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# Interleukin-1 Receptor Antagonist Decreases the Number of Necrotic Neurons in Rats with Middle Cerebral Artery Occlusion

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***Marked increases in the brain expression of interleukin (IL)-1 have been reported in rats after permanent occlusion of a large cerebral artery. Interactions between endothelial cells and leukocytes have been implicated in the pathogenesis of several types of ischemic injury to the myocardium and other organs. In this study we asked whether inhibiting the effects of IL-1 would affect the outcome of an experimental brain infarct. Adult male Wistar rats (n = 13) with permanent occlusion of the middle cerebral artery were given IL-1 receptor antagonist. A second group (n = 13) with the same type of brain injury was given a placebo. A third group, subjected to a sham operation, was given either IL-1 receptor antagonist (n = 2) or a placebo (n = 2). Experiments were terminated after either 24 hours or 7 days. Compared with the control group, animals treated with IL-1 receptor antagonist improved their neurological score (P < 0.05), experienced less pronounced changes in body weight (P < 0.05), and had fewer necrotic neurons (P < 0.001) and fewer leukocytes in the ischemic hemisphere (P < 0.001) as well as a smaller area of pallor (P < 0.05) in the ischemic hemisphere. The results suggest that inhibiting the proinflammatory effects of IL-1 with a receptor antagonist is an effective way of influencing the leukocyte responses elicited by an arterial occlusion. Such leukocyte inhibition seemingly attenuates the number of necrotic neurons resulting from the occlusion of a large brain artery. (Am J Pathol 1995, 147:1477–1486)***

The pathogenesis of the tissue necrosis that develops several hours after occluding a large artery, such as the coronary or the middle cerebral artery, may involve the activation of leukocytes, in particular polymorphonuclear neutrophils (PMNs).[1,2] The influx of PMN leukocytes into the brain of rats with middle cerebral artery (MCA) occlusion begins as early as 30 minutes after the arterial occlusion.[3] The chemoattractants mediating the arrival of PMN leukocytes into the ischemic territory are not clearly defined, but marked increases in the expression of interleukin (IL)-1 have been reported after making the rat brain ischemic either by means of bilateral carotid clamping[4] or by occluding a large intracranial artery.[5]

Some of the proinflammatory effects of IL-1, a cytokine biologically active in the low picomolar range, include leukocytosis, increased expression of endothelial cell adhesion molecules, and neutrophil tissue infiltration.[6] Because PMN leukocytes may actively participate in the brain injury that develops after a MCA occlusion,[2,7] we designed experiments aimed at answering the following questions. In rats with permanent MCA occlusion, does the administration of IL-1 receptor antagonist (IL-1ra) have beneficial effects on the clinical or the structural consequences of the arterial occlusion and are these effects accompanied by detectable decreases in PMN leukocyte sequestration in the ischemic territory?

## Materials and Methods

All experiments were conducted according to the guidelines issued by the institutional Animal Care

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Committee and in compliance with regulations formulated by the U.S. Department of Agriculture.[8]

Thirty outbred male Wistar rats (body weight 270 to 290 g) purchased from Charles River Laboratories (Wilmington, MA) and fed Agway rat chow during the 4 to 6 quarantine days were used in this study. Each animal was housed in an individual cage. Animals included in this study were divided into three experimental groups: (A) those with permanent MCA occlusion and treated with IL-1ra (n = 13); (B) those with permanent MCA occlusion and treated with CSE buffer (10 mmol/L citrate, 140 mmol/L NaCl, 0.5 mmol/L EDTA), or placebo group (n = 13); and (C) sham-operated animals treated either with IL-1ra (n = 2) or CSE (n = 2). Experiments were terminated by cardiovascular perfusion either 24 hours or 7 days after the arterial occlusion.

## Surgical Procedure

The origin of the right MCA was occluded by inserting, through the external carotid artery, a short segment (18 ± 0.77 mm) of a nylon monofilament (4-0). The surgical procedure is an adaptation of the method originally described by Koizumi et al[9] and Zea-Longa et al[10]; additional details on the methods used in this laboratory including anesthetic procedures and methods to maintain constant core temperature (37°C) have been published elsewhere.[3,11,12] The same surgical procedure as in the group with permanent MCA occlusion was applied to sham-operated animals but the occluding monofilament was withdrawn less than 60 seconds after it had been inserted via the external carotid artery.

## Treatment

For the IL-1ra treatment group, human recombinant IL-1ra (Synergen,, Inc., Boulder, CO) was diluted in CSE buffer; a dose equivalent to 100 mg/kg was given at each time. This was chosen after completion of a dose-response study.[13] The initial IL-1ra dose was given, via the right femoral vein, immediately before the monofilament was inserted into the external carotid artery. Booster doses (100 mg/kg each) were subcutaneously given three times per day (at 4-hour intervals) during the initial 24 hours. Additional subcutaneous injections (three times per day) were given to rats included in the 7-day survival group. Among those receiving IL-1ra, there were 5 rats in the group surviving 24 hours and 6 rats in the group surviving 7 days. The same frequency of injections was given to 13 rats in group B (0.3 ml of CSE buffer, three times per day); however, IL-1ra was not included in the injected material, which consisted of CSE buffer at a pH of 6.5. In addition, 2 animals in each of the groups (A and B) were subjected to sham operations.

## Clinical Evaluation

Counts of white blood cells were done in peripheral blood samples drawn once immediately before injecting the treatment and again immediately before terminating the experiments; the white blood cell count was done by a manual method described in detail elsewhere.[14] On the day of surgery and thereafter at daily intervals, body weight was measured always in the late afternoon hours utilizing the same calibrated scale for each measurement. At the time of the daily neurological evaluation each animal was given a neurological score ranging from 3 (worst) to 18 (best). This was done after subjecting each animal to six separate motor sensory tests, according to procedures validated in a separate group of experiments.[15] The examiner conducting the neurological tests was blinded to the type of treatment that each animal had received.

## Histological Preparation

On the last day of each experiment, under the analeptic effects of ketamine and xylazine, each rat underwent cardiovascular perfusion with 0.9% saline (USP Baxter, McGaw Park, IL) containing 1000 U of heparin, followed by perfusion with 4% paraformaldehyde in 0.2 mol/L phosphate-buffered saline (4% PBS). After removing the brain and overnight fixation in 4% PBS, five coronal sections (each 3.0 mm thick) were obtained with the use of a rat brain matrix (Activational Systems, Inc., Warren, MI). The brain slabs were labeled A (frontal) to E (occipital). Caudal surfaces of slabs A, B, D, and E were embedded in paraffin and cut into 6-μm-thick sections that were stained with hematoxylin and eosin (H&E). The 6-μm-thick sections from slab B were selected for glial fibrillary acidic protein (GFAP) immunohistochemical demonstration; additional details of the methods used in this procedure have been published elsewhere.[11,12] The rostral surface of slab C, corresponding to the level of the anterior commissure, was embedded in araldite; four 1.0-μm-thick sections representing the entire hemisphere ipsilateral to the occluded MCA were stained with toluidine blue. After selecting the appropriate region of interest, ultrathin sections were examined in a Philips 300 electron microscope. Two brain specimens from each of the two groups (A and B) were selected for

ultrastructural evaluation. Both of these experiments had been terminated at 24 hours; electron micrographs were obtained to verify the nature of cellular inflammation by monocytes and PMN leukocytes.

### *Histopathological Evaluation*

Calculating the volume of the area of pallor in each of the ischemic hemispheres, in H&E-stained histology slides, was accomplished with an image analysis system (IMAGIST, PGT, Princeton, NJ) at a magnification of ×2.5. The examiner was blinded to the conditions of individual specimens. The results are expressed as percentages of the hemisphere's surface where a pale area developed minus the area composed of the lateral ventricles. Differential counts of either intact or necrotic neurons within the territory of the occluded MCA were completed with an image measure system (Microscience, Inc., Seattle, WA). The Im 2500 morphometry program of this system was chosen to calculate the number of either necrotic or intact neurons utilizing 1.0-μm-thick sections stained with toluidine blue. Twenty nonoverlapping microscopic fields (at a magnification of ×400), each comprising an area 150 × 200 μm, were collected from the neocortex and caudoputamen in each specimen. Additional details of this method and definition as well as illustrations of intact and necrotic neurons have been published elsewhere.[16] For leukocyte infiltrates, numbers of PMNs and monocytes either in the ischemic parenchyma or in the lumen of venules and capillaries were counted in twenty nonoverlapping microscopic fields (as defined above) utilizing the Im 2500 morphometry program in 1.0-μm-thick sections stained with toluidine blue. Immunoreactivity of GFAP was measured in histology sections derived from coronal section B. Using the IMAGIST system, area fraction (or portion of the microscopic field occupied by the brown reaction product) was measured in five separate sites inside the lesion and in five separate sites outside the lesion. The results are expressed as a ratio of comparisons with similar measurements obtained from homotopic areas in the contraleteral hemisphere.

Ultrathin sections for random evaluation of ultrastructural features were obtained from four specimens (selected at random) from experimental group A (n = 2) and group B (n = 2). All electron microscopy samples were derived from experiments in which the MCA occlusion lasted 24 hours. The examiner quantitating the histological features was blinded to the type of treatment each animal had received.

### *Statistical Analysis*

To evaluate differences in neurological scores and body weight, mean values and standard deviations were obtained for each of the treatments and control groups. The Wilcoxon rank test was applied to these values. Analysis of variance followed by Bonferroni-corrected Student's *t*-test was applied for comparisons involving counts of necrotic neurons, volume of the area of pallor, GFAP immunoreactivity, and number of leukocyte infiltrates.

## *Results*

Two rats from the original thirteen in group A, treated with IL-1ra, died at 36 and 48 hours after the MCA occlusion. In group B, one rat of the original thirteen receiving placebo died on day 5 after MCA occlusion. Because brain fixation was different (immersion) from the rest of the experiments, data from these three animals are not included in the statistical analysis.

### *Clinical Evaluation*

#### *Body Weight*

Mean body weight for group B, injected with CSE buffer, was 277.57 ± 3.9 before MCA occlusion; a progressive decline started on the day of the experiment and by day 7 mean body weight was 188.42 ± 44.8 ($P < 0.05$). In the group treated with IL-1ra, the preoperative mean body weight of 280.80 ± 3.9 decreased to 241.0 ± 18.1 on the day of the experiment; however, on day 7 after MCA occlusion the mean body weight of 236.8 ± 70, was significantly different ($P < 0.05$) from the mean body weight of those in group B (Figure 1).

#### *Neurological Score*

The preoperative neurological scores for the two experimental groups (A and B) were 17.91 ± 0.3 and 17.92 ± 0.3, respectively. Four days after MCA occlusion, the mean neurological score in group A rats improved from 11.25 ± 4.0 to 13.25 ± 2.9, whereas the mean neurological score of those in group B (placebo) progressively decreased to 8.00 ± 4.3 ($P < 0.05$) and did not recover (Figure 2).

Counts of white blood cells in peripheral blood were not significantly different in the two experimental groups (Table 1).

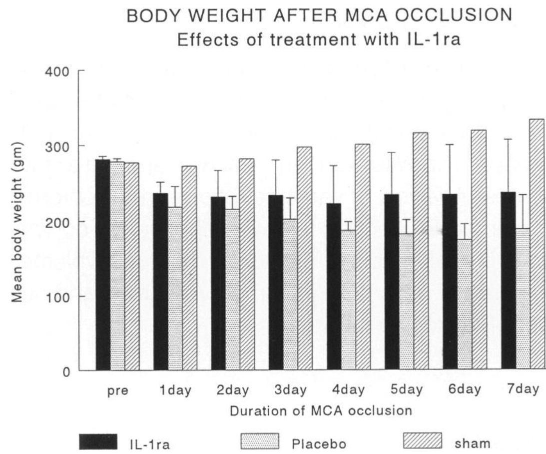


**Figure 1.** *Histogram showing differences in body weight, according to treatment, in three experimental groups.*

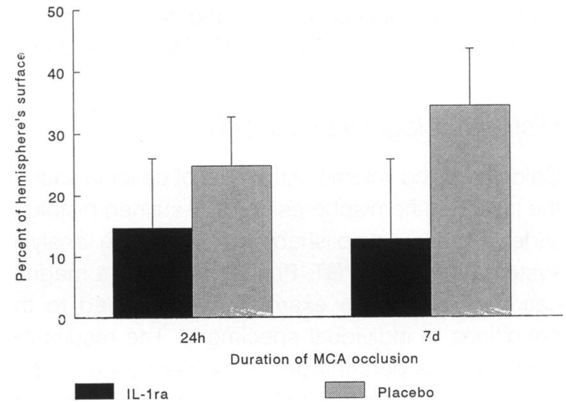


**Figure 3.** *Bar graph illustrating the differences in area of pallor surface (as measured in histology slides stained with H&E), according to treatment. Differences observed at 24 hours became more pronounced on day 7.*

## Histopathological Evaluation

### Area of Pallor

Expressed as a percentage of the hemisphere's surface, the mean values in group A were 14.69 ± 11.3 and 12.73 ± 13.0 at 24 hours and 7 days, respectively, after MCA occlusion. These figures were significantly different ($P < 0.05$) from those obtained in group B (Figures 3 and 4). There were fewer necrotic neurons in the group treated with IL-1ra, compared with the placebo group 24 hours and 7 days after the MCA was occluded ($P < 0.0001$; Figure 5). Conversely, there were more intact neurons after 7 days in the group treated with IL-1ra compared with the placebo group ($P < 0.05$). PMN leukocytes were rarely visible either in the ischemic parenchyma or in the microvessels of rats treated with IL-1ra. The mean number of PMN leukocytes was significantly different ($P < 0.05$) in the two groups (Table 1).

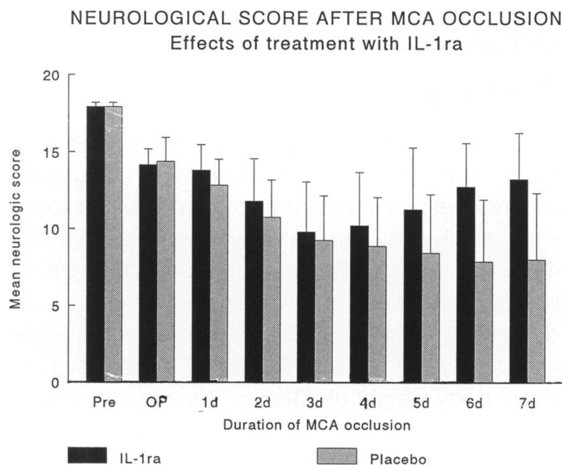


**Figure 2.** *Histogram depicting mean neurological scores and corresponding differences between groups of rats treated with IL-1ra and those receiving a placebo.*

### GFAP Reactivity

At 24 hours, immunoreactivity in the cortex was slightly decreased in the placebo group compared with the IL-1ra-treated group (Figure 6, A and B). After 7 days, the area of lost immunoreactivity is larger and more homogeneous in the placebo group

**Table 1.** *Leukocytes in Peripheral Blood and Ischemic Hemisphere after MCA Occlusion*

| Duration of MCA occlusion (hours) | No. of rats | Peripheral blood* | | Ischemic hemisphere† | |
|---|---|---|---|---|---|
| | | Before treatment | After treatment | PMN | Monocyte |
| IL-1ra treatment | | | | | |
| 24 | 5 | 7100 ± 1460 | 5880 ± 2040 | 0.18 ± 0.4 | |
| 168 | 5 | 7500 ± 1100 | 6820 ± 1050 | | 6.4 ± 7.4 |
| Placebo (CSE buffer) | | | | | |
| 24 | 5 | 7260 ± 712 | 6440 ± 1457 | 0.85 ± 1.1 | |
| 168 | 5 | 6700 ± 1013 | 6300 ± 866 | | 17.29 ± 15.4 |

*No significant differences in the number of white blood cells were noted in either group as a result of treatment.
†Mean numbers of PMNs and monocytes were significantly different ($P < 0.05$) as a result of the treatment with IL-1ra.

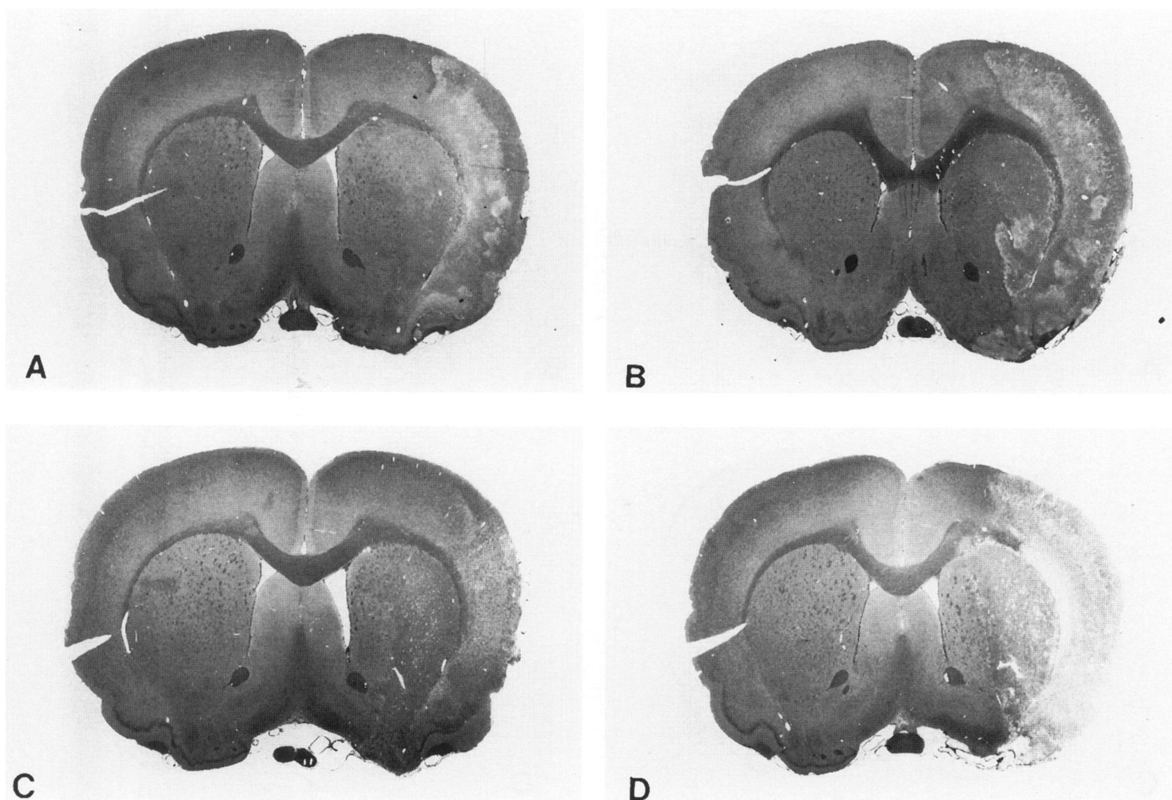


**Figure 4.** *Light micrographs demonstrating the area of pallor in representative samples obtained at 24 hours (***A** *and* **B***) and 7 days (***C** *and* **D***) after MCA occlusion.* **A** *and* **C***: IL-1ra injected.* **B** *and* **D***: Placebo. H&E; magnification, ×2.5.*

(Figure 6, C and D). No significant differences were noted in the area fraction measurements (Figure 7).

## Ultrastructural Evaluation

In the group treated with IL-1ra, necrotic neurons were not surrounded by inflammatory cells except for an occasional microglial cell; erythrocytes or leukocytes were rarely seen either in the lumen of microvessels or in the parenchyma (Figure 8). In contrast, PMN leukocytes filled the lumina of many microvessels in most samples from animals injected with placebo. In addition to PMN leukocytes, erythrocytes and monocytes were frequently detected in the vessel lumina; also, PMN leukocytes were easily found in the necrotic parenchyma (Figure 8).

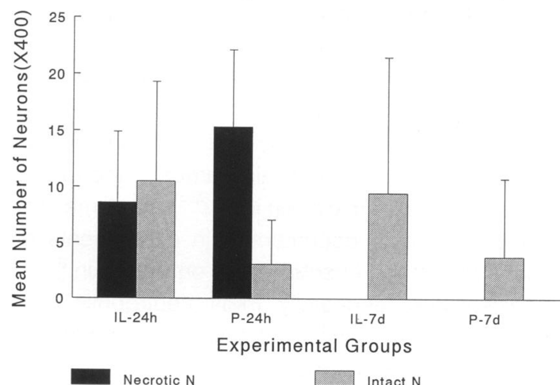


**Figure 5.** *Histogram showing differences in mean numbers of necrotic neurons, depending on the treatment administered. After 7 days, only intact neurons were counted.*

## Discussion

Brain ischemia, secondary to a single-artery occlusion, is associated with a prompt inflammatory response characterized by the early influx of polymorphonuclear leukocytes; this response increases as a function of time and peaks 48 to 72 hours after the arterial occlusion.[3,11] The inflammatory mediators involved in this type of brain ischemia are still unknown.[5]

IL-1 and tumor necrosis factor are two potent cytokines that are promptly released after septic events characterized by endotoxemia.[6] IL-1 and tumor necrosis factor responses to endotoxin can be greatly enhanced by hypoxemic conditions.[17,18] This

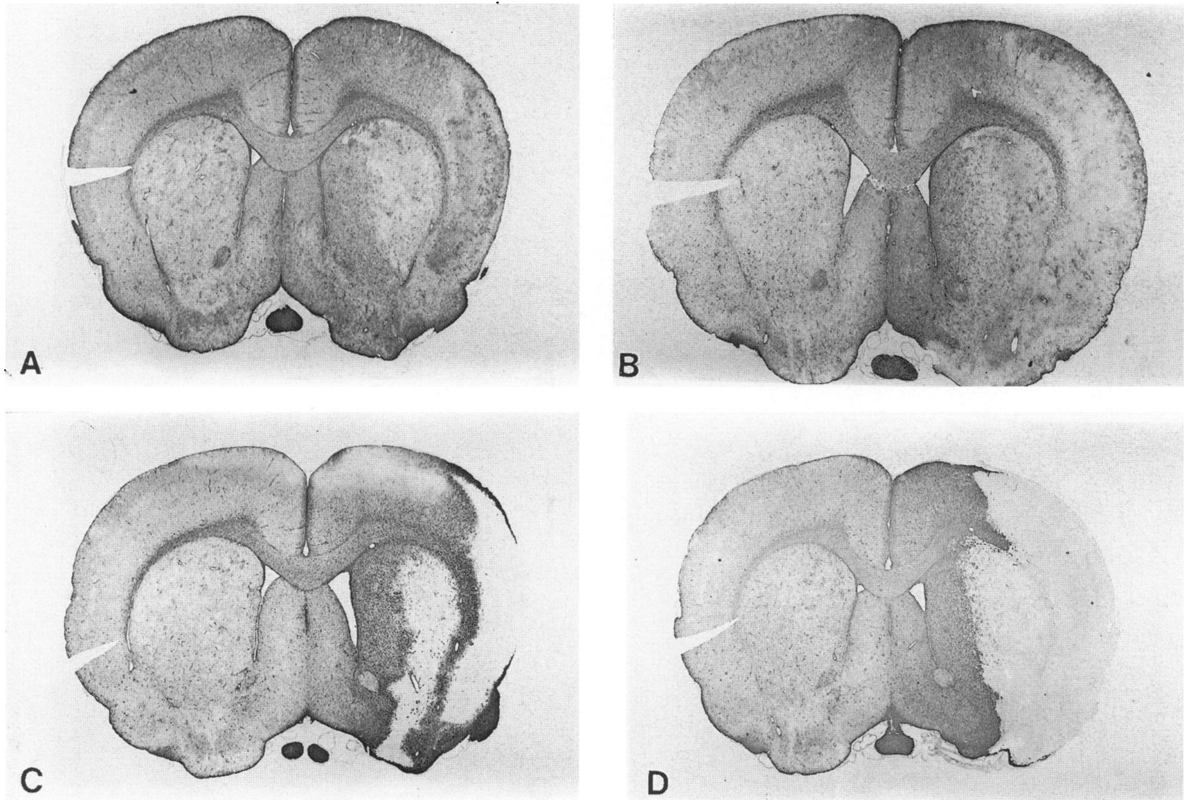

Figure 6. *Area of pallor in experiments terminated at 24 hours* (A *and* B) *and 7 days* (C *and* D) *after MCA occlusion.* A *and* C: *IL-1ra.* B *and* D: *Placebo. GFAP; magnification,* ×*2.5.*

prompted us to ask whether the ischemic conditions created by an arterial occlusion might be the stimulus that upregulates IL-1 and initiates PMN leukocyte responses in the ischemic brain. Expression of IL-1$\beta$ mRNA in rat brain was demonstrated after forebrain ischemia and reperfusion in experiments in which both vertebral arteries were cauterized and the two carotid arteries were transiently clamped.[4] By *in situ* hibridization the signals were observed (30 minutes after forebrain ischemia) on glial cells including their perivascular processes.[19] Expression of IL-1$\beta$ mRNA was also markedly increased in the ischemic areas of the brain as early as 1 to 6 hours after the occlusion of a MCA; the increase was especially marked after 12 hours and the effect persisted for the following 5 days. Hypertensive rats, compared with normotensive controls, had an even more pronounced expression of IL-1$\beta$ under similar conditions of MCA occlusion.[5]

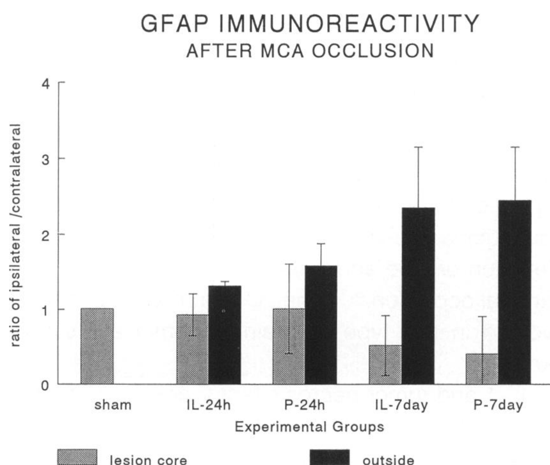


Figure 7. *Histogram showing absence of significant differences in GFAP immunoreactivity in the two experimental groups.*

Monocytes are the main source of IL-1, but glia, neurons, and endothelial cells are all known to synthesize IL-1 as well.[6] Leukocytosis,[20] brain edema,[21] breakdown of the blood-brain-barrier,[22] and exacerbation of the ischemic brain injury[4,23] are some of the biological effects documented in experiments designed to test the effects of IL-1 on the brain.[6]

Leukocytes, especially PMN neutrophils, have been implicated in the mechanisms leading to brain infarction several hours after an ischemic stroke.[2,24–26] PMN leukocytes are attracted, by as yet unknown cytokines, into a territory made ischemic by MCA occlusion long before there is morpho-

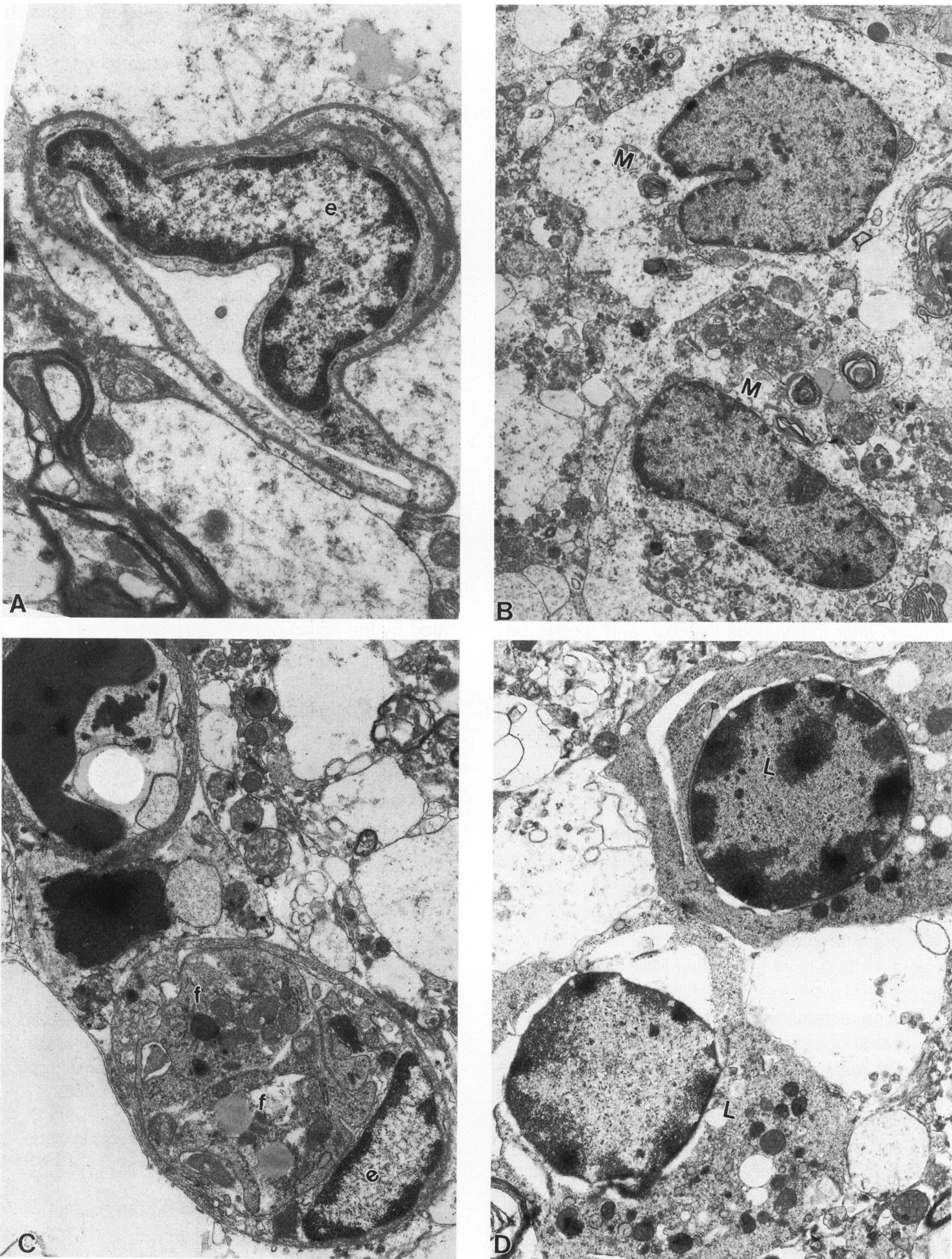


Figure 8. *Electron micrographs from the rat cerebral cortex at 24 hours after MCA occlusion. In the group treated with IL-1ra* (A *and* B), *microvessels show narrow, deformed lumen with swelling of endothelial cell nucleus (e); most surviving cells visible in the parenchyma were microglia (M). Original magnification:* A, ×15,400; B, × 7,200. *In the group treated with placebo* (C *and* D), *many microvessel lumina were filled with erythrocytes or leukocytes (f) shown next to an endothelial cell nucleus (e). PMN leukocytes (L) were easily found in the brain parenchyma.Original magnification:* C, ×8,100; D, ×8,500.

logical evidence of neuronal necrosis.[3] Consequently, we hypothesized that inhibiting IL-1 receptors might influence the outcome of an ischemic brain lesion. This beneficial effect, we thought, would be either mediated or accompanied by a decreased influx of PMN leukocytes, which would be activated by an upregulation of intercellular adhesion molecules (ICAMs). Upregulation of ICAMs has been demonstrated in nonhuman primates with transient MCA occlusion,[27] and such increased upregulation should facilitate the adhesion of the PMN leukocyte receptors to the appropriate endothelial cell ligands.

IL-1ra is a human monocyte-derived 17-kd polypeptide structurally similar to IL-1 and produced by the same cells as IL-1; IL-1ra acts as an antagonist by binding to the receptor sites for IL-1.[28,29] Several pathophysiological events characteristic of many inflammatory diseases are likely to be a reflection of a balance between the actions of IL-1 and IL-1ra.[6] Radioactively labeled IL-1ra is transported from blood to brain across the blood barrier by a saturable system. The percentage of the dose of IL-1ra calculated to enter the brain peaks 30 to 40 minutes after intravenous injection and ranges between 0.33 and 0.65% of the circulating IL-1ra.[30]

The mechanism of action for IL-1ra may involve inhibition of IL-1 that prevents endothelial cells from secreting IL-8, a cytokine necessary for the transendothelial migration of PMN leukocytes.[31]

The subcutaneous injection of IL-1ra to rats that had a MCA occluded according to the Tamura method[32] resulted in two beneficial effects. First, the volume of the brain area that failed to stain with a 2% solution of triphenyl tetrazolium chloride was 50% smaller at 24 hours after the MCA occlusion.[13] Second, brain edema measured by the wet weight/dry weight method at 24 hours after MCA occlusion was decreased by the injection of IL-1ra, even when the beginning of treatment was delayed 30 minutes after the arterial occlusion. The beneficial effects on decreased water retention in rat ischemic brains were not observed when the treatment began 1 hour after the arterial occlusion.[33]

In our experiments, injecting human recombinant IL-1ra to rats with permanent MCA occlusion significantly decreased the number of necrotic neurons in experiments terminated both at 24 hours and 7 days after the arterial occlusion. These beneficial effects, which were also reflected in improved neurological scores, occurred simultaneously with a decrease in the number of PMN leukocytes sequestered in the ischemic hemisphere. Previously, Mori et al[34] had demonstrated that microvascular patency improved as a result of treating primates subjected to transient MCA occlusion with monoclonal antibodies directed against the CD11b/18 PMN leukocyte receptor. Rats with transient MCA occlusion treated with RP-3 (an anti-neutrophil monoclonal antibody) had decreased myeloperoxidase activity in the brain, which the authors interpreted as an indication of decreased influx of PMN leukocytes 24 hours after MCA occlusion.[35] Compared with the controls, the RP-treated animals also had less brain edema and smaller area of pallor (on triphenyl tetrazolium chloride-stained slabs).[35] Decreases in the volume of area of pallor in rat brains with transient MCA occlusion (2 hours) and 2-day survival were also noted in animals treated with antibodies directed either against CD11b/18 receptor[36] or the ICAM-1 ligand.[37] The effects of anti-IL-1$\beta$ neutralizing antibodies were studied in rats with MCA occlusion (60 minutes); the authors observed decreased brain edema, decreased number of inflammatory cells, and decreased area of pallor on H&E-stained sections of the brain.[38] Collectively, these observations support the hypothesis that some of the biological events responsible for neuronal necrosis, under conditions of arterial ischemia, may be mediated by the action of PMN leukocytes.

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