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Progression from Ischemic Injury to Infarct Following Middle Cerebral Artery Occlusion in the Rat

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Focal brain ischemia induced in rats by occlusion of an intracranial artery is a widely used paradigm of human brain infarct. Details of the structural changes that develop in either the human or the rat brain at various times after occlusion of an intracranial artery are incompletely characterized. We studied, in 48 adult Wistar rats, structural alterations involving the cerebral hemisphere ipsilateral to an arterial occlusion, at intervals ranging from 30 min to 7 days. Microscopic changes developed over time in separate areas of the corresponding cerebral hemisphere in a predictable pattern, appearing as small lesions in the preoptic area (30 minutes), enlarging to involve the striatum, and finally involving the cerebral cortex. Two types of neuronal responses were noted according to the time elapsed; acute changes (up to 6 hours) included scalloping, shrinkage, and swelling, whereas delayed changes (eosinophilia and karyolysis) appeared later ($\geq$ 12 hours). Three types of astrocytic responses were noted. 1) Cytoplasmic disintegration occurred in the preoptic area at a time and in a place where neurons appeared minimally injured. 2) Nuclear and cytoplasmic swelling were prominent responses in the caudoputamen and cerebral cortex at a time when neurons showed minimal alterations. 3) Increased astrocytic glial fibrillary acidic protein reactivity was noted at the interface between the lesion and the surrounding brain tissue after 4 to 6 hours. The gross pattern of the brain lesion and the maturation of neuronal changes typical of a brain infarct have a predictable progression. Focal brain ischemia of up to 6-hour duration does not induce coagulation necrosis. (Am J Pathol 1993, 142:623–635)

Several investigators[1–8] have made histological analyses of brain lesions induced by middle cerebral artery (MCA) occlusion at random times. The intervals studied ranged from a few hours (3 to 6 hours) to several months. To our knowledge there are no publications detailing the sequence of structural abnormalities secondary to MCA occlusion as early as 30 min and at frequent subsequent intervals.

In this study we aimed to answer several questions. What are the morphological features of the ischemic lesion, ie, the condition that precedes an infarct? When does the ischemic lesion become an infarct? Do structural changes in neurons and astrocytes follow a predictable time-dependent sequence? The information derived from these experiments could be important for developing a logical time-table that may aid understanding of the physiopathological mechanisms operative in focal ischemic lesions of the brain.

We describe selected morphological alterations sequentially observed in the rat brain 30 min to 7 days after occlusion of a major intracranial artery. Cytological responses clearly separate into two temporally distinct events; acute changes are detectable as early as 30 min, whereas delayed changes become visible about 12 hours after the arterial occlusion. The brain lesion induced in Wistar rats by MCA occlusion grows and matures as a function of time; this growth spreads from a core located in the supraoptic area (first seen after 30 min) to adjacent regions; eventually (about 72 hours) the same type of structural abnormalities involve the entire territory supplied by the MCA, or the area at risk. In addition to the topographic spread of the lesion, neuronal

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changes mature or ripen so that the histological abnormalities evolve over a period of several hours from an ischemic lesion to an infarct.

Materials and Methods

Fifty-eight adult male Wistar rats (body weight, 270 to 310 g) were used in this study; 48 were experimental and 10 were control subjects (Table 1). Rats had been outbred and were purchased from Charles River Laboratory (Wilmington, MA), where they were fed Purina rat chow; upon arrival in Detroit they were fed Agway rat food, identical to the Purina chow except for a 1.3% higher protein content. After 1 day of fasting, each animal was anesthetized with 3% halothane and spontaneously respired with 1.0 to 2.0% halothane in a 2:1 N_2O:O_2 mixture, using a face mask. A PE-50 catheter in the femoral artery served for monitoring of arterial blood pressure and sampling of blood for analysis of blood gases, glucose, and hemoglobin concentration. Body core temperature of each rat was maintained at constant 37 C with a recirculating pad and K module and was monitored via an intrarectal type T thermocouple.

The surgical procedure to occlude a large intracranial artery followed the methods described by Zea Longa et al.[9] Under the operating microscope, the right common carotid artery was exposed through a midline incision. The external carotid artery (ECA) and the occipital artery were then ligated with a 5–0 silk suture. The internal carotid artery was isolated from the adjacent vagus nerve. Further dissection identified the origin of the pterygopalatine artery, at which point a silk suture was tied loosely around the mobilized ECA, and a microvascular clip was placed across the common carotid artery. An 18- to 19-mm segment of 4–0 nylon monofilament, its tip rounded by heating, was introduced into the ECA at the branching point of the occipital artery. The silk suture placed around the ECA was tightened around the intraluminal nylon monofilament to prevent bleeding, and the clip on the common carotid artery was removed. The nylon monofilament was gently advanced from the ECA into the internal carotid artery lumen; the skin incision was closed.

Two types of controls were included in the study, ie, animals that did not undergo either surgery or ischemia (n = 3; group A) and rats subjected to the same experimental procedures as described above but in which the nylon monofilament was removed within <1 minute (group B). Survival time for the group B control animals was either 1 hour (n = 4) or 24 hours (n = 3) (Table 1).

For fixation, under general anesthesia with intraperitoneal ketamine (44 to 80 mg/kg) and xylazine (13 mg/kg) the animal was transcardially perfused either with 4% paraformaldehyde (PF) in 0.1 mol/l phosphate buffer, for routine histological, histochemical, and immunohistochemical studies, or with 3% glutaraldehyde in 0.1 mol/l phosphate buffer, for electron microscopy. Brains fixed with 4% PF were allowed to fix overnight, removed from the skull, photographed, and immersed again in PF at 4 C overnight. Next, each brain was cut into seven coronal slices of 2-mm thickness each. These were labeled A (frontal) through G (occipital). Sections (approximately 6-μm thick) were obtained from paraffin blocks and stained with hematoxylin and eosin.

At least one animal from each group (Table 1) was selected at random for immersion fixation of the brain in 4% PF. This was done for the purpose of enhanc-

Table 1. *Experimental Protocol for Permanent MCA Occlusion*

	Time after MCA occlusion	Number of rats	Fixation procedures		
			PF*	PF†	GA‡
Control group A		3	1	1	1
Control group B (sham operated)	1 hour	4	2	1	1
	24 hours	3	1	1	1
Experimental group	30 minutes	2	1	0	1
	1 hour	3	2	1	0
	2 hours	3	1	1	1
	3 hours	3	1	1	1
	4 hours	7	3	2	2
	6 hours	4	3	0	1
	12 hours	4	2	1	1
	24 hours	4	3	0	1
	48 hours	4	2	1	1
	72 hours	4	2	1	1
	96 hours	4	2	1	1
	7 days	6	4	1	1
Totals		58	30	13	15

* PF, perfusion fixation with 4% paraformaldehyde.
† PF, immersion fixation in 4% paraformaldehyde.
‡ GA, perfusion fixation with 3% glutaraldehyde.

ing immunohistochemical reactions. In these animals, after death by an overdose of anesthetics, the brain was promptly removed and placed in PF fixative. For immunohistochemical demonstration of glial fibrillary acidic protein (GFAP), we used the avidin-biotin peroxidase complex (ABC) method of Hsu et al.[10] The sections were counterstained with hematoxylin and examined by light microscopy. Appropriate controls were used in each instance.

One of the coronal brain slices (either level C or D) from the group fixed by perfusion with glutaraldehyde was immersed in 3% glutaraldehyde and processed for electron microscopy. Each coronal brain slice was further trimmed into four or five sample pieces (each one measuring 1.0 × 1.0 × 2.0 mm). These samples were postfixed in 1% osmium tetroxide for 3 hours, dehydrated through graded ethanols, and embedded in araldite. Semithin sections (~1-µm thick) were stained with toluidine blue, and ultrathin sections of the areas of interest, stained with uranyl acetate and lead citrate, were examined with a Philips 300 electron microscope.

The quantitation of microscopic features is based on the evaluation of an average of 20 histology slides per experimental animal and a total of 600 electron micrographs obtained from ultrathin sections of the preoptic area, the caudoputamen, and the cerebral cortex of the hemisphere ipsilateral to the arterial occlusion. Control samples were derived from the contralateral hemisphere, the areas supplied by the ipsilateral anterior cerebral artery, and the two groups of control animals described above.

In each of the 48 experimental animals included in the study, coronal sections C or D (Figures 1 and 2) were used to calculate the percentage area of the hemispheric surface involved in the lesion, utilizing an IMAGIST-2 image analysis system (PGT, Princeton, NJ). Each lesion was identified in 6-µm-thick sections stained with hematoxylin and eosin (Figure 2). Histological features used to identify the lesion included vacuolation (sponginess) of the neuropil, diffuse pallor of the eosinophilic background, and alterations in the shape and stainability of both neuronal perikarya and astrocytic nuclei. Surface area (mm^2) and percentage volume of the hemisphere involved in the lesion were calculated from tracings of images collected with a charged coupled device (CCD) video-imaging microscope system.

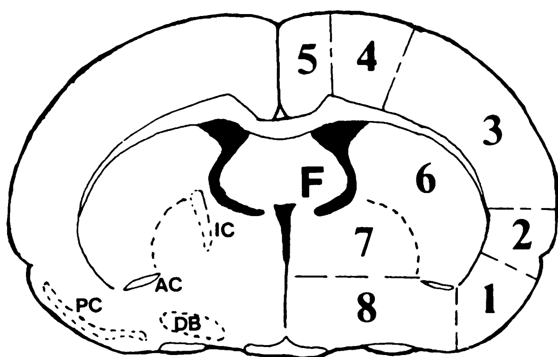

Figure 1. *Anatomical areas identified at the level of the anterior commissure of the rat brain.[11] Area 1, piriform cortex; area 2, insular cortex (granular and agranular); area 3, parietal cortex; area 4, hind- and forelimb areas of cortex; area 5, frontal and cingulate cortex; area 6, caudate putamen; area 7, globus pallidus; area 8, preoptic area (medial and lateral). IC, internal capsule; F, fornix; DB, diagonal band; PC, piriform cortex; AC, anterior commisure.*

Results

Arterial blood pressure, blood glucose, and blood gases, obtained during surgery, were within the normal range in all 48 experimental animals (Table 2).

In all experimental animals (n = 49), the tip of the monofilament was located in the proximal segment of the anterior cerebral artery, as confirmed by autopsy. The predominant neurological deficits included failure to extend the left forepaw, circling to the left, and tendency to fall to the left when animals attempted to walk across a high beam. No attempts were made to grade the neurological deficit as a function of time elapsed after MCA occlusion. Neurological evaluation was not carried out in rats sacrificed before 2 hours, because they were still under the influence of the anesthetic.

One of 59 rats died spontaneously 44 hours after surgery; this animal had a large hemispheric infarct and ipsilateral hemispheric brain edema. Death was attributed to the effects of cerebral herniation. Measurements derived from this animal's brain are not included in the data reported in this study; this leaves a total of 48 experimental rats. The brains from rats in both control groups (n = 10) were histologically normal and neurological deficits were not detected.

Table 3 depicts the chronology of the acute and delayed histological alterations observed in neurons and astrocytes after permanent occlusion of a MCA in 48 rats. Five histological events started within 30 to 60 minutes, and four different ones became detectable beginning some time 6 to 12 hours after the arterial occlusion.

Acute Changes (<12 Hours after MCA Occlusion)

Thirty minutes after MCA occlusion, the lesion was confined to the supraoptic area, as identified in an

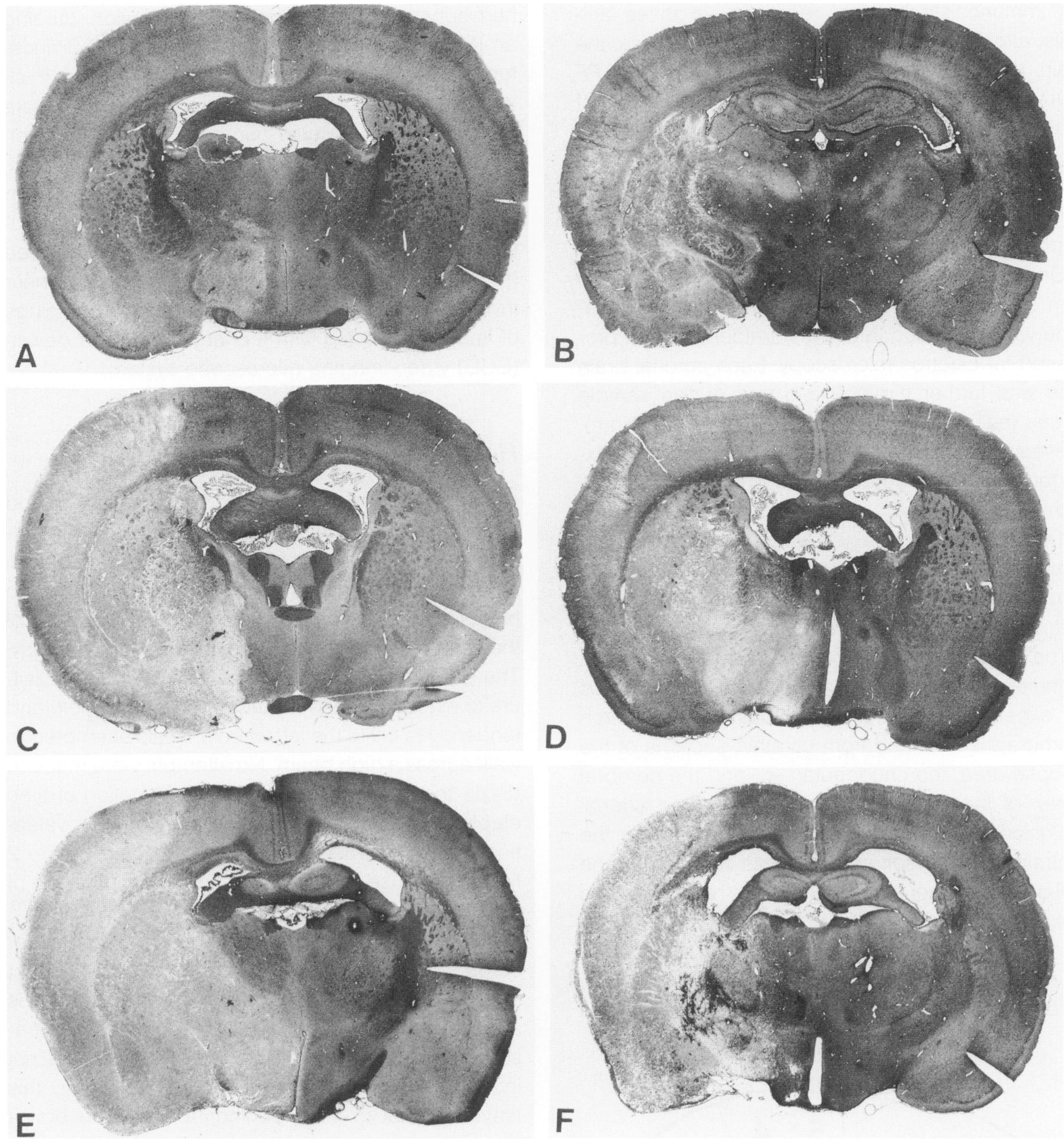

Figure 2. *Ischemic lesion at various time intervals after MCA occlusion. A notch in the cerebral cortex identifies the contralateral (left) side (hematoxylin and eosin, ×63).* A: *3 hours after MCA occlusion. A well circumscribed lesion exists in the medial portion of area 8. Areas 1 and 2 and the lateral portion of area 8 appear pale, compared with the opposite hemisphere.* B: *6 hours. Larger lesion; pale and spongy changes involve the basal ganglia and cortex. Pallor in the cerebral cortex on the left hemisphere is an artifact of staining.* C: *12 hours.* D: *24 hours. Areas 7 and 8 are more severely affected than area 6; the cortex shows a heterogeneous pattern of involvement.* E: *3 days. The lesion homogeneously involves the cortex, basal ganglia, and a portion of thalamus.* F: *7 days. The process of reabsorption is well underway.*

atlas of the rat brain[11] and as shown in Figures 1 to 3. Most vacuoles in the neuropil correspond to both swollen astrocytic processes (Figure 4B) and swollen vacuolated presynaptic terminals. Condensed neuronal perikarya surrounded by swollen cellular processes adopted a scalloped appearance. Minimal intrinsic changes affected neuronal perikarya; these included increased electron density of the cytosol and swelling of rough endoplasmic reticulum and Golgi apparatus cisternae (Figure 5A). In contrast to the mild nature of the neuronal changes, astrocytes in the supraoptic area disintegrated; cytoplasmic processes became fragmented, nuclear pyknosis was prominent, and GFAP diffused into the extracellular space (Figure 3, B and C). Astrocytes in this area also showed marked abnormalities in nuclear membranes and electron-dense deposits in their mitochondrial matrices (Figure 4A).

Table 2. *Results of Blood Gas Analysis, Serum Glucose, and pH Values Before MCA Occlusion**

Time intervals	pH	$PaCO_2$	PaO_2	Glucose (mg/dl)
30 minutes (n = 2)	7.47 ± 0.01	30.0 ± 2.6	148.60 ± 2.50	223.50 ± 68.50
1 hour (n = 3)	7.45 ± 0.02	35.13 ± 2.42	152.45 ± 6.82	180.33 ± 14.85
2 hours (n = 3)	7.42 ± 0.01	39.30 ± 2.30	117.43 ± 8.69	151.75 ± 13.17
3 hours (n = 3)	7.40 ± 0.02	40.87 ± 4.91	134.37 ± 10.23	155.0 ± 21.0
4 hours (n = 7)	7.41 ± 0.02	33.80 ± 2.63	195.32 ± 25.19	128.20 ± 15.12
6 hours (n = 4)	7.38 ± 0.03	37.86 ± 3.43	141.92 ± 6.09	153.50 ± 12.93
12 hours (n = 4)	7.41 ± 0.01	36.58 ± 1.20	153.70 ± 16.72	152.00 ± 16.56
24 hours (n = 4)	7.42 ± 0.02	31.53 ± 1.15	134.00 ± 10.62	180.0†
48 hours (n = 4)	7.43 ± 0.02	33.88 ± 3.74	110.98 ± 10.42	163.00 ± 29.57
72 hours (n = 4)	7.41 ± 0.02	38.63 ± 1.27	146.87 ± 6.00	169.67 ± 29.10
96 hours (n = 4)	7.41 ± 0.02	42.10 ± 1.89	122.30 ± 16.07	181.33 ± 35.97
168 hours (n = 6)	7.43 ± 0.01	33.64 ± 2.71	136.34 ± 17.12	160.60 ± 16.18

* Temperature, 36 to 37 C; mean arterial blood pressure, 110 to 130 mm Hg.
† Only one sample was collected.

After 1 to 3 hours, the lesion spread so that in the cortex astrocytic nuclear swelling and neuronal shrinkage were prominent throughout layers II to VI (Figure 3, D and E). The boundary zone between the territories of the middle and the anterior-cerebral arteries showed, for the first time, neuropil sponginess and astrocytic changes in those animals in which lesions extended to the entire area at risk. Neuronal scalloping (Figures 3D and 5A) and swollen pale neuronal perikarya abounded in the basal ganglia (Figure 5B). The rostral and lateral margins of the lesion in the territory of the lenticulostriate arteries were marked by a rim of tissue having spongy and markedly vacuolated tissue.

At 4 to 6 hours the lesion extended further so that increasing numbers of neuronal perikarya showed acute changes in more regions of the area at risk (Figure 2). The brain regions involved in the ischemic lesion at this time were not identical in all animals, but the following hierarchy prevailed: the supraoptic area was consistently affected and the basal ganglia displayed less severe injury than the supraoptic area and was involved in all cases, whereas in a few animals the cortex showed only lesions having focal or laminar distribution. The extent of cortical changes was smaller than that observed in the basal ganglia. Degenerative astrocytic changes spread from the supraoptic area to the medial striatum. Hypertrophic astrocytic processes and marked reactivity of the GFAP stood out in the cortex for the first time.

Delayed Changes (>12 Hours after MCA Occlusion)

In rats with MCA occlusion lasting at least 12 hours, new histological features developed. Axonal swelling was first seen at the edge of the lesion in the supraoptic area, and cytoplasmic eosinophilia affecting both neuronal and glial cells became evident at the same site after 24 hours (Figures 3, G to I, and 6A). These changes progressed to pan-necrosis at 48 hours.

In the basal ganglia (12 hours) a few red neurons appeared at scattered sites. These changes became

Table 3. *Chronology of Histological Events after Permanent MCA Occlusion in Rats**

	30 Minutes	1 Hour	2 Hours	3 Hours	4 Hours	6 Hours	12 Hours	24 Hours	2 Days	3 Days	4 Days	7 Days
Neurons												
Shrinkage and scalloping	I—	—	—	—	—	—	—	—I				
Swelling		I—	—	—	—	—	—	—I				
Vacuoles in dendrites		I—	—	—	—	—	—	—I				
Axonal swelling							I—	—	—	—	—I	
Red neurons							I—	—	—	—	—	—→
Ghost neurons										—	—	—→
Astrocytes												
Swelling	I—	—	—	—	—	—	—	—I				
Degeneration of GFAP+ cells	I—	—	—	—	—	—	—	—	—I			
Increased GFAP						I—	—	—	—I			
Ghost cells								I—	—	—	—	—→

* Lines show beginning and ending times.

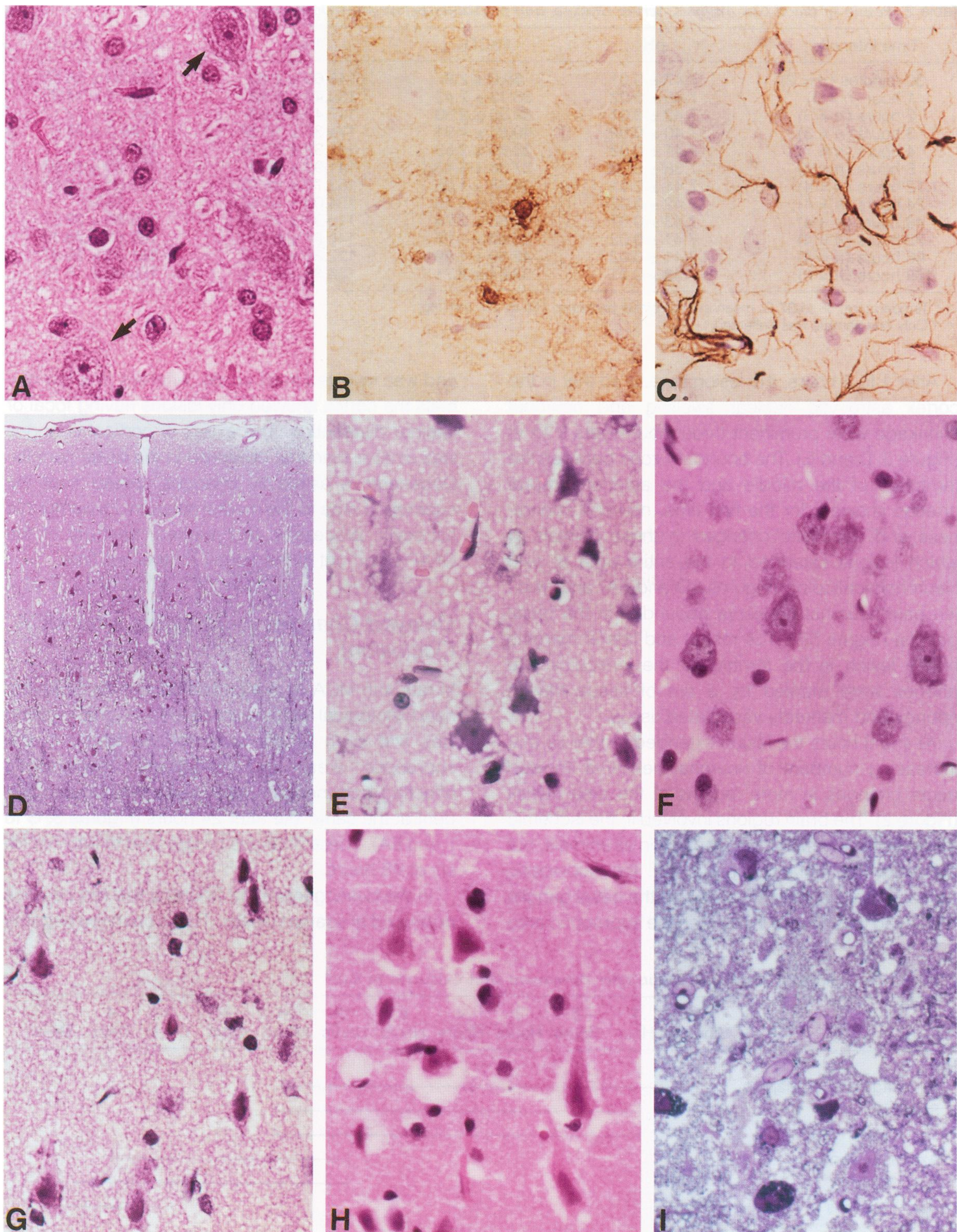

Figure 3. *Histological features of the lesion induced by MCA occlusion.* **A-C:** *Area 8, 30 min after MCA occlusion. A few vacuoles are seen in the neuropil; neurons (arrows) are structurally intact by hematoxylin and eosin staining* **(A)**. *GFAP-positive cells in the same area show fragmented processes and reaction product in the nucleus* **(B)**. *GFAP reactivity in area 8 of the contralateral hemisphere is normal* **(C)**. **A-C**, *×460.* **D-F:** *Area 3, 1 hour after MCA occlusion. Multifocal sponginess is apparent* **(D)**. *Shrunken angular neurons with perineuronal vacuoles appear scalloped* **(E)**. *The contralateral cortex is intact* **(F)**. **D**, *×90;* **E** *and* **F**, *×460.* **G-I:** *Area 3, at 24 hours* **(G)**, *48 hours* **(H)**, *and 4 days* **(I)** *after MCA occlusion. Most neurons are red after 24 hours* **(G)** *and become ghost cells at 48 hours* **(H)**; *granular appearance in 1.0-μm-thick section stained with toluidine blue* **(I)** *indicates breakdown of cytoplasm.* **G-I**, *×460.*

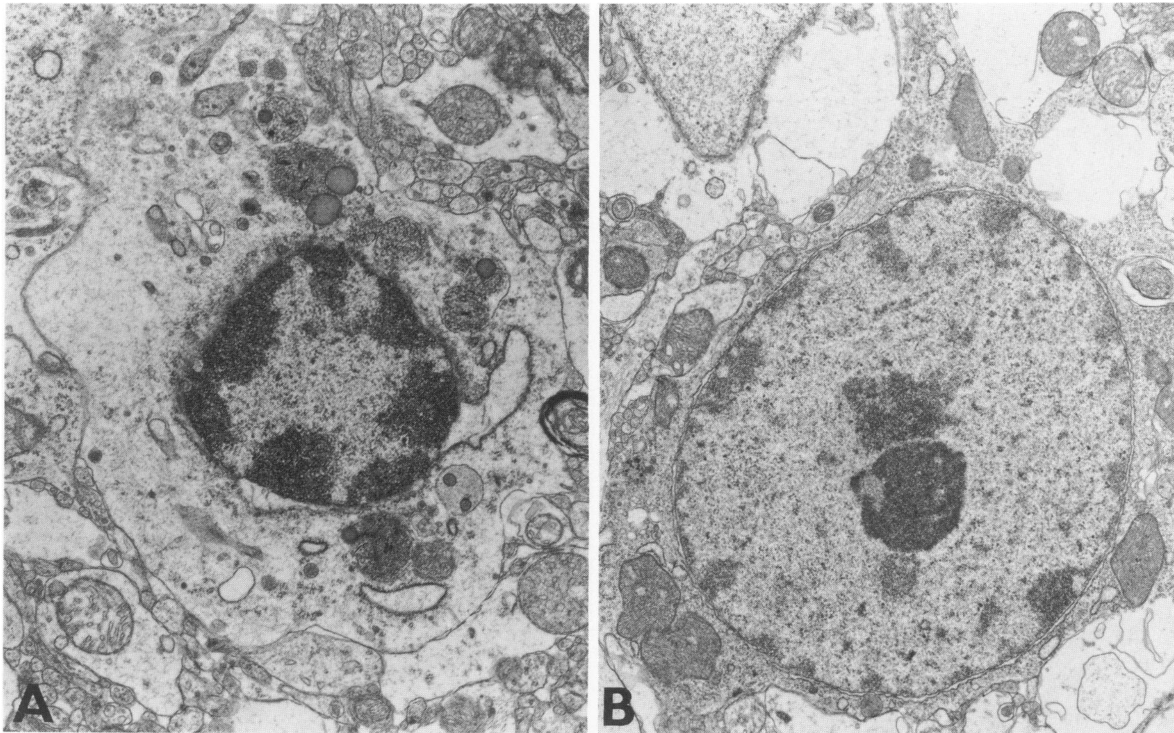

Figure 4. *Astrocytic changes in area 8 at 30 min.* **A**: *An astrocyte shows prominent swelling of endoplasmic reticulum cisternae and nuclear membrane evaginations; also, there are electron-dense bodies in mitochondria with condensed matrix; clumping of nuclear chromatin is apparent.* ×15,400. **B**: *A neuron in the same area is intact except for minimal swelling of endoplasmic reticulum cisternae.* ×10,400.

more numerous at 24 hours, when most cells transformed into ghosts (ie, cells without stainable nucleic acid materials) (Figure 3, H and I). GFAP-positive cells disappeared from the basal ganglia. In most rats the lesion involved the entire territory supplied by the MCA (Figure 2), whereas in a few the cortical involvement remained restricted to layers II to V. In addition to neuronal shrinkage and astrocytic swelling, scattered cortical neurons and glia showed cytoplasmic eosinophilia and nuclear pyknosis at 12 hours; after 24 hours an increasing number of cells became eosinophilic and at 48 hours most neurons and glia had lost their affinity for hematoxylin staining (ghost cells), as shown in Figure 3, G and I.

At 3 to 7 days after MCA occlusion, pan-necrosis was limited to the supraoptic area. For the first time (72 hours), the entire area at risk adopted a homogeneously pale appearance that became clearly discernible from the surrounding brain (Figure 2*E*). However, even at this time scattered cells within the pale area retained normal stainability, and several neurons outside the pale area showed eosinophilia. Neuronal eosinophilia, karyolysis, and diffusely pale neuropil were prominent after day 3; in addition, infiltration by inflammatory cells (mostly polymorphonuclear leukocytes) was profuse. Neuronal and glial changes (of the acute type) were no longer visible after 3 days. Prominent changes at day 4 included abundant ghost neurons and extensive injury to the microvasculature (Figure 6).

Data Analysis

The mean of the surface area measurements obtained in group 1 (30 min to 6 hours after MCA occlusion) was compared with the same in group 2 (12 hours to 7 days after MCA occlusion) (Table 4). Standard *t*-tests determined whether significant differences existed in the lesion size during the acute (<12 hours) stage, compared with the delayed (≥12 hours) stage ($P = < 0.002$), and whether the size of the lesions at 6 hours differed significantly from those measured at 12, 24 48, 72, 96, and 168 hours. The difference was statistically significant only for the comparison between the 6-hour and 72-hour groups ($P < 0.005$). The pattern of growth of the lesion induced in 48 rats by MCA occlusion is shown in Figure 7.

Discussion

We outline the chronological sequence of selected tissue responses in the brain territory supplied by a permanently occluded MCA in anesthetized rats.

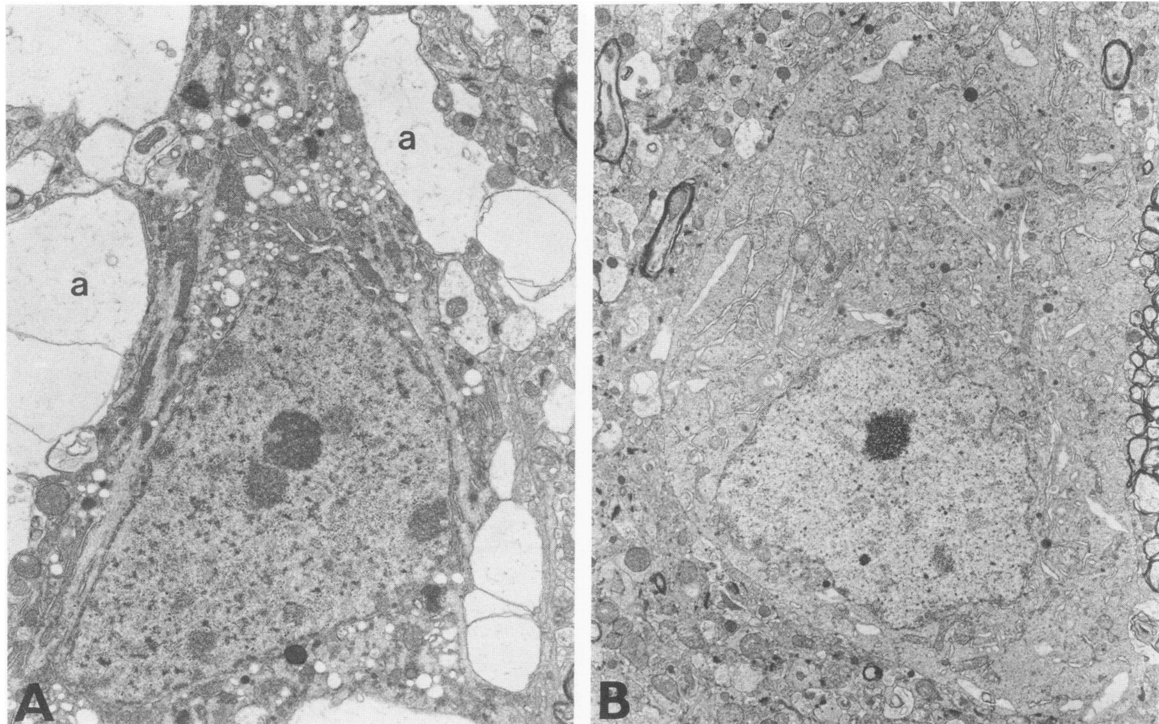

Figure 5. *Neuronal changes at 1 hour after MCA occlusion.* **A**: *A scalloped neuron (in the cortex) shows condensed cytoplasm with vesicular swelling of organelles. Perineuronal astrocytic processes (a) are prominent. ×8100.* **B**: *A minimally swollen neuron from area 6 shows electron-lucent cytoplasm with minimal dilatation of organelles; swelling of astrocytic processes is not prominent. ×4800.*

The methods to occlude the MCA without craniectomy were described by Koizumi et al[12] and Nagasawa and Kogure.[13] We applied the innovations suggested by Zea Longa et al,[9] who verified that inserting the monofilament according to their technique occludes the origin of the MCA. This produces a brain infarct limited to the territory of the occluded vessel, as was shown by autopsy 72 hours after the arterial occlusion.[9] Using the same model of MCA occlusion in rats, *in vivo* brain abnormalities were detected by magnetic resonance imaging as early as 30 min after the arterial occlusion, at a site that corresponded to the area where a brain infarct was demonstrated 24 hours later.[14] Again, based on studies of the same model in the rat, ie, intracarotid insertion of a monofilament, Nagasawa and Kogure[13] reported low cerebral blood flow (CBF) values localized to the territory supplied by the occluded MCA; despite differences in local CBF values in various brain regions, the CBF values were lower at 6 hours than they were after 3 hours.[13] Brain infarcts (or areas of pallor) developed in all 30 rats whose MCA had been occluded 24 hours before death.[15]

Thus, the procedure we used to occlude a MCA decreases the local CBF to levels sufficiently low to result in an infarct.[13] Our experience with the 48 rats analyzed in this study verifies that this method of MCA occlusion is highly reproducible; all 48 experimental animals developed brain lesions appropriate to the site of the arterial occlusion, and the supraoptic area was injured in all animals.

Local CBF changes in 45 rats with MCA occlusion fluctuated significantly as a reflection of both site and time elapsed after the arterial occlusion.[16] These investigators also reported that the lesion produced by MCA occlusion in rat brain appears to be "expansive during the first week . . . and that neuronal damage may still occur several days after the insult . . ."[17] This suggests that the histopathological changes in areas of focal brain ischemia change significantly as a function of both site and postocclusive time. Our observations are in agreement with this premise; at least two temporally separate sets of neuronal alterations were recorded at 30 minutes and 12 hours (Table 3), and the brain lesion grew as a function of time, especially between the period of 6 to 72 hours (Table 4 and Figure 7).

Swanson et al[18] conducted a systematic analysis to determine the minimum time required, after MCA occlusion in rats, for an infarct to become discernible; in that study an infarct, defined as a pale area sharply circumscribed from the surrounding brain, was not clearly visible until 3 to 5 days after a MCA

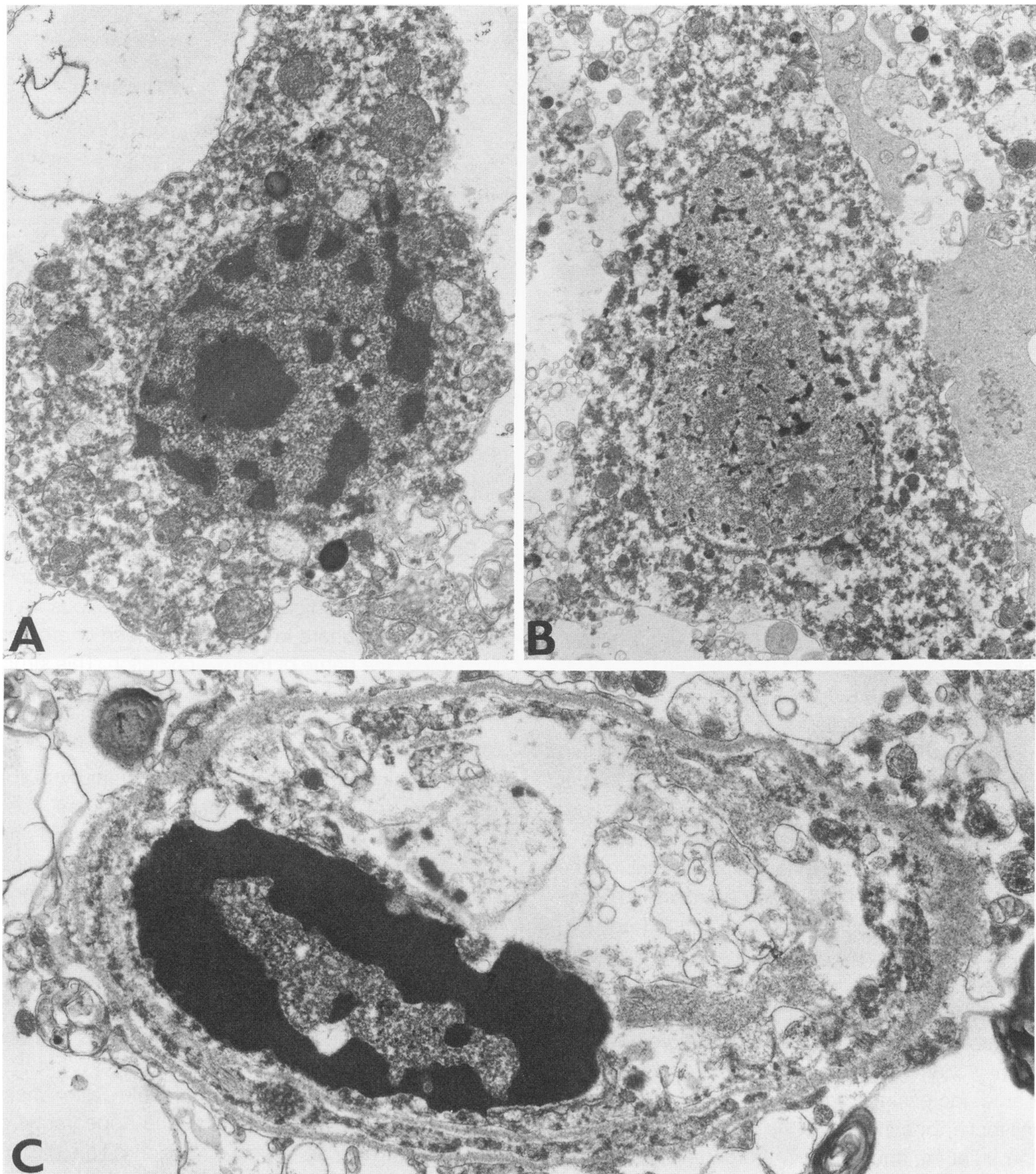

Figure 6. *Delayed injury of neurons and blood vessel.* **A:** *A neuron in area 6 (24 hours) shows cytoplasmic and nuclear disintegration, dense mitochondrial bodies, aggregation of cellular organelles, and marked nuclear chromatin clumping with discontinuities in nuclear membranes.* ×*15,400.* **B:** *4 days after MCA occlusion, a ghost neuron shows breakdown of cytoplasmic and nuclear membranes.* ×*6900.* **C:** *Four days after MCA occlusion, a capillary from area 8 shows extensive injury to endothelium and pericyte; the basal lamina remains intact.* ×*18,200.*

occlusion. Bederson et al[19] defined a brain infarct as a site where stainability with 2,3,5-triphenyltetrazolium chloride is lost as a reflection of irreversible mitochondrial injury; they reported that such an event occurred 24 hours after MCA occlusion.

Despite the absence of readily visible brain changes, numerous physiological and metabolic abnormalities are demonstrable both in human and in animal brains with intracranial arterial occlusions of <24-hour duration.[20] The changes preceding the development of an infarct (or area of coagulation necrosis) are called ischemic, to differentiate them from the irreversible changes of the mature infarct.[20] The morphological features of such ischemic alterations, as revealed by our study, include neuronal swelling, scalloping, and neuronal shrinkage; the

Table 4. *Growth of the Ischemic Lesion as a Function of Time**

	Time	Ischemic area (mm^2)	Ischemic area as percentage of hemisphere area
Group 1	30 minutes (n = 2)	0.59 ± 0.19	1.61 ± 0.66
	1 hour (n = 3)	3.91 ± 1.22	10.23 ± 3.49
	2 hours (n = 3)	8.38 ± 3.02	21.87 ± 9.65
	3 hours (n = 3)	14.20 ± 2.16	38.96 ± 8.72
	4 hours (n = 7)	16.37 ± 7.51	43.30 ± 19.41
	6 hours (n = 4)	13.49 ± 3.00	38.38 ± 9.72
Group 2	12 hours (n = 4)	17.95 ± 5.02	49.80 ± 10.11
	24 hours (n = 4)	22.90 ± 7.34	59.07 ± 18.21
	48 hours (n = 4)	18.55 ± 7.70	49.37 ± 15.00
	72 hours (n = 4)	24.83 ± 4.35	74.88 ± 9.26
	96 hours (n = 4)	22.96 ± 1.80	71.04 ± 5.42
	168 hours (n = 6)	18.61 ± 7.08	59.73 ± 23.38

* Values are mean ± standard deviation. $P < 0.002$; comparison between 22 animals in group 1 and 26 in group 2. $P < 0.005$; comparison between four animals with MCA occluded for 6 hours and four animals with MCA occluded for 72 hours.

process of acute swelling involves both the perikaryon and the dendritic processes. Ultrastructural alterations visible during the acute stage include moderate clumping of nuclear chromatin granules, dilatation of a few rough endoplasmic reticulum cisternae, and swelling of mitochondria without calcium salts precipitates. All of these reflect cellular injury of a reversible type.[21–23]

Time-dependent changes typical of cell death, induced by ischemia, have been carefully characterized in liver cells both *in vitro* and *in vivo* by Farber and colleagues[21,22] under the designation of "coagulative (or coagulation) necrosis." Two types of histological abnormalities define this kind of injury; the cytoplasm of the dead cell becomes intensively eosinophilic while the nucleus turns pyknotic (red neurons) and later the cellular nucleic acids completely lose their affinity for hematoxylin (ghost neurons). These types of cellular changes were not visible in the neurons of the affected territory before 12 hours of focal ischemia. Thereafter, red and ghost neurons became increasingly more numerous at isolated small foci appearing first in the supraoptic area, next in the striatum, and finally in the cortex. Pan-necrosis, or coagulation necrosis of neurons, glia, capillaries, and myelinated fibers, involved the area at risk only 72 to 96 hours after the arterial occlusion. This is in agreement with observations based on gross staining abnormalities described by others who timed the appearance of an infarct (or pan-necrosis) in experiments of MCA occlusion in rats.[13,24–29] This is also in agreement with estimates made, in human specimens, of the minimal time required before a brain infarct becomes grossly visible.[30] Dereski and associates[30a] reported a study of 59 Fisher rats with tandem MCA/carotid artery occlusion in which eosinophilic neurons were seldom seen in experiments terminated before 8 hours.

Some of the changes of acute neuronal injury in experimental models of hypotension in the baboon[31] and after combined carotid ligation plus exposure to nitrogen atmosphere in rats[32] have been characterized under the name of microvacuolation. This structural change involving scattered neurons in areas of focal ischemia is the result of dilatation of endoplasmic reticulum cisternae and mitochondria.[33]

The original description of neuronal injury of the delayed type, ie, eosinophilic change, has been attributed to Spielmeyer, who first described this alteration in human brains of patients whose medical history is unclear.[33a] This same type of neuronal alteration developed in mouse cerebellar tissue completely devoid of circulation only when the ambient temperature was kept at 37 C and the pH was maintained alkalotic (≥7.4). A minimum of 24 hours of incubation was needed before eosinophilic neurons appeared in this system.[34]

Our observations are compatible with those derived from these experiments; the appearance of red neurons after an arterial occlusion is time dependent (about 12 to 14 hours as a minimum) and coincides with the time when ischemic tissues become alkalotic. In rats subjected to forebrain ischemia and reperfusion, brain tissue pH changed from acidotic to alkalotic (7.27 ± 0.12) 24 to 48 hours after the ischemic injury.[35]

The temporal progression of acute neuronal changes to the stage of coagulation necrosis (infarct) shares similarities with the evolution reported in the structure of myocytes after the occlusion of a coronary artery.[23] In this model, appearance of reversible and irreversible injury to myocytes was

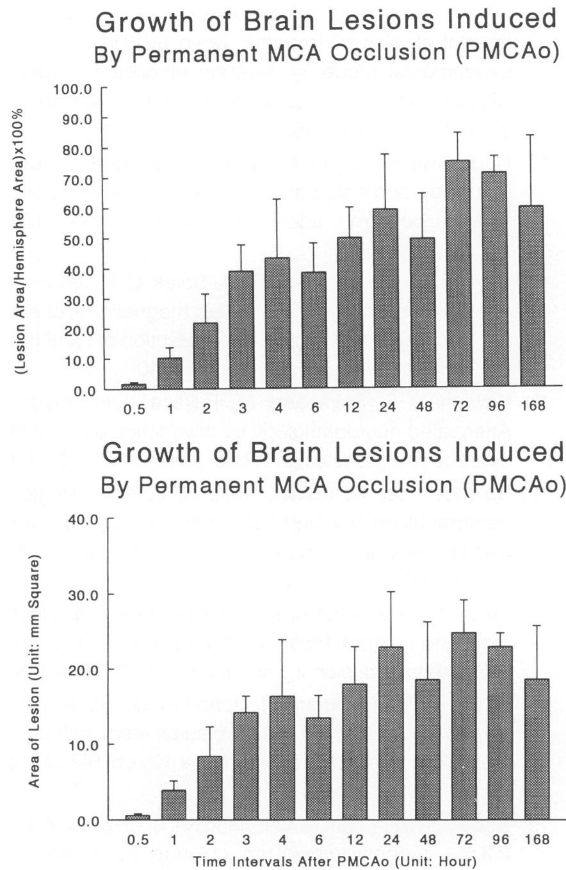

Figure 7. *Pattern of spread of the lesion induced in 48 rats by MCA occlusion, expressed in mm^2 and as a percentage of the area of the hemisphere.*

time dependent[23]; the earliest and most dependable sign of irreversible (lethal) injury to myocytes was the appearance of amorphous calcium salt precipitates in mitochondrial matrix, together with the appearance of discontinuities in the sarcoplasmic membranes.[23] In most experimental models of focal ischemia in the brain and heart, the timing of the irreversible changes is delayed by several hours depending on the degree of ischemia and the region of the lesion being studied.

Increasing numbers of hippocampal red neurons were described in a rodent model of forebrain ischemia (ie, bilateral carotid ligation) lasting 5 to 10 minutes, followed by 1 to 4 days of reperfusion.[36,37] The process by which these neuronal changes develop has been called maturation phenomenon or delayed neuronal injury. In our experiments based on permanent MCA occlusion, and therefore in the absence of reperfusion, we note the existence of comparable maturation effects. Many neurons, in basal ganglia and cortex, that stained normally during the acute stage (6 hours or less) turned necrotic after 12 hours. An obvious explanation for this phenomenon is not readily available, although several authors have suggested that excitatory neurotransmitters, in particular glutamate, may be involved in progressive neuronal death.[38] As for the growth of the ischemic lesion, Cavanaugh et al[39] reported their observations from a large number of experiments based on MCA occlusion in rats. Growth of the brain lesion was evident by a progressive increase in the surface of the pale area (in 2,3,5-triphenyltetrazolium chloride-stained preparations) and by the continuous breakdown of spectrin. Both of these peaked 12 hours after MCA occlusion.[39]

Astrocytic responses of two types have been reported in rodents whose brains were injured by forebrain ischemia (four-vessel ligation), followed by variable times of reperfusion. Cytoplasmic swelling begins during the acute stage of the lesion, whereas increased reactivity to GFAP is a phenomenon delayed by several hours. The latter reaction persists only at sites where neuronal injury of an irreversible type has developed.[40] A third type of astrocytic response in focal brain ischemia was uncovered by our experiments with MCA occlusion, ie, disintegration of the glial stroma occurred before neurons showed necrotic changes. A detailed analysis of selected astrocytic responses in areas of evolving focal ischemia (without reperfusion) is currently being completed.

In conclusion, this time-dependent study reveals three important features of the lesion created by permanent occlusion of the MCA in the rat. 1) In eight animals in which the arterial occlusion lasted <2 hours, the histological abnormalities were qualititatively minimal but always involved the supraoptic area. Over a period of about 12 hours histological changes of a comparable type spread to the entire territory at risk. Gross changes in the stainability of the lesion became prominent and well demarcated only after 48 to 72 hours. Pan-necrosis (or coagulation necrosis involving all cell types) was demonstrable for the first time after 48 to 72 hours, but only in the core of the lesion. In other regions of the area at risk isolated groups of cells retained normal stainability as late as 3 days after MCA occlusion. 2) Two types of neuronal responses developed depending on the time elapsed after the arterial occlusion. The neuronal changes in animals sacrificed <12 hours after MCA occlusion included scalloping, shrinkage, and swelling of the perikaryon, as well as dendritic vacuolation. Additional neuronal changes at 12 hours included nuclear pyknosis and cytoplasmic eosinophilia (red neurons), accompanied by neu-

ronal karyolysis with complete loss of hematoxylinophilia (ghost neurons). 3) In selected areas of the territory supplied by the occluded artery, astrocytes disintegrated before neurons showed structural alterations suggestive of necrosis.

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