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Endogenous Interleukin-1 Receptor Antagonist is Neuroprotective

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Interleukin-1 (IL-1) has been implicated in chronic and acute cerebral neuropathologies. IL-1 receptor antagonist (IL-1ra), a naturally occurring protein that binds to IL-1 receptors without inducing signal transduction, blocks several actions of IL-1. IL-1ra acts at the local level and it also circulates in the bloodstream. We now report evidence for a biological function of IL-1ra in the brain as an endogenous neuroprotective molecule. Cerebral expression of IL-1ra mRNA is induced rapidly by focal cerebral ischemia in rats, and inhibition of the action of IL-1ra, by passive immunoneutralization, markedly enhances ischemic damage. To our knowledge this is the first report of an action of endogenous IL-1ra in the brain. Control of IL-1ra expression or action may therefore provide a useful therapeutic strategy to limit acute neurodegeneration.

Interleukin-1 (IL-1) exerts direct actions on the central nervous system (see 1 for review), mediated via specific receptors (2-4). IL-1 is present in normal adult brain and it is constitutively expressed at low levels in specific brain areas (5, 6), but is markedly induced by traumatic (7, 8) ischemic (9) or excitotoxic (10) injury in the rat. It has been proposed that excessive IL-1 production contributes to acute and chronic cerebral neurodegeneration (11-15).

IL-1 receptor antagonist (IL-1ra) is a member of the IL-1 family, which acts as a selective, competitive receptor antagonist for IL-1 receptors (16). Recombinant IL-1ra inhibits or abolishes most known actions of IL-1 *in vitro* and *in vivo* in many species including humans (16). In peripheral tissues, endogenous IL-1ra is synthesized by similar cell types to IL-1, and appears to be produced in response to the same stimuli that activate IL-1 expression, although its synthesis is usually delayed compared to that of IL-1 (16). Endogenous peripheral IL-1ra is secreted into the peripheral circulation (17). A recent report (18) demonstrated that in peripheral tissues neutralization of endogenous IL-1ra exacerbates inflammatory bowel disease in the rabbit, but no function has yet been identified for IL-1ra in the brain.

We now propose that IL-1ra acts in the brain as an endogenous inhibitor of IL-1 action and of neurodegeneration. In order to test this hypothesis, expression of IL-1ra mRNA was assessed in rat brain in response to permanent focal cerebral ischemia, induced by middle cerebral artery occlusion (MCAo) (19). The potential function of brain IL-1ra was investigated by testing the effects of blocking its action on neuronal damage induced by MCAo.

MATERIALS AND METHODS

Studies were approved by the University of Manchester under British Home Office law and by the National Institute of Mental Health Animal Care and Use Committee.

Male Sprague–Dawley rats (240-260 g) were used in all experiments. MCAo was induced by permanent unilateral occlusion of the middle cerebral artery proximal to the lenticulo-striate branch, in halothane-anaesthetized rats (19). Sham operated animals were submitted to exposure of the artery only.

IL-1ra mRNA was detected by in situ hybridization in 15 μm thick frozen brain sections, as previously described by Wong *et al.* (20). Ribonucleotide probes were generated from the rat IL-1ra cDNA (provided by Dr Ronald Hart, Rutgers University, Newark, NJ) (21). Probes (~500 nt) were directed against the 5′ end of the rat IL-1ra sequence (21). IL-1ra insert was sequenced by the dideoxynucleotide chain termination method (22), and had no homology to other sequences deposited at Genbank. Transcription was carried out using the Riboprobe System (Promega Biotech) in the presence of [α-^{35}S]-UTP (specific activity: 1000-1500 Ci/mmol, NEN). Controls were gen-

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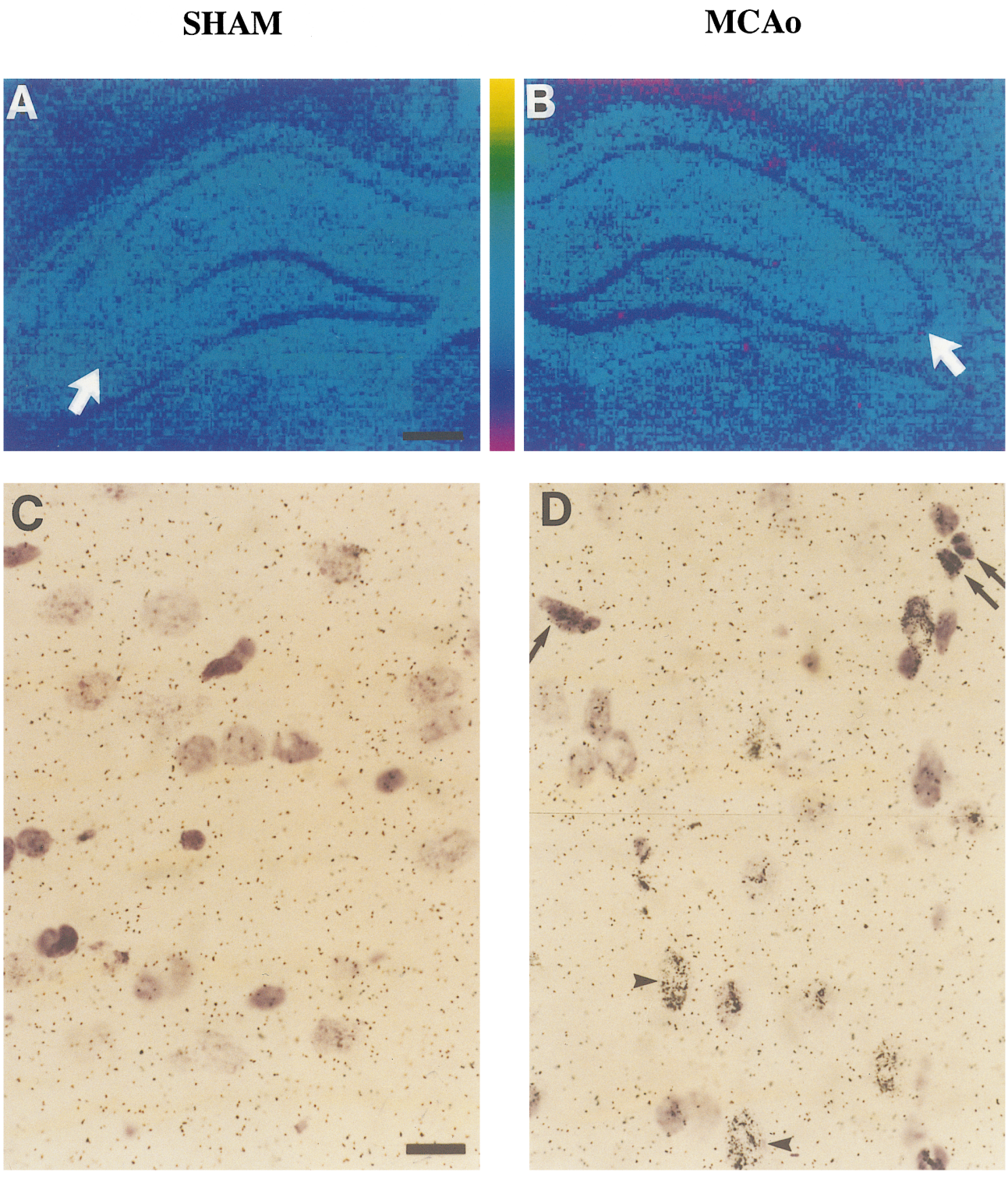

FIG. 1. Early induction of IL-1ra gene expression in focal ischemia. (A) Darkfield photomicrographs of IL-1ra mRNA hybridization in hippocampus of sham-operated animals (scale bar, 500 μm). (B) 60 min after MCAo, IL-1ra mRNA levels were increased in the pyramidal cell layer, particularly in the CA_3 region (arrows). Color scale is in the centre of the figure; top colors represent low, and bottom colors represent high hybridization levels. (C) Brightfield photomicrographs of IL-1ra mRNA hybridization in cortex of sham-operated rats. Note that there is only background hybridization (scale bar, 10 μm). (D) Brightfield photomicrographs of IL-1ra mRNA hybridization in cortex 60 min after MCAo. Note that deposits of silver grain overlying IL-1ra mRNA (black dots in the picture) are found either tightly packed in glial-like cells (arrows), which are densely counterstained with Cresyl violet and have relatively small nucleus, or less abundantly in neuron-like cells (arrowheads). Photomicrographs are representative of the results observed in 6–8 rats per group.

erated by using either excess cold probe (100×) or labeled sense probe.

Hybridization and post-hybridization treatments were carried out concomitantly on all sections. Anatomical localization of the probe at the cellular level was performed by dipping the slides in NTB-2 nuclear track emulsion, exposing for 8 weeks, developing in D19, and counterstaining with Cresyl violet. IL-1ra mRNA levels were semi-quantitatively assessed with the use of the NIH Image Program (version 1.55) (23). Images were digitized in a Macintosh computer, background was subtracted and the number of silver grains overlying individual cells was counted in ten different cortical fields per group. Values were averaged and expressed as percent of control (sham-operated) animals.

Blocking of endogenous brain IL-1ra was achieved by passive immunoneutralization in vivo. Rabbit pre-immune serum or rabbit anti-rat IL-1ra antiserum was injected (5.0 μl) into the lateral ventricle of rats (icv), 18 h and 30 min before MCAo, via cannulae implanted seven days prior to experiment. The anti-IL-1ra antiserum binds to rat IL-1ra but not related proteins (unpublished data) and binding to rat brain is blocked by preadsorption with rat IL-1ra (Toulmond, personal communication). Animals were killed by decapitation 24 h after MCAo, and 500 μm fresh coronal brain sections were stained with tetrazolium for measurement of the area of brain damage in each section. An "indirect" approach was used to compensate for the effect of edema. Total volume of damage was calculated by integration of these areas (see ref. 13).

All data are presented as mean values ± SEM. Statistical analysis was performed unpaired Student's *t*-test.

RESULTS

IL-1ra mRNA expression, assessed by *in situ* hybridization, was detectable in the hippocampus of sham-operated rats (fig. 1A), but was markedly increased 60 min after MCAo (fig. 1B), particularly in the CA_3 layer, where no signal was observed after sham-surgery. Because no signal was observed in the CA_3 layer after sham surgery a quantitative comparison between sham and MCAo groups was not performed for the hippocampus. In the cortex of sham-operated animals, a weak background hybridization signal was observed (fig. 1C). In rats sacrificed 60 min after MCAo (fig. 1D), signal induction was observed and localized in scattered neurone-like and glia-like cells.

An assessment of silver grain counts overlying IL-1ra mRNA in rat cerebral cortex shows that after MCAo the numbers of silver grains overlying IL-1ra mRNA were 470% higher than those in sham-operated animals ($P < 0.0001$) (Fig. 2). Control hybridization of adjacent sections with labelled IL-1ra sense riboprobes produced a uniform, low signal, which was barely visible on film. Excess cold probe (100 ×) was also used as control, and yielded only background hybridization (not shown).

The hypothesis that endogenous brain IL-1ra influences neurodegeneration was tested by blocking its action by passive immunoneutralization with anti-IL-1ra antiserum. ICV injection of preimmune serum had no effect on infarct volume compared to untreated animals (data not shown). Similarly in separate studies, we have shown that antiserum from animals immunized

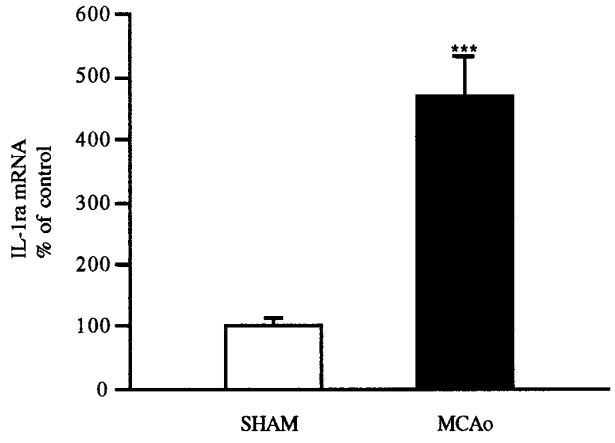

FIG. 2. Effects of MCAo on silver grains overlying cerebral cortical IL-1ra mRNA. Levels are expressed as % of control. Mean values ± SEM ($n = 6$); ***, $P<0.0001$.

with irrelevant antigens does not affect damage caused by MCAo (Loddick & Rothwell, unpublished observations). In marked contrast, ICV injection of rabbit anti-rat IL-1ra antiserum (5 μl, 18 h and 30 min before surgery) dramatically enhanced infarct size measured 24h after MCAo (Fig. 3A, 3B); this time was chosen as infarct volume is maximal at 24 h after MCAo. The exacerbation of brain damage was apparent at all levels throughout the lesion (Fig 3A). In rats subjected to MCAo, total infarct volume (v) was increased by 71% in IL-1ra antiserum treated animals (v = 175.0 ± 12.3 mm^3, n=7), compared to pre-immune serum treated animals (v = 102.0 ± 5.5 mm^3, n= 8, $P < 0.001$) (Fig. 3B). No apparent histological damage was observed after icv infusion of IL-1ra antiserum in non-operated rat brain, and icv infusion of rabbit pre-immune serum did not modify the extent of damage caused by MCAo (data not shown).

DISCUSSION

Our data indicate that IL-1ra gene expression, previously identified in normal rodent brain (24, 25), is induced rapidly after ischemic damage, and that inhibition of endogenous brain IL-1ra actions by icv treatment with IL-1ra antiserum markedly enhances ischemic damage. Our separate studies reveal that expression of IL-1ra proteins (assessed by immunocytochemistry) is markedly induced by brain injury in the rat in the same pattern as IL-1ra mRNA (26). Therefore, we propose that IL-1ra is a potent endogenous inhibitor of ischemic brain damage.

In view of the specificity of IL-1ra, it is likely that its actions on experimentally induced neurodegeneration reflect inhibition of the effects of brain IL-1. Both of these molecules are induced rapidly after damage to the brain, and are expressed in resident brain cells,

but the mechanisms controlling their expression are largely unknown. It has been shown that IL-1 induces IL-1ra expression in monocytes (27) and fibroblasts (28). Furthermore, it has been suggested that IL-1-induced resistance to gram-negative infections in granulocytopenic mice is due in part to an increase in IL-1ra expression, stimulated by pre-treatment with IL-1 (29).

Yabuuchi *et al.* have shown that IL-1β gene expression is induced in the brain 30 min after the onset of ischemia (30). Our own *in situ* hybridization studies yielded similar results to those reported by Yabuuchi *et al.* (31), indicating that the increase in IL-1ra mRNA levels after brain injury occurs at 60 min and follows that of IL-1β by 30 min (unpublished data). It is therefore possible that increased IL-1ra expression after brain injury is a natural response to an increase in cerebral IL-1 levels, and that IL-1ra acts as an inhibitor of IL-1 actions and facilitates restoration of physiological conditions.

The locus of action of IL-1 in the events leading to neuronal death is not known. Toxic effects of IL-1, and inhibition by IL-1ra, may be due to actions on non-neuronal cells such as glial and endothelial cells, leading to production of neurotoxic factors, and/or direct actions on neurons. *In vitro* experiments have suggested that IL-1 could be a modulator of neuronal and glial growth during development, and could also control neuronal plasticity in the adult brain (see 1 for review). It has also been shown that IL-1 induces nerve growth factor (NGF) expression by astrocytes (31), suggesting a potential beneficial effect of IL-1 for neuronal regrowth after brain injury. Nevertheless, IL-1 also stimulates astrocytes to produce potentially neurotoxic substances such as nitric oxide (32, 33) and arachidonic acid metabolites (1). Furthermore, intracerebroventricular infusion of IL-1 increases ischemic brain damage (14), whilst inhibition of endogenous IL-1 reduces ischemic damage. It is therefore likely that, while low (physiological) levels of IL-1 allow the maintenance of neuronal plasticity in the adult brain, excessive and sustained production of IL-1, which can occur within 30 min following brain injury, dramatically impairs not only neuronal, but also non-neuronal cell functions. We have previously shown that the gene encoding for IL-1ra is expressed in neurons in non-pathological conditions (24); neurons may therefore exhibit some resistance to direct, neurotoxic actions of IL-1. Thus, the level of constitutive IL-1ra expression and the rate and extent of its synthesis after damage may significantly influence the sensitivity of specific neurons to insults which induce IL-1 synthesis. Understanding the mechanisms of induction of IL-1ra and IL-1 expression in the brain after injury may therefore offer therapeutic benefit for the treatment of neurodegenerative disorders.

To our knowledge this is the first report of an action of endogenous IL-1ra in the brain, where it acts as a potent neuroprotective molecule. The search for endogenous neuroprotective molecules represents a promising therapeutic strategy for neurodegeneration.

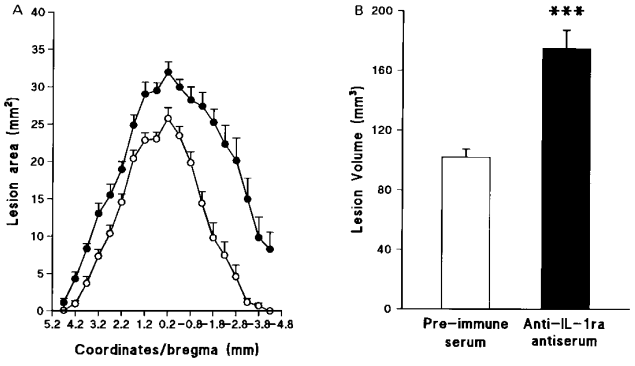

FIG. 3. Exacerbation of brain damage by passive neutralization of endogenous IL-1ra. (A) Blocking endogenous IL-1ra action by icv injection of anti-rat IL-1ra antiserum before MCAo markedly exacerbated damage measured 24 hours after surgery when compared to icv injection of pre-immune serum. This exacerbation was apparent at all levels throughout the lesion (-●-●- pre-immune serum, and -○-○- anti-rat IL-1ra anti serum). (B) Total infarct volume (*v*) was increased by 71% in IL-1ra antiserum-treated animals (*** $P < 0.001$), compared to pre-immune serum-treated animals.

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