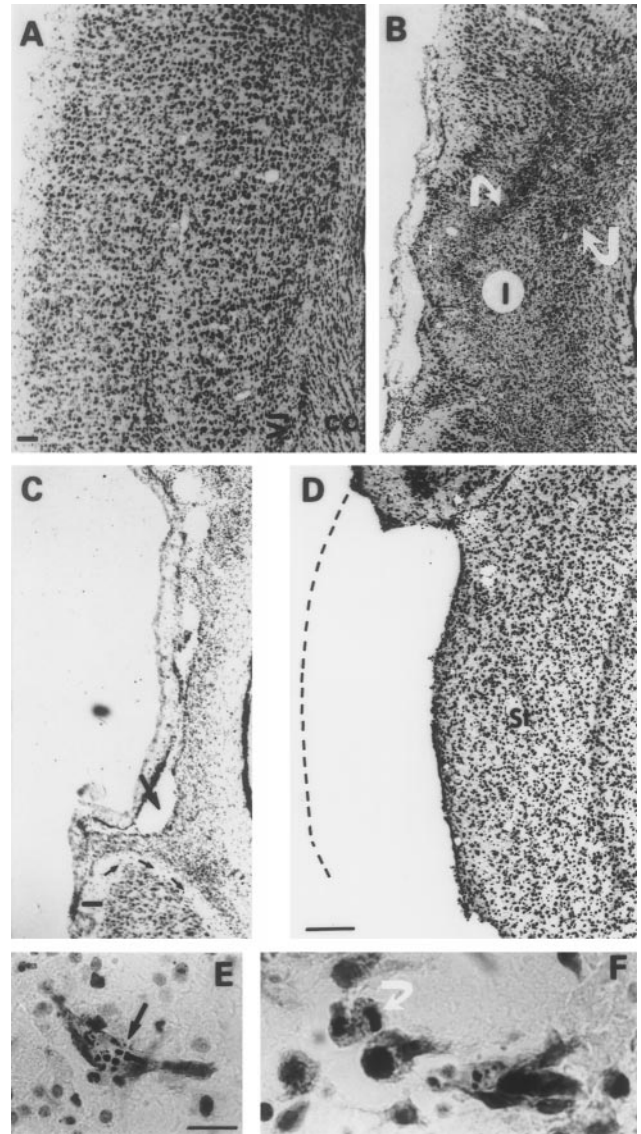


Figure 1. Histopathological changes after neonatal ischemia. Cresyl violet-stained sections (bregma 0.2 mm) show frontoparietal cortex of control rat (A) and rats subjected to unilateral permanent electrocoagulation of the MCA associated with 1-hour common carotid occlusion followed by various recovery intervals (B through F). A, General view of the cortex of a 7-day-old rat pup showing normal cytoarchitecture. B, Note the important cortical thickness at 72 hours of reperfusion with the formation of a gliotic wall (curved white arrows) surrounding the infarcted zone (I). C, Loss of the upper cortical layers and formation of a cavity in the infarcted area (black arrow) at 7 days of reperfusion. Note the arrangement of the molecular layer I of adjacent noninfarcted cortical area (small black arrows). D, At 3-month recovery, complete loss of the frontoparietal ischemic cortex is shown. E, F: Details of panel B at higher magnification. E, Dying neuron showing pyknotic nucleus (black arrow). F, Glial cell at the periphery of the infarct showing mitotic figure (white arrow). I through VI indicate layers of the cerebral cortex; cc, corpus callosum; and St, striatum. Bar represents 50 μm in A through D and 10 μm in E and F.

the avidin-biotin peroxidase (Elite ABC kit, Vectastain Vector, Byosis). The peroxidase activity was evidenced with the use of 0.1 mol/L phosphate buffer containing 0.025% 3,3'-diaminobenzidine (DAB) and 0.02% hydrogen peroxide. Nonspecific peroxidase activity was abrogated by incubating the sections in 2% hydrogen peroxide in 10% methanol at the appropriate stage. Some immuno-

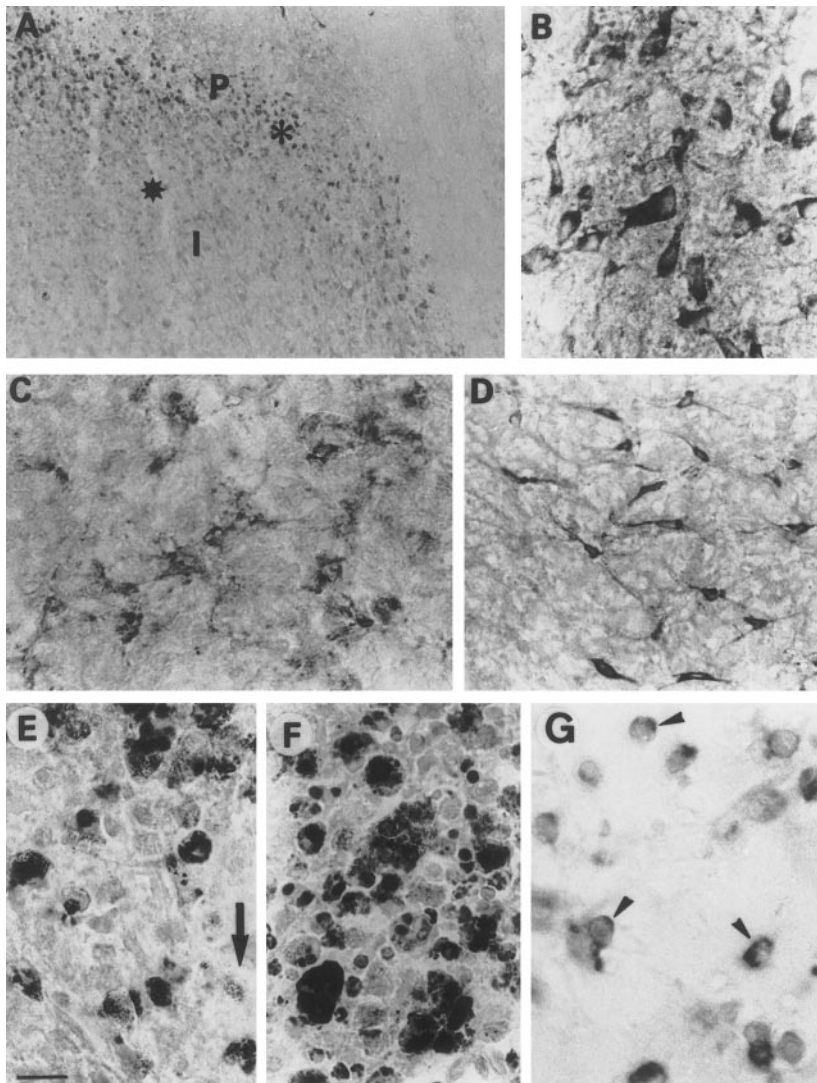


Figure 2. Status of the BBB and infiltrating cells after ischemia/reperfusion in neonatal rats. A, Rat immunoglobulin immunostaining demonstrating the leakage at the BBB at 72 hours after ischemia. Note that the leakage area is bigger than the infarcted zone (I). Stars (*, *) indicate area enlarged in B and C, respectively. B, Neurons within the infarct immunostained by anti-rat immunoglobulins. C, Cells exhibiting glial phenotype localized at the periphery of the infarcted area immunostained by anti-rat immunoglobulins. D, Rat immunoglobulin-immunostained cells within the substantia nigra in the ipsilateral hemisphere. E, F, PMNL with typical segmented nuclei (MPO positive). E, Polymorphonuclear cells in the upper layers of the infarcted cortex at 48 hours of reperfusion. Arrow indicates that MPO-positive cells invaded the cortical layers from layer I to layer VI. F, Polymorphonuclear cells in large number within the infarct at 96 hours of reperfusion. G, Lymphocytes in the hemisphere ipsilateral to the ischemic lesion at 24 hours of reperfusion. Sections in E and F have been counterstained by hematoxylin. I indicates infarct; P, periphery. Bar represents 40 (A) and 10 (B through G) μm .

stained sections were counterstained with hematoxylin for localization of the labeled cells. In double-labeling experiments, sections were first incubated with antibodies against GFAP, OX-42, or ED1 and revealed by the avidin-biotin alkaline phosphatase (ABC kit, Vectastain Vector) with the Substrate Kit III (Vector Blue). They were then incubated with anti-Bax and visualized with peroxidase and DAB.

In Situ Labeling of Fragmented DNA

Coronal cryostat sections were processed for terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) assay, as previously reported.¹⁴ Briefly, sections were incubated with terminal deoxynucleotidyl transferase (0.2 U/ μL ; Gibco) and biotin-16-dUTP (20 $\mu\text{mol/L}$; Boehringer Mannheim), then visualized with streptavidin-biotin-peroxidase complex and diaminobenzidine.

Quantification of Neutrophils

Quantification of neutrophils was performed with the use of a $\times 40$ objective and a camera lucida attached to the microscope. At different times after ischemia, in the 5 ischemic animals, intraparenchymal MPO-positive neutrophils were counted in a 0.3-mm² circular area on 3 sections containing the infarct. The statistical significance of the results was evaluated by the unmatched Student's *t* test.

Results

Sections used in the figures are representative of the 5 animals analyzed in each group at each time period.

Histopathology

Figure 1 shows the cytoarchitecture and cell morphology of the cortex from control (Figure 1A) and ischemic (Figure 1B through 1D) rat pup brains after examination of cresyl violet-stained sections. At 72 hours of reperfusion (Figure 1B), the infarcted area collapsed and demonstrated cells with coarse chromatin clumping and nuclear pyknosis (black arrow in Figure 1E). At the periphery of the infarct, a more marked hypercellular band (white curved arrows in Figure 1B) appeared and showed several mitotic figures (white curved arrow in Figure 1F). One week after the ischemic insult, the infarcted area cleared progressively of neuronal perikarya. Cortical upper layers disappeared, and a cavity was formed (Figure 1C, black arrow). By 2 weeks of reperfusion, the ipsilateral hemisphere appeared reduced compared with the contralateral hemisphere (not shown). The loss of the frontoparietal cortex was completed 3 months after recovery (Figure 1D).

Early BBB Impairment, PMNL, and Lymphocyte Infiltration

Endogenous immunoglobulin extravasation was only observed in the ischemic ipsilateral hemisphere and appeared as

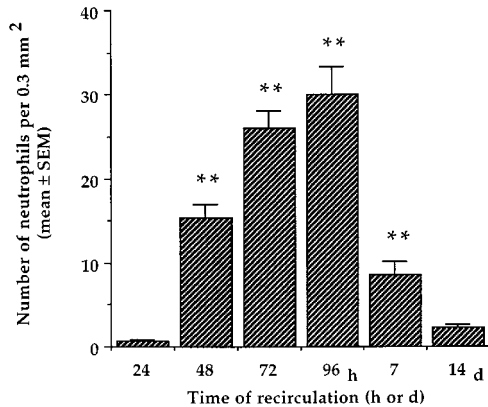


Figure 3. Density of intraparenchymal neutrophils in the ipsilateral cortex at different times of reperfusion. Student's *t* test indicates a significant increase of neutrophils at 48, 72, and 96 hours and 7 days of recirculation compared with that found at 24 hours (** $P < 0.0001$). MPO-positive cells were counted in 12- μm -thick sections; $n = 5$ to 8 sampling areas from 5 rats at each time point.

early as 2 hours after reperfusion in the core of the infarct. At 72 hours, the area of BBB leakage was larger than the infarcted area (Figure 2A). Altered cells within the infarct (Figure 2B), glial cells at the periphery of the infarct (Figure 2C), and nonaffected neurons located in regions without the BBB and occasionally in the substantia nigra (Figure 2D) were immunostained by anti-rat immunoglobulins. Anti-rat immunoglobulin immunostaining was still observed 1 month after reperfusion in the ipsilateral hemisphere, but not thereafter.

PMNL were detected in the ischemic tissue at 24 hours of reperfusion. At this point, they were essentially observed in arachnoid spaces and associated with intraparenchymal blood vessels within and near the lesioned area (not shown). Progressively, PMNL invaded the upper layers of infarcted cortex from 48 to 96 hours of recovery (Figure 2E and 2F). Intraparenchymal neutrophils peaked at 72 to 96 hours of reperfusion (Figure 3). No PMNL were observed after 2 weeks of reperfusion. Infiltrating lymphocytes (pan-T immunoreactive cells) were transiently observed between 24 and 96 hours of recovery and were preferentially located in the white matter of the ipsilateral hemisphere, notably the corpus callosum (Figure 2G), the internal capsule, and often in close vicinity to blood vessels.

Time Course of Microglia/Macrophage Responses

Activated OX-42-immunostained microglia/macrophages in neonatal ischemic rat brain were detected at the periphery of the infarcted region at 72 hours of reperfusion (Figure 4A). They increased in number and progressively invaded the injured tissue between 96 hours and 1 week of reperfusion (Figure 4B) and expressed the survival promoter Bcl-2.¹⁵ Bcl-2-immunoreactive cells showed cell bodies and numerous long, thin processes (called ramified microglia) (Figure 4C and 4D) or stout processes (called amoeboid microglia) (Figure 4E). Quiescent microglia in sham pups was not immunostained by Bcl-2 antibodies (not shown). OX-42-immunostained microglia persisted around the cavity several weeks after ischemia.

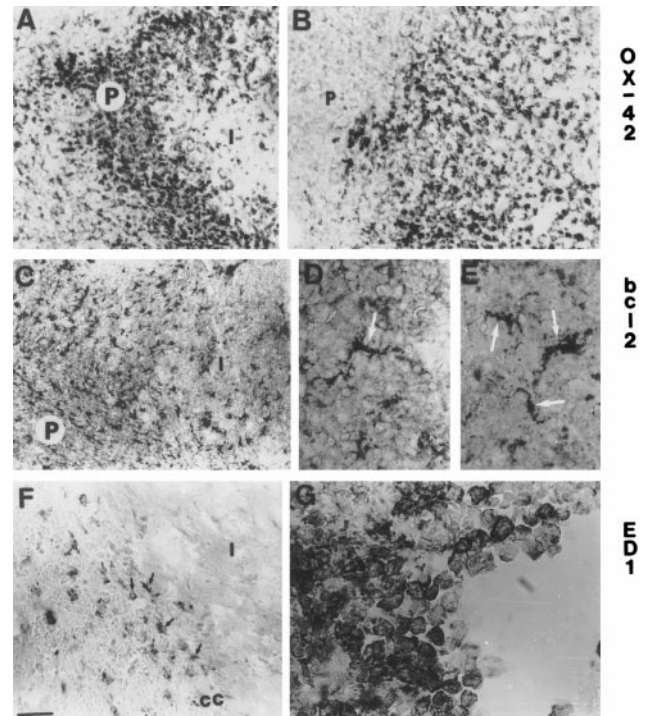


Figure 4. Microglia/macrophage activation in neonatal ischemia/reperfusion identified by immunocytochemistry using the OX-42 antibody. A, Reactive microglial cells (OX-42 positive) at the periphery of the infarct at 72 hours of reperfusion. B, Progression of OX-42 immunostaining from the periphery to the core of the infarct at 7 days of reperfusion. C through E, Bcl-2-immunostained cells showing morphology similar to that of microglial cells at 72 hours of reperfusion. D, E, Higher magnification of Bcl-2-positive cells showing a typical punctiform labeling. Note ramified type of activated microglial cells (white arrow in D) and pseudopodic type of activated microglia located in the subcortical white matter (white arrows in E). F, ED1-positive macrophages at the periphery of the infarct at 24 hours of reperfusion. G, Round ED1-positive cells within the infarct at 7 days of reperfusion. P indicates periphery; I, infarct; and cc, corpus callosum. Bar represents 40 (A through C, F), 25 (D, E), and 10 (G) μm .

ED1 antibody identified macrophages, round cells that had a smooth surface and lacked processes; they appeared first in the lateral ventricle and migrated along the corpus callosum at 24 hours of recovery (Figure 4F). A prominent increase in their accumulation was observed between 72 hours and 1 week of reperfusion, and these cells invaded the infarcted area (Figure 4G). Pseudopodic and ramified ED1-positive microglia/macrophages remained present in the infarcted area at later times of reperfusion.

Time Course of Astroglial Cell Responses

During the first month of life, control rat brains showed GFAP-positive astrocytes predominantly in the corpus callosum and the internal capsule. The molecular layer of the cortex and perivascular spaces showed weak immunostained GFAP-positive astrocytes (Figure 5A through 5C). The infarcted cortical area of ischemic rats showed increased GFAP immunoreactivity 24 to 48 hours after reperfusion. Reactive astrocytes formed a dense network delineating the infarcted area (Figure 5D and 5E). Reactive GFAP-immunostained

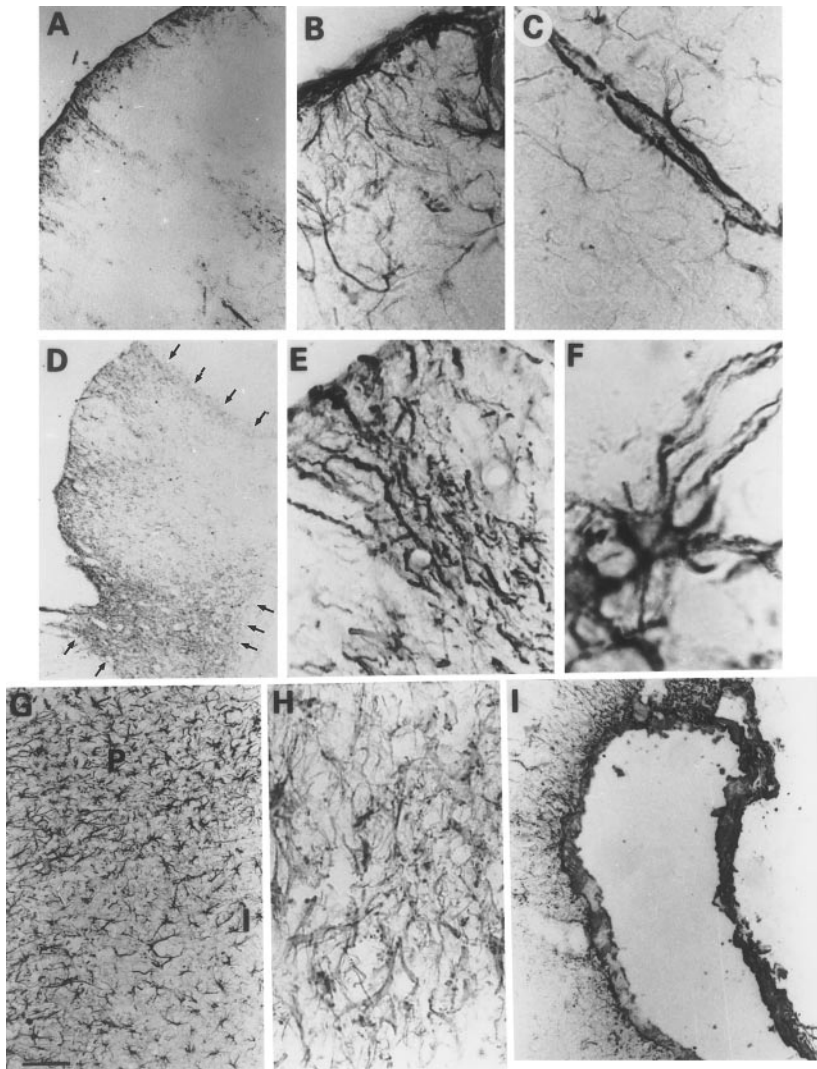


Figure 5. Morphological remnants of astrocytes in neonatal ischemia/reperfusion identified by immunohistochemistry using the GFAP antibody. A, Distribution of GFAP-positive astrocytes in the frontoparietal cortex from 7-day-old control rat. Note that GFAP-positive cells are localized in subpial spaces and around vessels. B, Astrocyte processes directed toward subpial spaces. C, Astrocyte processes surrounding a vessel in the cortex. D, The infarcted area at 72 hours of reperfusion. Note that reactive astrocytes delineate the infarcted zone (small arrows). E, F, Higher magnification of reactive astrocytes. E, Strongly GFAP-immunoreactive astrocyte processes directed toward subpial spaces in the infarcted cortex. F, GFAP-positive reactive astrocyte at the periphery of the infarct with a small soma and long processes. G, GFAP-positive reactive astrocytes increased at 7 days of reperfusion and showed large soma and short processes. H, Astrocytes within the infarct showing decreased GFAP immunoreactivity at 14 days of reperfusion. I, The astrocytic processes delineate the cavity at 1 month of reperfusion. I indicates infarct; P, periphery. Bar represents 50 (A and D), 25 (B and E, G through I), and 10 (C and F) μm .

cells have a small soma and long processes directed toward the subpial spaces and the core of the infarct (Figure 5F). One week after the ischemic insult, strongly GFAP-immunostained astrocytes were observed at the periphery and within the infarct (Figure 5G). After the second week of recovery, reactive astrocytes within the infarct showed features of degradation, including a decrease in GFAP immunostaining in fragmented processes and residual star-shaped cell elements (Figure 5H). From 14 days until 2 months after reperfusion, astrocyte processes closely surrounded the smooth-walled cavity (Figure 5I).

Cell Death: Did Glial Cells Undergo Apoptosis?

Cells exhibiting DNA fragmentation were shown as early as 6 hours; they increased and peaked at 24 to 96 hours.¹¹ During the next 2 weeks, the infarct developed a smooth-walled cavity (Figure 1), and TUNEL-positive cells were observed around this cavity (Figure 6A). These cells, with different morphology, exhibited the death promoter gene Bax, expressed by dying cells,¹⁶ as demonstrated by immunostaining of adjacent sections (Figure 6B). At 1 month of recirculation, Bax-positive cells were detected at the periphery of the infarct (Figure 6C), but these cells displayed a glial

morphology (Figure 6D). The staining of sections with both Bax and GFAP or OX-42 antibodies revealed the presence of double-labeled astrocytes (Figure 7A) and microglia (Figure 7B) at the periphery of the infarct between 14 and 30 days of recovery.

Discussion

The present study shows that ischemia/reperfusion in immature rats induces progressive tissue destruction, leading to a well-delineated lesion in the ipsilateral cortex at 3 months after recovery. BBB impairment followed by gliosis and infiltration of leukocytes provides evidence of a strong local inflammatory response (summarized in the Table). At later times of reperfusion, cells integrated in the inflammatory response were eliminated through an apoptotic pathway.

The BBB was reported to contribute to the progression of the cellular injury, as suggested in other models of cerebral ischemia (for review, see Reference 15). The labeling of neurons by anti-rat immunoglobulins within the ipsilateral hemisphere may reflect a nonspecific uptake of extravasating immunoglobulins rather than specific recognition, as previously described in other pathological processes¹⁶; that of

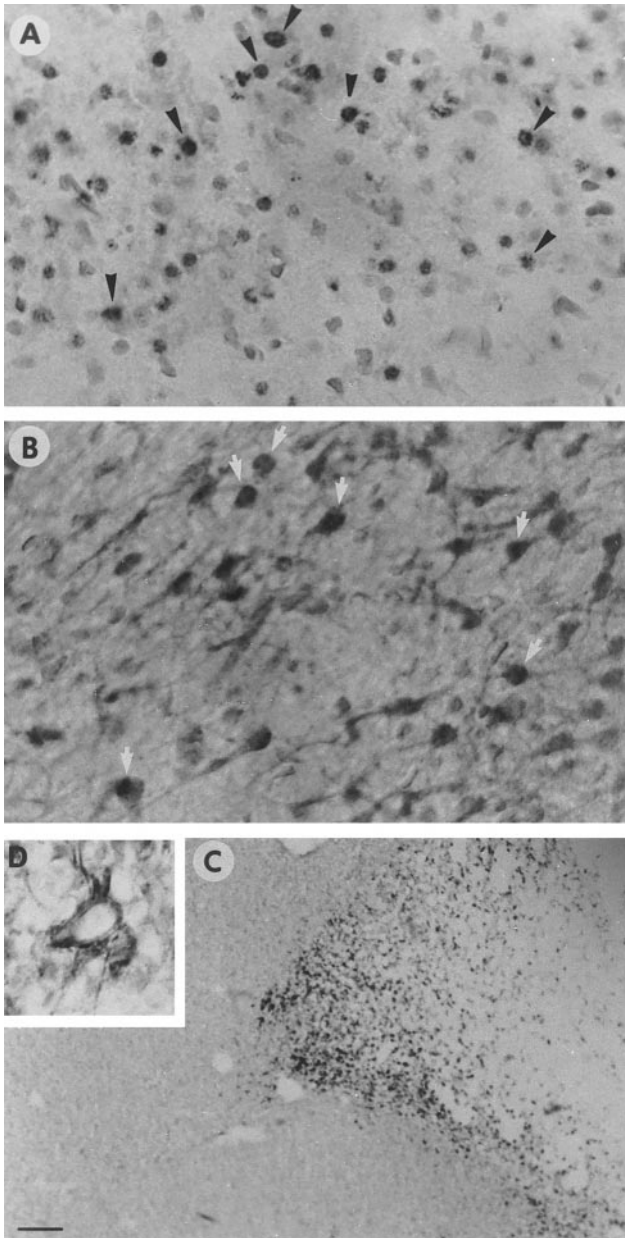


Figure 6. Cell death in the ipsilateral cortex demonstrated by the TUNEL assay and immunostaining with anti-Bax antibodies. A, Apoptotic nuclei (arrowheads) in the deep layers of the ipsilateral cortex at 14 days of reperfusion. B, Adjacent section to that seen in A, immunostained with the cell death promoter Bax; white arrows indicate Bax-positive cells, corresponding to TUNEL-positive nuclei. C, Bax-positive cells localized at the periphery of the infarct at 1 month of reperfusion. Enlarged panel (D) shows a Bax-positive cell exhibiting glial morphology. Bar represents 25 (C) and 10 μm (A, B, and D).

microglial cells may be due to the overexpression of the Fc receptor during their activation.¹⁷ BBB disruption may also induce an entry of toxic molecules, such as albumin and excitatory amino acids. There has been increasing recognition that the cerebrovascular endothelium participates in immune responses as a target for cytokine action and is a source of arachidonic acid metabolites,¹⁸ free radicals, and nitric oxide.¹⁹ Furthermore, endothelial cells participate in immune responses by the regulation of intercellular adhesion molecule

1,²⁰ which plays a key role in leukocyte recruitment and subsequent brain injury.²¹

The first immune cells to appear in the ischemic immature tissue are T lymphocytes and PMNL. Abundant²² and small²³ T-cell infiltrates have been noted after MCA occlusion in the adult rat brain. PMNL were observed to be accumulated in the subarachnoid spaces and blood vessels 24 hours after reperfusion and progressively invaded the different layers of the infarcted cortex. Perivascular intraparenchymal neutrophil migration into the ischemic brain has been reported to begin as early as 6²⁴ and 24²⁵ hours after recirculation following ischemia/reperfusion in adult rat. In contrast, minimal neutrophilic response⁹ and lack of neutrophil extravasation during the evolution of the infarct²⁶ were reported following neonatal hypoxia/ischemia. Nevertheless, neutrophil depletion in such a model reduced brain swelling at 42 hours of recovery by approximately 70%, which was comparable to the protective effect of allopurinol.²⁶ PMNL are known to be integrated into the acute inflammatory response and possess extensive cytotoxic capacities, supporting their pathological role in destructive processes. They represent an important biological source of oxygen-derived free radicals and generate nitric oxide underlying oxidant-mediated tissue injury.^{27,28} We recently reported that perivascular reactions mediated by nitric oxide and peroxynitrites in T lymphocytes and polymorphonuclear cells are important in the cascade of events that lead to brain oxidative stress in neonatal ischemia.²⁹ In addition, *N*^G-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase, reduced infarct volume in a filament model of transient MCA occlusion in the rat pup.³⁰

Our results demonstrated a microglial activation elicited by ischemia in immature rats that was first detected at 3 days after injury. The presence of ED1-positive microglia-macrophages in the ipsilateral periventricular area has been previously reported after hypoxia/ischemia in P7 rats.³¹ A rapid, relatively widespread microglial response was also demonstrated after hypoxia/ischemia by the use of major histocompatibility complex II (OX-6) and complex I (OX-18) antibodies,^{10,32} whereas macrophage infiltration may be delayed or absent.³² A relationship between delayed neuronal loss in tissues neighboring sites of ischemia and cytotoxin secretion has been suggested.³³ Activated microglia may release a variety of cytokines, including interleukin-1 β and tumor necrosis factor- α , known to activate astrocyte proliferation,^{34,35} leukocyte infiltration,^{36,37} and brain edema.³⁸ However, their role in the progression of ischemic lesion remains uncertain.³⁹ The association of activated microglia with nuclear ghosts in the infarcted area also suggests that they contribute to the removal of cell death-associated debris induced by ischemic injury. Activated microglia after ischemia may act as mediators of further injury and/or as cleansing scavengers and repair agents.⁴⁰ Microglial cells seem to express the death inhibitor protein Bcl-2. Furthermore, the delayed expression of the cell death promoter Bax from 2 weeks until 1 month after injury suggests their death by apoptotic features, as demonstrated in human atherosclerotic lesions.⁴¹ Therefore, the balance between proapoptotic (Bax proteins) and antiapoptotic (Bcl-2) elements may also account for death or survival of glial cells, respectively.

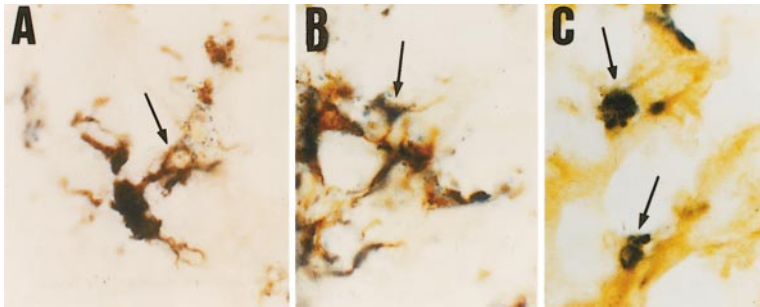


Figure 7. Double staining of dying astrocytes (A), microglia (B), and macrophages (C) identified by immunocytochemistry using Bax, GFAP, OX-42, and ED1 antibodies. GFAP, OX-42, and ED1 antibodies were visualized by peroxidase (light brown staining), whereas Bax antibodies were detected by alkaline phosphatase (blue staining), giving a dark brown color. Note that the structure of dying cells (black arrow) is difficult to distinguish. Bar represents 10 μm .

Staining Patterns of Glia and Inflammatory and Dying Cells in the Infarcted Brain

Cellular Markers	2 h	6 h	24 h	48 h	72 h	96 h	7 d	14 d	1 mo
IgG	±	±	+	+	++	++	++	+	±
MPO	–	–	±	+	++	+++	+	–	–
Pan-T	–	–	±	+	++	+	–	–	–
OX-42	–	–	±	+	++	+++	+++	+	±
ED1	–	–	+	+	++	+++	+++	+	±
GFAP	+	+	+	++	+++	+++	+++	++	+
Bcl-2	–	–	+	+	++	++	++	+	–
Bax	–	±	±	+	++	+++	+++	+++	+
TUNEL	–	±	±	+	++	+++	+++	+++	+

– indicates no staining; ±, low intensity of staining on scattered occasional cells; +, low intensity of staining throughout the whole infarct; ++, strong staining; and +++, very strong staining.

Evidence of interleukin-1 β -converting enzyme-like immunoreactivity in microglial cells was recently reported, which suggests a potential role for caspases in ischemic damage through mediation of an inflammatory response.⁴² Apoptosis, through its several steps, thus appears to be one mechanism by which activated microglia are gradually eliminated after cerebral ischemia and by which steady state of microglial cell numbers is achieved in vivo.

Reactive astrocytes are believed to reestablish a protective barrier, the glial-limiting membrane.⁴³ Astrocytic changes in the present model of neonatal stroke could be directed to newly formed glia limitans around the cortical cavity. The temporal pattern of astrocyte responses indicates that reactive astrocytes may assist ischemic brain repair by stabilizing the tissue surrounding neuronal injury.⁴⁴ Early morphological remnants of resident astrocytes may reflect changes in ionic concentrations and excitatory amino acids in the area of neuronal depletion. It is presently unclear whether these astrocytes migrate into injured areas or locally proliferate. However, our previous data suggested that glial cells at the periphery of the infarct expressed proliferating cell nuclear antigen,¹² known to be involved in protective and proliferative processes. At later times of recirculation, astrocytes exhibited DNA fragmentation and expressed the cell death promoter Bax, suggesting that their downregulation occurred through apoptosis, compared with microglial cells.

In conclusion, our results show that ischemia without hypoxia in immature animals triggers microglial cell activation, astrocytic response, and granulocytic infiltration, similar to those seen in adult brain (for comparison, see References 45 through 48). The main discrepancy between our results

and that found after neonatal hypoxia/ischemia was neutrophil involvement. Our results, however, did not establish a cause-and-effect relationship between the inflammatory markers and the progression of histological damage. To investigate such functional outcome, experiments with inflammatory mediator antagonists (free radical scavengers, anti-adhesion molecule antibodies, cytokine antagonists)⁴⁹ would be of interest.

References

1. Younkin D. Hypoxic-ischemic brain injury of the newborn: statement of the problem and overview. *Brain Pathol.* 1992;2:209–210.
2. Gray P, Tudehope T, Masel J, Burns Y, Mohay H, O'Callaghan M, Williams G. Perinatal hypoxic-ischaemic brain injury: prediction of outcome. *Dev Med Child Neurol.* 1993;35:965–973.
3. Volpe J. Neurology of the newborn. In: *Neurology of the Newborn*. Philadelphia, Pa: WB Saunders; 1987.
4. Friede R. Perinatal lesions of gray matter. In: Friede RL, ed. *Developmental Neuropathology*. Göttingen, Germany: Springer-Verlag; 1989: 82–97.
5. Feuerstein G, Liu T, Barone F. Cytokines, inflammation and brain injury: role of tumor necrosis factor. *Cerebrovasc Brain Metab Rev.* 1994;6: 341–360.
6. Fellman V, Raivio K. Reperfusion injury as the mechanism of brain damage after perinatal asphyxia. *Pediatr Res.* 1997;41:599–606.
7. Rice J, Vannucci R, Brierley J. The influence of immaturity on hypoxia-ischemic brain damage in the rat. *Ann Neurol.* 1981;9:131–141.
8. Towfighi J, Yager J, Housman C, Vannucci R. Neuropathology of remote hypoxic-ischemic damage in the immature rat. *Acta Neuropathol (Berl).* 1991;81:578–587.
9. Towfighi J, Zec N, Yager J, Housman C, Vannucci R. Temporal evolution of neuropathologic changes in immature rat model of cerebral hypoxia: a light microscopic analysis. *Acta Neuropathol (Berl).* 1995;90: 375–386.
10. McRae A, Gilland E, Bona E, Hagberg H. Microglia activation after neonatal hypoxia-ischemia. *Dev Brain Res.* 1995;84:245–252.

11. Renolleau S, Aggoun-Zouaoui D, Ben-Ari Y, Charriat-Marlangue C. A model of transient unilateral focal ischemia with reperfusion in the P7 neonatal rat: morphological changes indicative of apoptosis. *Stroke*. 1998; 29:1454–1461.
12. Renolleau S, Benjelloun N, Ben-Ari Y, Charriat-Marlangue C. Regulation of apoptosis-associated proteins in cell death following transient focal ischemia in rat pup. *Apoptosis*. 1997;2:368–376.
13. Milligan C, Cunningham T, Levitt P. Differential immunochemical markers reveal the normal distribution of brain macrophages and microglia in the developing rat brain. *J Comp Neurol*. 1991;314:125–135.
14. Charriat-Marlangue C, Margaill I, Represa A, Popovici T, Plotkine M, Ben-Ari Y. Apoptosis and necrosis following reversible focal ischemia: an in situ DNA fragmentation analysis. *J Cereb Blood Flow Metab*. 1996;16:186–194.
15. Betz A, Dietrich W. Blood-brain barrier: dysfunction in cerebral ischemia. In: Ginsberg M, Bogousslavsky J, eds. *Cerebrovascular Disease: Pathophysiology, Diagnosis and Management*. Philadelphia, Pa: Blackwell Science; 1998:358–370.
16. Rhodes R. Evidence of serum-protein leakage across the blood-brain barrier in the acquired immunodeficiency syndrome. *J Neuropathol Exp Neurol*. 1991;50:171–183.
17. Vedeler C, Ulvestad E, Grundt I, Conti G, Nyland H, Matre R, Pleasure D. Fc receptor of immunoglobulin for IgG (FcR) an rat microglia. *J Neuroimmunol*. 1994;49:19–24.
18. Maurer P, Moskowitz M, Levine L, Melamed E. The synthesis of prostaglandins by bovine cerebral microvessels. *Prostaglandin Med*. 1980;4: 153–161.
19. Beckman J, Ye Y, Chen J, Conger K. The interactions of nitric oxide with oxygen radicals and scavengers in cerebral ischemic injury. In: Siesjö BK, Wieloch T, eds. *Advances in Neurology: Cellular and Molecular Mechanisms of Ischemic Brain Damage*. Philadelphia, Pa: Lippincott-Raven; 1996:339–354.
20. Chopp M, Zhang R, Chen H, Li Y, Jiang N, Rusche JR. Postischemic administration of an anti-Mac-1 antibody reduces ischemic cell damage after transient middle cerebral occlusion in rats. *Stroke*. 1994;25: 869–876.
21. Hallenbeck J, Kochanek P. Inflammatory responses in cerebral ischemia: role of leukocytes. In: Ginsberg M, Bogousslavsky J, eds. *Cerebrovascular Disease: Pathophysiology, Diagnosis and Management*. Philadelphia, Pa: Blackwell Science; 1998:489–506.
22. Schroeter M, Jander S, Witte O, Stoll G. Local immune responses in the rat cerebral cortex after middle cerebral artery occlusion. *J Neuroimmunol*. 1994;55:195–203.
23. Morioka T, Kalehua A, Streit W. Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. *J Comp Neurol*. 1993;121:123–132.
24. Clark R, Lee E, White R, Jonak Z, Feuerstein G, Barone F. Reperfusion following focal stroke hastens inflammation and resolution of ischemic injured tissue. *Brain Res Bull*. 1994;35:387–392.
25. Garcia J, Liu K, Yoshida Y, Lian J, Chen S, Del Zoppo G. Influx of leukocytes and platelets in an evolving brain infarct. *Am J Pathol*. 1994; 144:188–199.
26. Hudome S, Palmer C, Roberts R, Mauger D, Housman C, Towfighi J. The role of neutrophils in the production of hypoxic-ischemic brain injury in the neonatal rat. *Pediatr Res*. 1997;41:607–616.
27. Traystman R, Kirsch J, Koehler R. Oxygen radical mechanisms of brain injury following ischemia and reperfusion. *J Appl Physiol*. 1991;71: 1185–1195.
28. Chan P. Role of oxidants in ischemic brain damage. *Stroke*. 1996;27: 1124–1129.
29. Coeroli L, Renolleau S, Arnaud S, Plotkine D, Cachin N, Plotkine M, Ben-Ari Y, Charriat-Marlangue C. Nitric oxide formation and perivascular nitrotyrosine following focal ischemia in immature rats. *J Neurochem*. 1998;70:2516–2525.
30. Ashwal S, Cole D, Osborne S, Osborne T, Pearce W. L-NAME reduces infarct volume in a filament model of transient middle cerebral artery occlusion in the rat pup. *Pediatr Res*. 1995;38:652–656.
31. Ivacko J, Sun R, Silverstein F. Hypoxic-ischemic brain injury induces an acute microglial reaction in perinatal rats. *Pediatr Res*. 1996;39:39–47.
32. Ohno M, Aotani H, Shimada M. Glial responses to hypoxic/ischemic encephalopathy in neonatal rat cerebrum. *Dev Brain Res*. 1995;84: 294–298.
33. Giulian D, Vaca K. Inflammatory glia mediate delayed neuronal damage after ischemia in the central nervous system. *Stroke*. 1993;24(suppl I):I-184–I-190.
34. Giulian D, Lachman L. Interleukin-1 stimulation of astroglial proliferation after brain injury. *Science*. 1985;228:497–499.
35. Giulian D, Woodward J, Young D, Krebs J, Lachman L. Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. *J Neurosci*. 1988;8:2485–2490.
36. Kishimoto T, Rothlein R. Integrins, ICAMs, and selectins: role and regulation of adhesion molecules in neutrophil recruitment to inflammatory sites. *Adv Pharmacol*. 1994;25:117–169.
37. Montefort S, Holgate S. Adhesion molecule and their role in inflammation. *Respir Med*. 1991;85:91–99.
38. Gordon C, Merchant R, Marmarou A, Rice C, Marsh T, Young H. Effect of murine recombinant interleukin-1 on brain edema in the rat. *Acta Neurochir (Wien)*. 1990;51:268–270.
39. Giulian D. Ameboid microglia as effectors of inflammation in the central nervous system. *J Neurosci Res*. 1987;18:155–171.
40. Giulian D, Chen J, Ingeman J, George J, Nojonen M. The role of mononuclear phagocytes in wound healing after traumatic brain injury to adult mammalian brain. *J Neurosci*. 1989;9:4416–4429.
41. Bjorkerud S, Bjorkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am J Pathol*. 1996;149:367–380.
42. Bhat R, DiRocco R, Marcy V, Flood D, Zhu Y, Dobrzanski P, Siman R, Scott R, Contreras P, Miller M. Increased expression of IL-1beta converting enzyme in hippocampus after ischemia: selective localization in microglia. *J Neurosci*. 1996;16:4146–4154.
43. Reier P. Gliosis following CNS injury: the anatomy of astrocytic scars and their influences on axonal elongation. In: Fedoroff S, Vernadakis A, eds. *Astrocytes, Cell Biology and Pathology of Astrocytes*. New York, NY: Academic Press; 1986:263–324.
44. Montgomery D. Astrocytes: from functions and role in disease. *Vet Pathol*. 1994;31:145–167.
45. Chen H, Chopp M, Schultz L, Bodzin G, Garcia J. Sequential neuronal and astrocytic changes after middle cerebral artery occlusion in the rat. *J Neurol Sci*. 1993;118:109–116.
46. Garcia J, Yoshida Y, Chen H, Li Y, Zhang Z, Lian J, Chen S, Chopp M. Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol*. 1993;142:623–635.
47. Levine S. Anoxic-ischemic encephalopathy in rats. *Am J Pathol*. 1960; 36:1–17.
48. Pulsinelli WA, Briery JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol*. 1982;11: 499–509.
49. Feuerstein G, Wang X, Barone F. Inflammation-related gene expression and stroke: implications for new therapeutic targets. In: Kriegstein J, ed. *Pharmacology of Cerebral Ischemia*. Stuttgart, Germany: Medpharm Scientific Publishers; 1996:405–419.

Editorial Comment

Benjelloun et al report in the accompanying article that an extensive inflammatory reaction in response to focal ischemia evolves in the neonatal rat brain. To those involved in investigations of immune and inflammatory reactions associ-

ated with brain injury, the data come as no surprise; over the past few years, numerous reports have pointed out in a quite detailed manner that inflammation and possibly immune components are markedly activated de novo in brain tissue

injured by trauma, ischemia, toxins, and infectious agents.¹⁻³ The report by Benjelloun et al extends the subject to neonatal life, as also reported by Hallenbeck and Kochanek¹ and others using various trauma models. Most welcomed in the present report are (1) long-term follow-up (months); (2) the inclusion of T-cell lymphocytes in the study (this cellular component of the immune system has not been frequently studied); and (3) the association of the Bcl-2/Bax system of cell death regulatory pathways along with the inflammatory reaction. Taken together, the report provides high-quality data that amplify and strengthen the fact that immune/inflammatory cells and mediators are part of the brain response to injury. The conclusion forwarded by the authors in the abstract and further reiterated in the article is that inflammatory responses may be involved in accelerating the destructive process in perinatal ischemia. With this in mind, it is of interest to point out that the conclusion by the authors of this article, as in all previous reports, is in marked contrast to the reputable role of inflammation in tissue injury as defined by medical dictionaries, such as the *Stedman's*,⁴ in which inflammation is described as "... a dynamic complex of cytologic and chemical reactions that occur in the affected blood vessels and adjacent tissues in response to an injury or abnormal stimulation ... including: 1) the local reactions ..., 2) ... removal of the injurious material, 3) the responses that lead to repair and healing." Similar definitions can be found in textbooks of inflammation⁵ and pathology.⁶ Clearly, the sense and purpose of the inflammatory reaction to repair, reconstruct, and heal have not been favored in the discussion sections of articles that describe the cellular and biochemical components of the inflammatory reactions to brain ischemia/trauma. This discrepancy is even more perplexing in view of several important reports that demonstrate the following: (1) lack of therapeutic benefits of glucocorticosteroids or NSAIDs in stroke or head injury; (2) experimental models with genetic deletion of cytokine signaling elements (eg, tumor necrosis factor- α receptor knockout) show no protection against ischemia or other injuries (on the contrary, a measure of vulnerability has been noted)⁷; (3) activated macrophages and T-cell lymphocytes are of therapeutic potential in brain and spinal cord injury⁸; and (4) some of the most "maligned" mediators in reference to ischemic brain injury, interleukin-1 and tumor necrosis factor- α , serve as

inducers of tolerance to certain experimental paradigms of brain injury.⁹

These comments are not to be taken as intentional "contrarianism" to the prevalent dogma on the role of inflammation in brain injury; rather, in my opinion, it is time to adopt a more sophisticated understanding that immune and inflammatory cells and mediators serve complex processes, including healing elements.

We should seek to identify the cells/mediators that serve in the specific functions of the reaction, aiming for suppression of particular cells/mediators that act in specific time frames to augment injury while enhancing cells/mediators (which could be similar) that mediate healing at other phases after injury. Such views may help to define better strategies for pharmaceuticals that may not target suppression of the inflammatory reaction at large, including the use of specific agents that may support regeneration/reconstruction exemplified by many functions of the inflammatory reaction itself.

Giora Z. Feuerstein, MD, *Guest Editor*
Department of Cardiovascular Sciences
DuPont Pharmaceuticals Company
Wilmington, Delaware

References

- Hallenbeck JM, Kochanek PM. Inflammatory responses in cerebral ischemia: role of leukocytes. In: Ginsberg MD, Bogousslavsky J, eds. *Cerebrovascular Diseases*. Philadelphia, Pa: Blackwell Science; 1998:489-506.
- Feuerstein GZ, Wang X, Barone FC. Inflammatory mediators and brain injury: the role of cytokines and chemokines in stroke and CNS diseases. In: Ginsberg MD, Bogousslavsky J, eds. *Cerebrovascular Diseases*. Philadelphia, Pa: Blackwell Science; 1998:507-531.
- Chopp M, Zhang RL, Chen H, Li Y, Jiang N, Rusche JR. Posts ischemic administration of anti-Mac-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in rats. *Stroke*. 1994;25:869-876.
- Stedman's Medical Dictionary*. 26th ed. Baltimore, MD: Williams & Wilkins; 1995.
- Fantone JC, Ward PA. *Pathology*. Rubin E, Farber JL, eds. 1994.
- Gallin JI, Goldstein IM, Snyderman R. *Inflammation: Basic Principles and Clinical Correlates*. New York, NY: Raven Press; 1988.
- Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, Carpenter MK, Holsberg FW, Mattson MP. Altered neuronal and microglial responses to brain injury in mice lacking TNF receptors. *Nat Med*. 1996;2:788-794.
- Moalem G, Leibovitz-Amit R, Yoles E, Mor F, Cohen IR, Schwartz M. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat Med*. 1999;5:49-55.
- Ohtsuki T, Ruetzler CA, Tasaki K, Hallenbeck JM. Interleukin-1 mediates induction of tolerance to global ischemia in gerbil hippocampal CA1 neurons. *J Cereb Blood Flow Metab*. 1996;16:1137-1142.