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Time Course of ICAM-1 Expression and Leukocyte Subset Infiltration in Rat Forebrain Ischemia

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ABSTRACT

The time course of ICAM-1 expression and leukocyte subset infiltration was studied in a model of CNS reperfusion injury in adult rats. Leukocyte adhesion and infiltration, mediated in part by intercellular adhesion molecule-1 (ICAM-1), appears to potentiate CNS reperfusion injury. The timing and relationship between ICAM-1 staining and leukocyte infiltration postglobal CNS ischemia is unknown. Reversible forebrain ischemia was produced in 32 adult Sprague-Dawley rats using the two-vessel occlusion model with histologic analysis performed at specific intervals postischemia: 1, 3, 6, 12, and 24 h, 4 and 7 d, or sham-operated controls (n = 4 each group). Monoclonal antibodies against ICAM-1 (1A29 and TM8), a specific granulocyte (PMN) (HIS48), and a specific monocyte/macrophage (MØ)(ED1) were used. No specific leukocyte and only rare ICAM-1 vessel immunoreactivity was observed in sham controls. ICAM-1: Significant expression in microvessels beginning at 1 h with additional diffuse CA1 pyramidal layer staining beginning at 4 d. Leukocytes: No PMN cells and rare MØ identified at 6 and 12 h. By 24 h: moderate infiltrate in areas of ICAM-1 expression of PMN and MØ. At 4 and 7 d: only MØ accumulation, cellular morphology now similar to microglia. The results

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of this study indicate that early and persistent ICAM-1 expression occurs following CNS ischemia with associated leukocyte infiltration.

Index Entries: ICAM-1; cerebral ischemia; reperfusion injury; leukocytes; microglia; macrophage.

INTRODUCTION

Recent evidence suggests that leukocytes are directly involved in the pathogenesis and extension of CNS ischemic injury (Engler et al., 1986; Schmid-Schonbein and Engler, 1986). Two proposed mechanisms of leukocyte involvement in ischemia are: direct microvascular occlusion from endothelial and basement membrane adhesion (Schmid-Schonbein, 1987; Mori et al., 1992); and transendothelial migration with secondary central nervous system (CNS) tissue infiltration and neuronal cytotoxic injury (Schmid-Schonbein and Engler, 1986). For initiation of either of these mechanisms, adhesion of leukocytes to microvascular endothelium is essential.

Leukocyte adhesion to the endothelium is primarily mediated by the leukocyte adhesion molecule (CD 18) and its endothelial ligand, the intracellular adhesion molecule (ICAM-1) (Smith et al., 1988; Argenbright et al., 1991). ICAM-1 expression on endothelial cells has been shown to be induced by inflammation, cytokines, and ischemia (Wilcox et al., 1990). Immunohistologic studies in endothelial cell cultures have demonstrated ICAM expression within 6 h of cytokine stimulation. Previous studies have demonstrated that ICAM-1 can be induced by various inflammatory mediators or ischemia (Smith et al., 1988; Argenbright et al., 1991). The importance of ICAM-1 and leukocyte infiltration in CNS ischemia is supported by experimental studies that demonstrate reduced CNS ischemic injury when leukocyte adhesion is blocked by anti ICAM-1 or CD-18 antibodies (Clark et al., 1991a,b). In the human CNS, increased ICAM expression on endothelial and parenchymal cells has been demonstrated in encephalitis, and multiple sclerosis plaques, and in one stroke case at 5 d (Sobel et al., 1990). Recent CNS experimental studies have produced conflicting results with transient ICAM-1 expression seen at 1 h in a focal reperfusion model (Okada et al., 1994), whereas persistent ICAM-1 expression beginning at 24 h was seen in a focal nonreperfusion model (Jander et al., 1995). However, the time course of ICAM-1 expression following global CNS ischemia with reperfusion and its topographical relationship to leukocyte infiltration is unknown.

In focal CNS ischemia in rats, granulocyte infiltration occurs as early as 4 h and peaks at 48–72 h (Dereski et al., 1992; Garcia et al., 1994). General leukocyte infiltration has also been observed in clinical stroke (Wang et al., 1993). However, the time course of granulocyte and monocyte/macrophage leukocyte subset infiltration as assessed by specific immunohistochemistry has not been well established.

In this study, we used immunohistochemistry staining to determine the time course of ICAM-1 expression and leukocyte subset infiltration in reversible CNS forebrain ischemia. This animal model shows preferential ischemic injury in the CA1 region of the hippocampus and variable ischemic areas throughout the cortex.

METHODS

Ischemic Animal Model

We used a variation of the two vessel occlusion model of transient reversible ischemia (Smith et al., 1984a,b; Wieloch, 1985). All procedures approved by OHSU animal care committee. Adult male Sprague-Dawley rats weighing 250–350 g were anesthetized with a constant flow of 3–5% halothane in O_2 for 5 min in a closed chamber. An endotracheal tube (PE-240) was inserted and respiration was mechanically controlled using a Harvard rodent ventilator (model 683) with a tidal volume of approximately 2 mL at a rate of 80–90/min. The tidal volume and rate were adjusted to provide an arterial PCO_2 of 30–40 torr and a pH of 7.35–7.45 as measured by an acid-base analyzer (Radiometer ABL 30). The ventral tail artery was catheterized for monitoring arterial pressure and obtaining blood samples. Temperature was monitored through a rectal probe and maintained at 36.5–37.5°C with a heating pad. Nitrous oxide (N_2O, 66%), halothane (0.3–1.5%), and oxygen as the balance was added to the respirator intake. Needle electrodes were positioned on the surface of the skull at the dorsal medial margin of the temporalis muscle to monitor the interhemispheric EEG. The carotid arteries and right jugular vein were identified through a horizontal incision in the lower cervical region. The carotids were separated from the vagus nerves and encircled with loose ligatures. A large bore silicone catheter (0.03'' ID) was inserted approx 3.8 cm into the right jugular vein, and 0.1 mL of heparin (300 U/mL) was injected into the jugular catheter. The mean arterial pressure was rapidly lowered to 25 torr by withdrawing 4–9 mL of blood through the jugular catheter and leaving the syringe attached to the catheter. Both carotid arteries were immediately clamped. Blood pressure was maintained at 25 torr via the syringe attached to the jugular catheter. The period of ischemia lasted 15 min and at the end of this period the carotids were unclamped and the withdrawn blood reinfused along with 25 mg of $NaHCO_3$ (0.5 mL). The jugular catheter was removed and neck incisions were sutured. The EEG, EKG, and BP were monitored for 30 min.

Tissue Preparation

At specified times postischemia (1, 3, 6, and 12 h, 1, 4, and 7 d), rats (n = 4) were anesthetized, given an intracardiac injection of 0.2 mL heparin (300 U/mL), and tissues were fixed via intracardiac perfusion of 10 mL

sodium phosphate buffer (100 m*M*, pH = 7.2) followed by 300 mL of 2% formaldehyde in phosphate buffer. Brains were removed and a coronal section was dissected between the stereotaxic coordinates of interaural + 2.5 mm – interaural + 8.0 mm. This section was immersion fixed with 2% formaldehyde in phosphate buffer for 30 min at room temperature. The tissue was equilibrated with a 15% sucrose solution containing 0.02% sodium azide followed by a 30% sucrose solution containing 0.02% sodium azide. The tissue was next cut into serial 50 μm thick sections on a freezing sled microtome. The sections were stored in phosphate buffered slaine (PBS pH = 7.4, 100 m*M*) containing 0.1% sodium azide at 4°C until used in immunohistochemistry.

Immunohistochemistry

Immunohistochemical staining was performed on free floating sections. Endogenous peroxidase activity was removed by the incubation of sections in PBS containing 15% ethanol and 1% H_2O_2 for 30 min followed by three 10-min washes in PBS. Sections were incubated for 1 h with PBS containing 10% horse serum, 0.5% Triton-X-100, and 0.02% sodium azide (blocking buffer), followed by overnight incubation with one of the following antibodies diluted into blocking buffer: 1A29 (mouse monoclonal antirat ICAM-1.1:300 dilution, Serotec, Oxford, UK) (Tamatani and Mlyasaka, 1989), TM8 (mouse monoclonal antirat ICAM-1.1:300 dilution. Athena Neurosciences, San Francisco, CA) (Athena, unpublished report), ED1 (mouse monoclonal antirat monocyte/M∅/dendritic cell 1:1000 dilution. Serotec) (Dijkstra et al., 1985), HIS48 (mouse monoclonal antirat granulocyte, 1:50 dilution, Serotec) (van Goor, 1991), MRC OX-42 (mouse monoclonal antirat complement receptor type 3. 1:1600 dilution, Serotec) (Robinson et al., 1986), or mouse serum control (1:2000 dilution). Sections were then incubated with biotinylated horse antimouse IgG, avidin biotin horseradish peroxidase (Vector, Burlingame, CA), and developed using 3,3-diaminobenzidine (Sigma, St. Louis, MO) as a substrate.

To assess corresponding neuronal changes, thionin staining was performed by incubating mounted sections in 0.1% thionin (Sigma) for 4–10 s at room temperature.

The intensity of immunoreactivity was estimated semiquantitatively by bright-field microscopy by two blinded observers and scored on a four-point scale (Chauhan et al., 1993; Liu et al., 1993). 0 = no staining, 1 = faint (e.g., Figs. 2A and 3C), 2 = moderate (e.g., Figs. 2E and 3E), and 3 = intense (e.g., Figs. 2F and 3H). Scores were averaged and the rank sum test was used to determine significant differences from sham control.

RESULTS

A total of 32 animals were studied. Successful forebrain ischemia was confirmed in all animals by a marked attenuation of EEG activity during

ischemia and by abnormal initial neurologic symptoms postischemia consisting of altered posture, decreased activity, impaired grooming, and weight loss.

Immunocytochemical Distribution of ICAM-1

Table 1 shows semiquantitative results and Fig. 1 illustrates the temporal profile of ICAM-1 staining with TM8 and 1A29 with representative TM8 sections shown in Fig. 2. In sham controls, a small number of microvessels showed weak lumenal ICAM-1 staining. Significant ICAM-1 expression occurs by 6 h postischemia and continues throughout 7 d. ICAM-1 staining is seen predominantly in the lumen of microvessels in both the cortex and hippocampus. At 6–24 h postischemia in the hippocampus, most of the ICAM-1 positive microvessels are seen within the CA1 stratum radiatum and oriens, and along the hippocampal fissure (Figs. 2A,C,E). At 6–24 h postischemia in the cortex, ICAM-1 positive microvessels are seen throughout the retrosplenial, occipital, parietal, and temporal cortecies (Figs. 2B,D,F). Both microvessels (7.5–30.0 μm) and larger 100 μm vessels were involved (as measured by an Image Pro Plus analysis program). At 4 and 7 d postischemia, in addition to specific microvessel staining, diffuse parenchymal staining is seen in the CA1 oriens and stratum radiatum of the hippocampus (Figs. 2G,H,J,K). In the cortex, specific microvessel ICAM-1 staining persists in the retrosplenial, occipital, parietal, and temporal lobes at 4 and 7 d (Figs. 2I,L). Overall, there was a good agreement between the time course of TM8 and 1A29 staining; in the hippocampus $r = 0.88$, in the cortex $r = 0.95$. The localization and time course of ICAM-1 staining in the hippocampus and cortex at 4 and 7 d was similar to that seen with ED1 (in the hippocampus $r = 0.90$; in the cortex $r = 0.86$), a monocyte-macrophage specific antibody, *see the discussion* in the next section (Figs. 3E,H,G,J), and with OX42, an antibody that binds CR3 receptor; positive staining and morphology used to identify microglia cells (Figs. 5C,D).

Immunocytochemical Distribution of Leukocytes

Semiquantitative results of ED1 staining are shown in Table 1 and Fig. 1, with representative sections shown in Fig. 3. No ED1-positive cells (monocytes/macrophages) were seen in sham controls. Significant macrophage-monocyte accumulation was observed by 6 h postischemia. These cells appeared to be individual round cells within the lumen of microvessels and are present equally in both hippocampus and cortex (Figs. 3A,B). At 1 d postischemia, clusters of ED1-positive round cells are seen in the lumen of microvessels and in the corresponding parenchymal regions of both hippocampus and cortex (Figs. 3C,D). At 4 (Figs. 3E,F,G) and 7 (Figs. 3H,I,J) d the number of ED1-positive cells has dramatically increased in both the hippocampus and cortex. In the cortex, ED1-positive cells are localized to ischemic regions in the retrosplenial and occipital cortecies.

Table 1
Intensity of Immunoreactivity of ICAM-1 and Leukocyte Subset Infiltration in Hippocampal and Cortex Regions of the Rat after Ischemia[a]

	TM8		IA29		EDI		HIS48	
	Hcamp	Cortex	Hcamp	Cortex	Hcamp	Cortex	Hcamp	Cortex
Sham	0.5 ± 0.5	0.5 ± 0.5	0.2 ± 0.4	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1 h	0.8 ± 0.3	0.8 ± 0.3	0.7 ± 0.3[b]	0.7 ± 0.3[b]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3 h	1.0 ± 0.0	1.2 ± 0.3	0.8 ± 0.6[b]	0.8 ± 0.3[b]	9.2 ± 0.3	0.0 ± 0.0	0.2 ± 0.3	0.2 ± 0.3
6 h	1.3 ± 0.3[b]	1.7 ± 0.6[b]	0.7 ± 0.3[b]	0.8 ± 0.8[b]	0.5 ± 0.0[b]	0.8 ± 0.3[b]	0.2 ± 0.3	0.3 ± 0.3
12 h	1.5 ± 1.0	1.7 ± 0.8[b]	1.2 ± 1.2[b]	1.3 ± 1.0[b]	0.5 ± 0.0[b]	0.5 ± 0.0[b]	0.2 ± 0.3	0.3 ± 0.6
24 h	2.1 ± 0.6[b]	2.6 ± 0.8[b]	0.8 ± 0.3[b]	1.8 ± 0.3[b]	0.6 ± 0.5[b]	1.1 ± 0.5[b]	1.0 ± 0.4[b]	1.6 ± 0.6[b]
96 h	2.7 ± 0.5[b]	2.4 ± 1.0[b]	2.0 ± 0.0[b]	1.9 ± 1.1[b]	2.0 ± 0.4[b]	1.9 ± 0.6[b]	0.0 ± 0.0	0.0 ± 0.0
168 h	2.6 ± 0.3[b]	2.4 ± 0.8[b]	2.3 ± 0.5[b]	1.6 ± 0.8[b]	2.6 ± 0.5[b]	2.3 ± 1.0[b]	0.0 ± 0.0	0.0 ± 0.0

[a] Values represent semiquantitative scores +/– SD.
[b] = Significant difference from sham control by rank sum test. 0 = No staining, 1 = faint, 2 = moderate, 3 = intense.

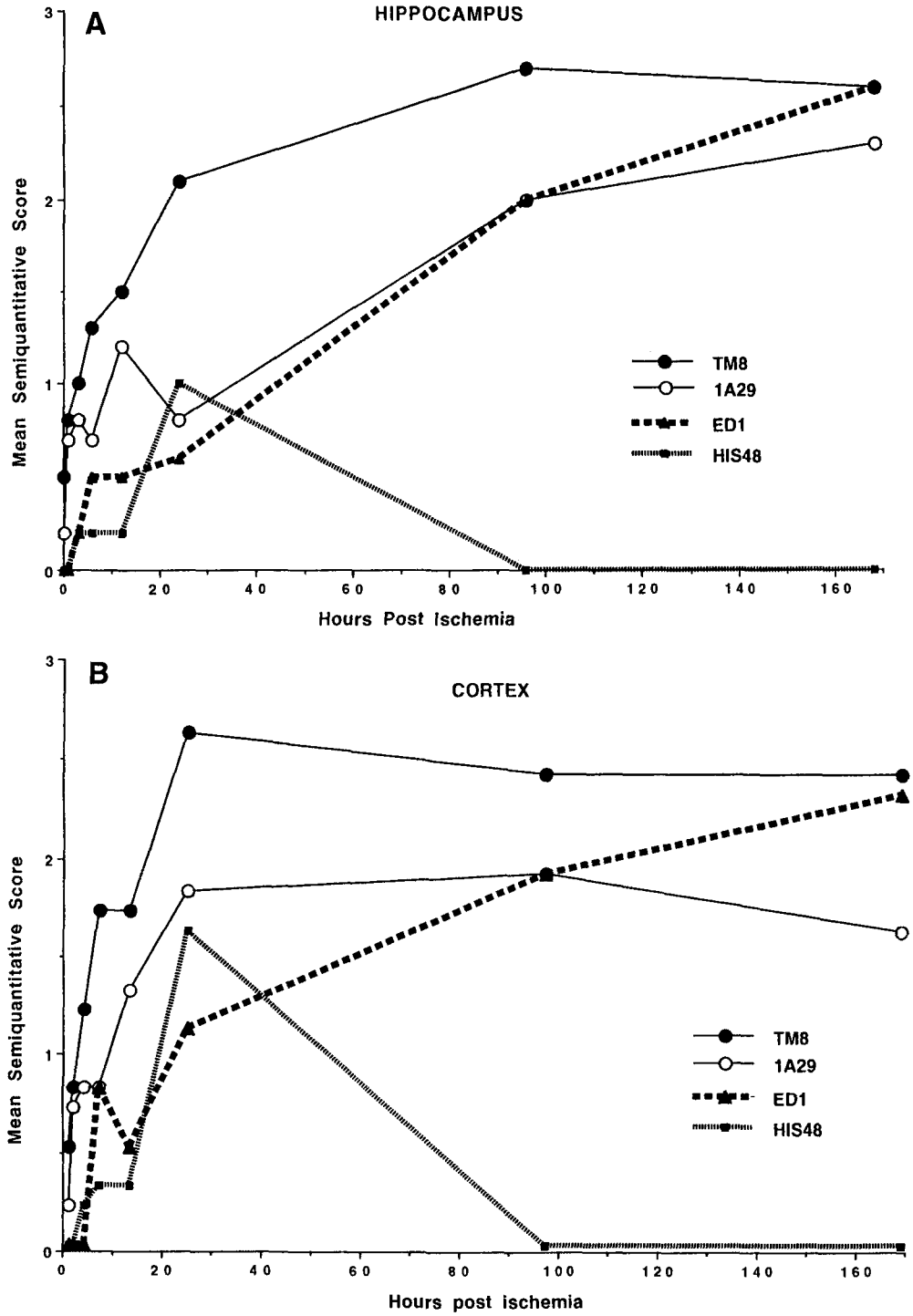

Fig. 1. Time course of ICAM-1 and leukocyte subset immunostaining in the (**A**) hippocampus and (**B**) cortex after ischemia. Values represent the mean semiquantitative immunohistochemical intensity score for all animals at each time point (n = 4).

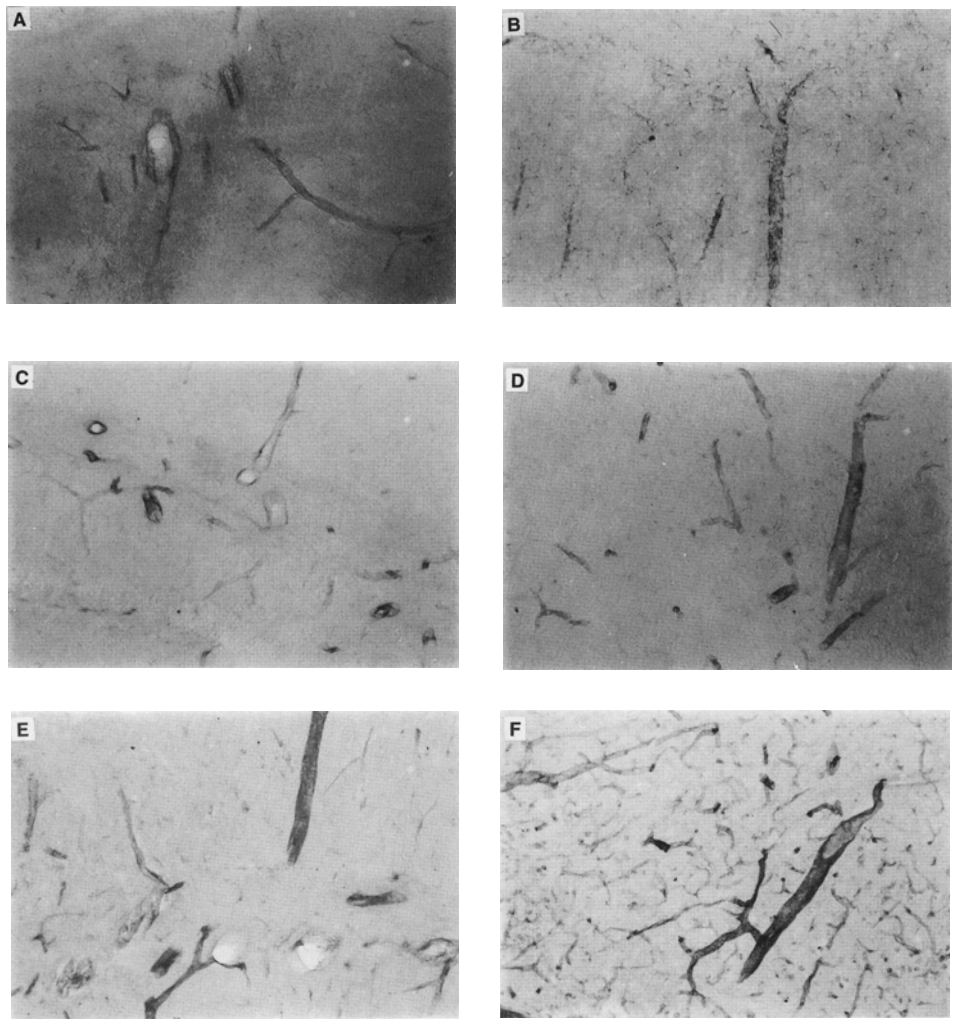

Fig. 2. Anti ICAM-1 immunohistochemistry: **TM8** 1:600 dilution. (**A**) 6 h postischemia hippocampus. (**B**) 6 h postischemic cortex. (**C**) 12 h postischemia hippocampus. (**D**) 12 h postischemia cortex. (**E**) 1 d postischemia hippocampus. (**F**) 1 d postischemia cortex.

In the hippocampus, ED1-positive cells are present primarily in the CA1 pyramidal cell layer, the oriens and stratum radiatum, and the dentate gyrus, with the largest number of cells seen in the CA1 pyramidal layer. The ED1-positive cells have now assumed an ameboid morphology with stout processes, similar to that of activated microglia cells (Figs. 3F vs 5A and 3I vs 5B). Double label staining with TM8 and ED1 revealed that areas with ED1 staining also had congruent ICAM-1 staining in the microvessels (Fig. 6).

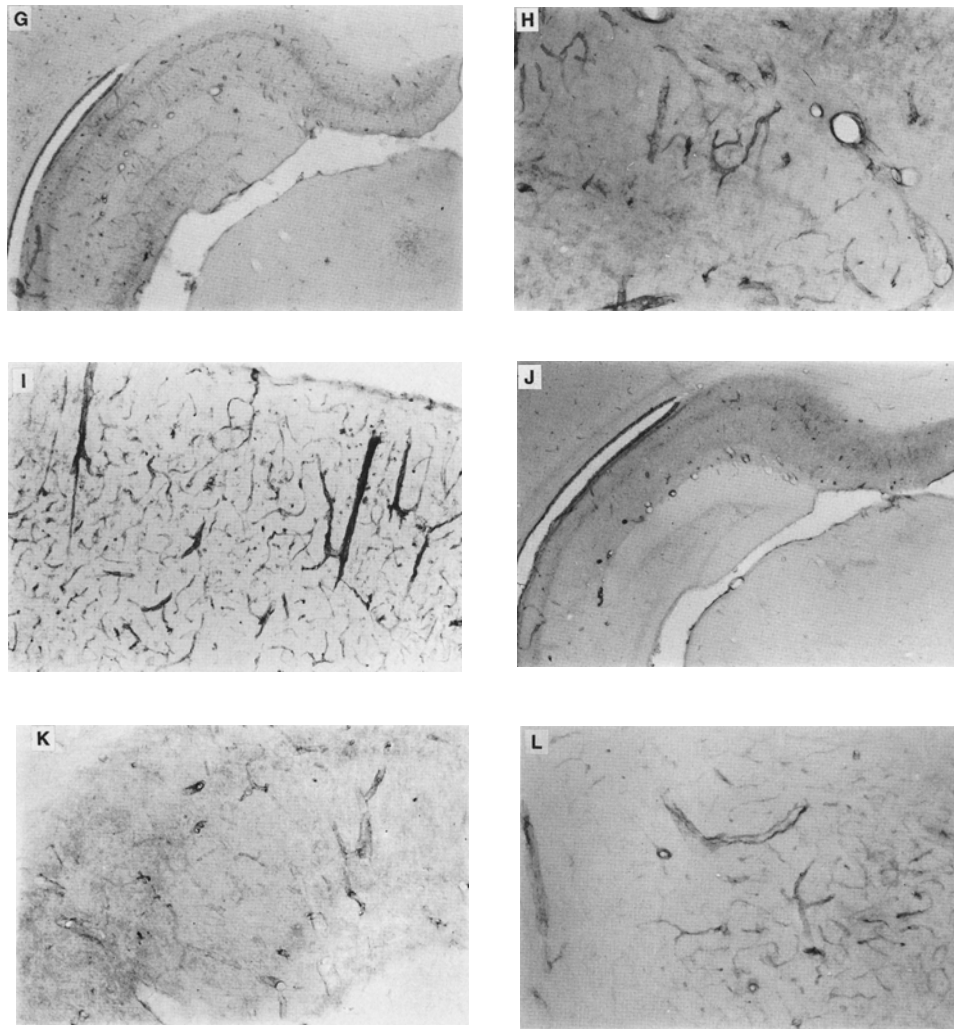

Fig. 2. (*cont'd*). (**G**) 4 d postischemia hippocampus. (**H**) 4 d postischemia hippocampus. (**I**) 4 d postischemia cortex. (**J**) 7 d postischemia hippocampus. (**K**) 7 d postischemia hippocampus. (**L**) 7 d postischemia cortex.

Semiquantitative results of His48 staining are shown in Table 1 and Fig. 1, with representative sections shown in Fig. 4. No His48-positive cells were seen in sham controls. In both hippocampus and cortex, significant granulocyte infiltration occurs only at 24 h postischemia (Figs. 4A,B). His48-positive cells were evenly distributed between the lumen of microvessels and the corresponding parenchyma. No His48-positive cells were seen at 4 and 7 d postischemia.

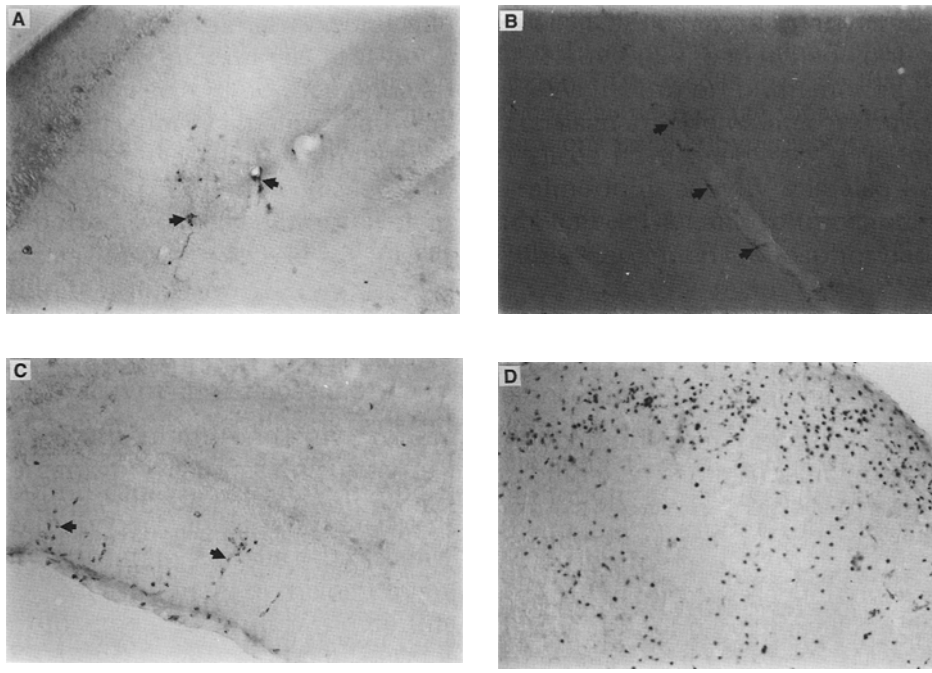

Fig. 3. Anti monocyte/macrophage immunohistochemistry: **ED1** 1:1000 dilution. (**A**) 6 h postischemia hippocampus (arrows). (**B**) 6 h postischemia cortex (arrows). (**C**) 24 h postischemia hippocampus (arrows). (**D**) 24 h postischemia cortex.

Immunocytochemical Distribution of Microglia

In sham controls, 1, 3, 6, 12, and 24 h postischemia brains, scattered OX42 positive cells are evenly distributed throughout the cortex and hippocampus (Figs. 5A,B). At 4 and 7 d postischemia, there was a dramatic increase in OX42 positive cells in the CA1, CA2, and CA3 pyramidal layers, stratum radiatum, oriens, and dentate of the hippocampus (Fig. 5C), and in ischemic areas of the cortex (Fig. 5D).

Immunocytochemical Distribution of Neurons

We performed qualitative assessments of neuronal changes using thionin staining. No evidence of neuronal loss was seen at time points up to 24 h. In contrast, at 4 and 7 d there was a marked decrease in thionin staining in the CA1 pyramidal layer implying a loss of viable neurons (Figs. 7A,B). This area of neuronal loss in the hippocampus corresponds to the area that exhibits increased ED1 staining at these time points (*see* Fig. 3E).

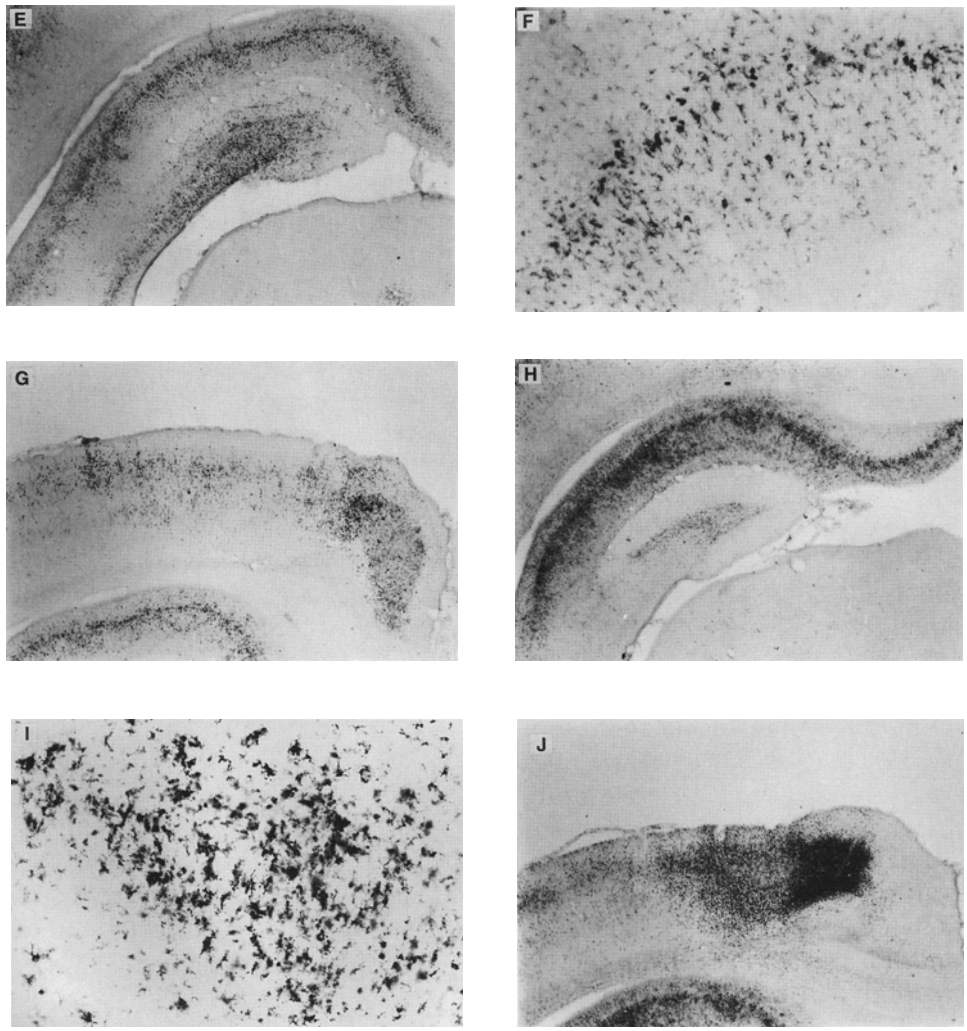

Fig. 3. (*cont'd*). (**E**) 4 d postischemia hippocampus. (**F**) 4 d postischemia hippocampus. (**G**) 4 d postischemia cortex. (**H**) 7 d postischemia hippocampus. (**I**) 7 d postischemia hippocampus. (**J**) 7 d postischemia cortex.

DISCUSSION

This study found that following CNS ischemia, ICAM-1 expression occurs early in microvessels with delayed parenchymal expression, and that this expression correlates both temporally and topographically with leukocyte accumulation.

Our study found that ICAM-1 expression occurs very early following CNS ischemia. Both 1A29 and TM-8 demonstrated increased ICAM-1

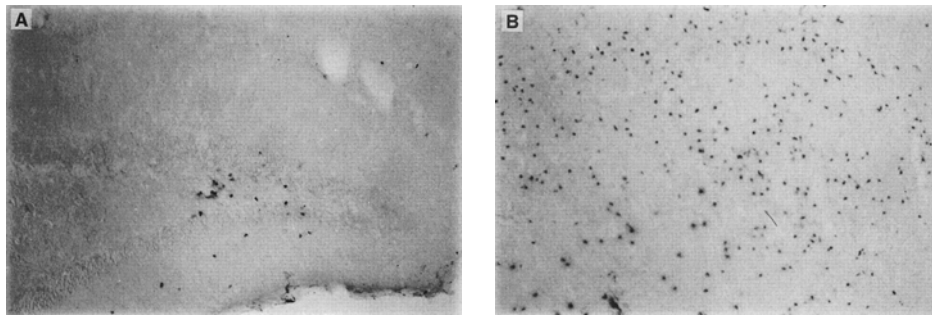

Fig. 4. Antigranulocyte immunohistochemistry: **HIS48** 1:50 dilution. (**A**) 24 h postischemia hippocampus. (**B**) 24 h postischemia cortex.

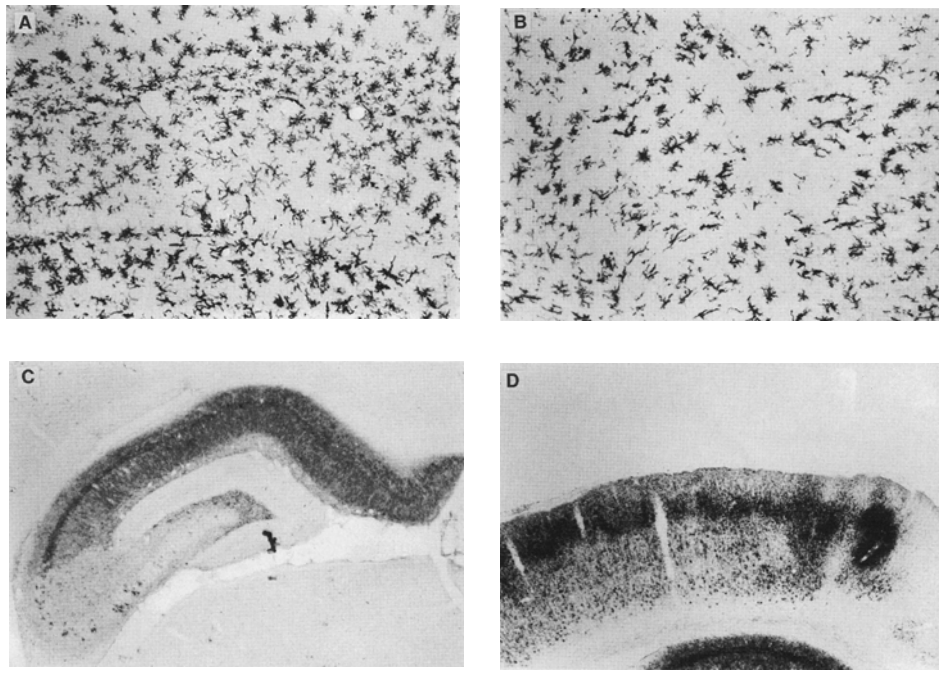

Fig. 5. Antimicroglia immunohistochemistry: **OX42** 1:1600 dilution. (**A**) 24 h postischemia hippocampus. (**B**) 24 h postischemia cortex. (**C**) 7 d postischemia hippocampus. (**D**) 7 d postischemia cortex.

staining beginning at 1 h although the TM-8 increase was not significant until 6 h because of higher baseline staining (*see* Table 1). This time course is similar to that seen in in vitro studies using human brain endothelial cells where ICAM-1 expression increased by 4 h following cytokine (IL-1) exposure and persisted for 72 h (Wong and Dorovini-Zis, 1992). Hypoxia with reoxygenation has also been shown to increase ICAM-1 expression in in vitro human brain endothelial cell cultures with significant increases

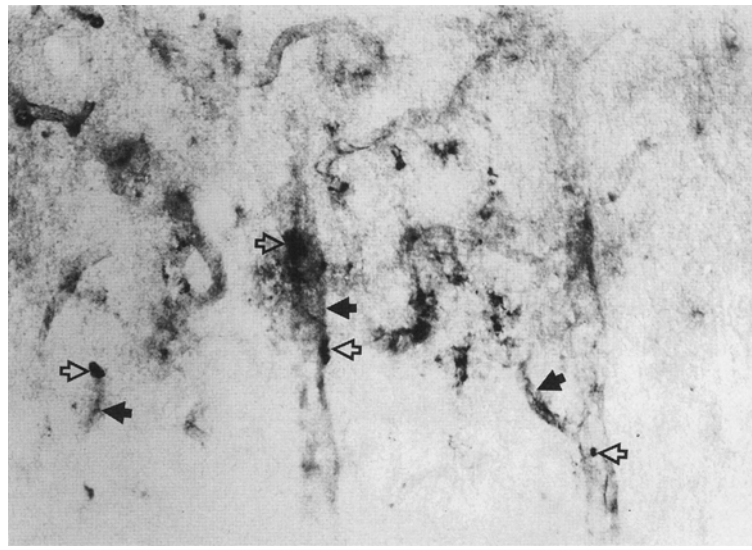

Fig. 6. Double label immunohistochemistry: **ED1** (1:1000) (open arrow)/ **TM8** (1:600) (closed arrow) 4 d postischemia cortex.

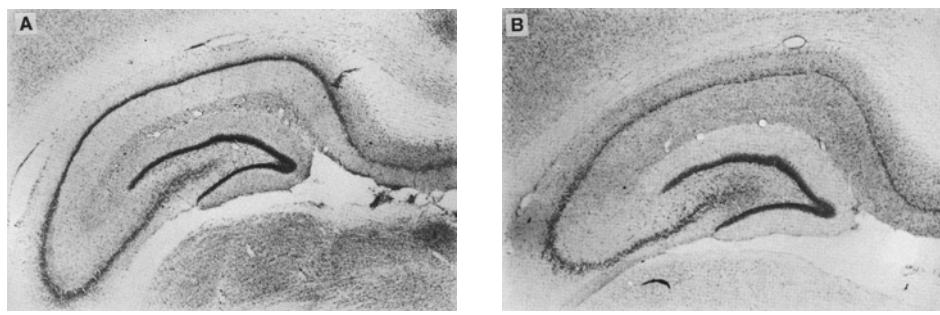

Fig. 7. Thionin staining: (**A**) 24 h postischemia hippocampus. (**B**) 4 d postischemia hippocampus.

seen at 4, 12, and 24 h (Hess et al., 1994). The very early ICAM-1 expression seen in our study is also similar to the findings of a recent experimental CNS ischemia study. In a primate MCA occlusion model, Okada et al. (1994) found a significant increase in ICAM-1 expression in brain microvessels at 1 and 4 h postreperfusion that returned to baseline at 24 h. This suggests that the stimuli required to upregulate ICAM-1 expression appear very early following reperfusion. Unlike the transient expression in the Okada study, we found persistent ICAM-1 expression in microvessels for up to 7 d. Both the number of involved vessels and the intensity of staining shows a marked increase between 1 and 4 d. Potential reasons for the difference in the duration of ICAM-1 expression between the studies

include differences in ICAM-1 assessment techniques (qualitative vs quantitative), species, antibodies, ischemic models, and the fact that the Okada study did not examine time points beyond 24 h. Our findings must also be compared with the results of the Jander et al. study (1995), which found persistent ICAM-1 expression beginning at 24 h with associated macrophage accumulation seen at 48 h in a focal nonreperfusion model. Similarities to our study include the findings of persistent ICAM-1 expression and extensive associated macrophage accumulation as assessed by ED1 staining. The main difference is that both the ICAM-1 and ED1 staining occurred later in their study. Possible explanations for this difference include the finding that leukocyte adhesion appears to be more important/robust in reperfusion injury (Kochanek and Hallenbeck, 1992), therefore ICAM-1 expression and leukocyte infiltration should be detected earlier in transient CNS ischemia models, and the fact that the Jander study did not assess 6- and 12-h time points.

Our results suggests that in our model the stimuli that induces ICAM-1 expression may persist for up to 7 d. The time frame of this delayed ICAM-1 expression corresponds to the period of delayed neuronal injury described in previous experimental CNS ischemic studies (Kirino et al., 1982; Petito et al., 1982; Jacobs et al., 1987). The finding that ICAM-1 expression continues to increase could have significant therapeutic implications. An agent that blocks leukocyte infiltration by targeting the ICAM-1 receptor may need to be administered for at least 4 d to produce maximal neuroprotective efficacy. The time course of ICAM expression in clinical stroke has not been determined. In the two stroke cases reported, ICAM staining was seen in 100% of the microvessels within the infarct zone at 2 and 5 d (Sobel et al., 1990).

Our study found that in the first 24 h ICAM expression appears to be confined to vessels. Both microvessels and larger 100-μm vessels were involved. These larger vessels morphologically appeared to be venules, although we can not exclude some arteriolar involvement. Previous intravital microscopic studies have demonstrated that leukocyte adherence takes place in postcapillary venules and not in arterioles (Lehr et al., 1993). Although this ICAM-1 expression persists on the vessels at 4 and 7 d, there also appears to be additional diffuse parenchymal involvement. The pattern of ICAM-1 staining was similar to that seen with a macrophage (ED1) antibody. This suggests that some macrophages or reactive microglia may be expressing ICAM-1 in these areas. Previous studies have suggested that ICAM-1 is expressed on cells of multiple lineages in the CNS, including microglia (Sobel et al., 1990; Wilcox et al., 1990). It is possible that ICAM-1 receptors on activated microglia could aide in the recruitment of additional leukocytes to the area of ischemia. The diffuse parenchymal ICAM-1 staining may also reflect soluble ICAM-1 accumulated in tissue related to shedding (Rothlein et al., 1991).

Our ICAM-1 results were confirmed using two separate ICAM-1 monoclonal antibodies. 1A29, A commercially available antibody has been used

in all prior published rat CNS ischemia studies. It is felt to block the Mac-1 domain on ICAM-1 (Zhang, 1994). TM8 is a newly developed antibody that appears to block the LFA-1 ICAM-1 interaction (Athena, unpublished report). In our study, the time course and distribution of ICAM-1 expression was similar for the two antibodies ($r > 0.88$). However, for all animals TM8 gave a more intense stain, even with ideal antibody concentrations.

Previous histologic studies have demonstrated that leukocytes are present early in CNS ischemic injury (Pozzilli et al., 1985; Kochanek and Hallenbeck, 1992; Garcia et al., 1994). The majority of these studies used standard H&E to estimate the cell type of the leukocyte infiltration. Although it is generally believed that granulocytes appear within the first 24 h and that mononuclear cells accumulate after this (Garcia and Kamijyo, 1974; McCormick, 1983) the actual time course of leukocyte subset accumulation is not well established. To our knowledge, ours is the first study that used specific immunohistochemistry to differentiate between granulocyte and mononuclear cell infiltration. Our study found monocyte/macrophage accumulation within 6 h in areas with concurrent ICAM-1 expression of vessels confirmed by double-label staining. The morphology of these cells was consistent with individual mononuclear cells. This suggests that these cells represent demarginated blood monocytes. Previous studies have not found significant mononuclear cell infiltration until 12–48 h in focal cerebral ischemia (Garcia et al., 1994; Jander et al., 1995) and 16 h in spinal cord ischemia (Giulian and Robertson, 1990). The early appearance in our study may reflect the greater sensitivity of immunohistology to identify cells. Early infiltration of blood monocytes has been observed in other diseases with accumulations seen at 1 h in dermal inflammation (Issekutz et al., 1981). By 4 d we observed a large number of ED1-positive cells in ischemic areas. The pattern and morphology of these cells was similar to that seen with a microglia marker, suggesting that these cells represent transformed microglia. Whether macrophage accumulation represents infiltration or transformation of resident cells is a subject of debate (Giulian and Robertson, 1990; Kochanek and Hallenbeck, 1992). Our finding suggest that both processes may play a role, depending on the time point. However, further studies using antibodies that block monocyte infiltration will be needed to answer this question.

The relationship between macrophage/microglia reaction and neuronal changes in reversible forebrain ischemia previously has been studied extensively by Morioka et al. (1991) and Gehrmann et al. (1992). In agreement with these studies, we found early accumulation of mononuclear cells/activated microglia prior to any morphologically apparent ischemia-induced neuronal damage. At later time points, the areas of increased ED1 staining corresponded to areas of apparent neuronal loss (Figs. 3E vs 7B).

Our study found significant granulocyte infiltration only at 24 h, with no evidence of granulocytes after 24 h. Previous studies have found evidence of very early granulocyte involvement, with plugging of the micro-

circulation demonstrated within 1 h following brain ischemia (del Zoppo et al., 1991). Hallenbeck et al. (1986) found ^{111}In-labeled granulocyte accumulation by 4 h in a canine stroke. The reason that our study did not detect significant accumulation before 24 h may be because of different ischemic models and also because we were examining infiltration and not just vessel plugging.

In conclusion, our study found early and persistent ICAM-1 expression with associated early and persistent mononuclear cell accumulation. If further studies confirm that mononuclear cells potentiate CNS ischemic injury, our results suggest that future antiadhesion therapies may need to be given for up to 7 d for maximal beneficial effect.

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