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Interleukin-10 modulates neuronal threshold of vulnerability to ischaemic damage

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Abstract

Interleukin-10 (IL-10) is a powerful suppressor of cellular immune responses, with a postulated role in brain inflammation. First, we have evaluated the role of this cytokine in ischaemic brain damage using IL-10 knockout (IL-$10^{-/-}$) mice. The middle cerebral artery (MCA) was occluded in either IL-$10^{-/-}$ or wild-type animals of corresponding strain (C57Bl/6) and age. Infarct volume was assessed 24 h later in serial brain sections. Brain infarct produced by MCA occlusion was 30% larger in the IL-$10^{-/-}$ than in wild-type mice (21.8 ± 1.2 vs. $16.9 \pm 1.0\,mm^3$, respectively; $P < 0.01$; Student's *t*-test). To further characterize these findings, studies were extended to *in vitro* models. Primary neuronal cortical cultures derived from IL-$10^{-/-}$ animals were more susceptible to both excitotoxicity and combined oxygen–glucose deprivation compared with cell cultures from wild-type mice. Moreover, when added to the culture medium, recombinant murine IL-10 (0.1–100 ng/mL) exerted a concentration-dependent prevention of neuronal damage induced by excitotoxicity in both cortical and cerebellar granule cell cultures taken from either strain. The accordance of *in vivo* and *in vitro* data allows us to suggest a potential neuroprotective role of IL-10 against cerebral ischaemia when administered exogenously or made available from endogenous sources.

Introduction

The brain is capable of mounting a pronounced inflammatory response to injury which is manifested in part by gliosis and driven by both centrally and peripherally derived immune cells. Such a response is the result of the orchestrated expression of the inflammatory and immune system gene products, which are also operative in the periphery, including cytokines, chemokines, inflammatory enzymes and adhesion molecules. The significance of the inflammatory response to brain injury is not fully understood although evidence has emerged to suggest that this reaction may be causally related to both neuronal damage and recovery.

Acute inflammation induced by cerebral ischaemia has provided a fertile ground for exploration of the contribution of inflammatory mediators to brain damage (Feuerstein *et al*., 1996, 1998). In particular, most of the research in this area has been focused on pro-inflammatory mechanisms, e.g. interleukin (IL)-1β, involved in this complex response (Rothwell & Hopkins, 1995; Merrill & Benveniste, 1996), hence contributing to a potentially fruitful area of therapeutic development. Less attention has been given to understanding the relevance of endogenous anti-inflammatory mechanisms that might counteract the inflammatory component associated with neurodegeneration and so representing additionally attractive therapeutic targets. Interleukin-10 (IL-10) represents such a candidate molecule. In the periphery, the cytokine is a powerful suppressor of various aspects of the cellular immune response. In particular, it specifically inhibits the synthesis of several pro-inflammatory cytokines by T cells and macrophages, including IL-1, IL-2, IL-6, tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), whilst inducing secretion of interleukin-1 receptor antagonist (IL-1ra), a competitive inhibitor of IL-1 activity (Kline *et al*., 1995; Fuchs *et al*., 1996). Recently, activated microglia have been shown to be a major source of IL-10 in the brain (Williams *et al*., 1996). Within a few hours following an ischaemic attack, increased levels of IL-10 in the cerebrospinal fluid (CSF) of patients have been observed (Tarkowski *et al*., 1997). In addition, high levels of IL-10 are also detected in experimental animal models of focal ischaemia (Zhai *et al*., 1997). More recently, Spera *et al*. (1998) have reported that exogenous administration of IL-10 induces neuroprotection in a rat model of cerebral focal ischaemia.

The aim of the present study was to investigate the role of endogenous IL-10 on the outcome of brain damage induced by cerebral ischaemia. For this purpose, we have taken advantage of the availability of mutant mice in which the IL-10 gene is inactivated by targeted gene disruption (Rennick *et al*., 1995, 1997). IL-$10^{-/-}$ mice have proved useful in defining the physiological relevance of the cytokine as an essential anti-inflammatory and immunosuppressant agent in the periphery, and in particular in the gastrointestinal tract (Kühn *et al*., 1993). Following permanent occlusion of the middle cerebral artery (MCA) we have measured the extension of necrosis, compared with wild-type mice of corresponding strain and age. Moreover, to further characterize the role of endogenous IL-10 in neurodegeneration, we have used primary cortical cultures from IL-$10^{-/-}$ and wild-type mice to evaluate differences in susceptibility to neuronal death following either an excitotoxic stimulus or oxygen–glucose deprivation (OGD).

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Materials and methods

Mice

IL-$10^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME, USA, strain JR 2251) were generated by targeted gene disruption in a C57Bl/6–129/Ola background and backcrossed 10 times to the C57Bl/6 strain to obtain a congenic strain, carrying the mutation on a definite genetic background. The care and the use of mice for all experimental procedures were carried out in accordance with the European Communities Council directive (86/609/EEC) so as to minimize pain and discomfort for animals.

Induction of focal cerebral ischaemia

The study was carried out using adult (3–4-month-old) male mice. A group of 47 mice was IL-$10^{-/-}$. The control group was 49 wild-type C57Bl/6 mice of corresponding age (Jackson Laboratories). During the period of the experiment, the mice were kept in specific pathogen free (SPF) conditions (Charles-River animal room facility, San Raffaele Science Park, Milan, Italy). On each day of the experiment two or three mice from each group were randomly chosen for surgery. They were anaesthetized using tribromethanol, 500 mg/kg i.p. (Sigma-Aldrich Chemie, Steinheim, Germany). After a skin incision made between the eye and the ear, and transection of the temporalis muscle, the left MCA was visible through the temporal surface of the skull. Craniectomy was performed by drilling, the inner layer of the skull was removed with fine forceps, and the MCA was exposed. The artery was occluded by electrocoagulation (Samed, Merlino, Milan, Italy). Body temperature was kept at about 37 °C during the surgery by means of a rectal probe connected to a heating pad (Harvard Apparatus, Edenbridge, UK) and monitored until recovery from anaesthesia (≈ 30 min). No difference in body weight was reported between the two groups (mean body weights ± SE were 29.4 ± 0.2 and 29.8 ± 0.8 g, in IL-$10^{-/-}$ and wild-type mice, respectively). A separate group of animals was used to assess physiological parameters relevant to the focal ischaemia surgical procedure. After anaesthesia, a skin incision was made in the neck, and the right carotid artery was isolated. After cannulation, 200-μL blood samples were withdrawn. Analysis of pH and gas (pO_2 and pCO_2) was carried out using a gas analyser (Stat Profile 5, Nova Biomedical, Waltham, MA, USA).

Determination of infarct volume

Twenty-four hours after MCA occlusion mice were decapitated, brains rapidly removed, and coronal sections (700 μm) were cut from the frontal pole, using a vibratome (Leica VT1000E, Nublock, Germany), and incubated in a solution containing 2% w/v triphenyl tetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) in saline, at 38 °C. Eight sections for each brain were stored in formaldehyde until measurements of infarct areas were carried out using an image analyser system (Image Pro-Plus, Media Cybernetics, Silver Spring, MD, USA). Infarct volume was obtained by multiplying each area by slice thickness. All measurements were performed on a blind basis. The amount of ischaemic damage was expressed both in absolute terms (mm^3) and as a percentage of left hemisphere. Due to different individual responsiveness to ischaemic stimulus, there were brains showing either limited necrosis or no necrosis at all. This is in accordance with what is reported in literature for similar models in the rat (Bederson *et al.*, 1986). To improve the homogeneity of the samples considered for analysis, we introduced *a priori* inclusion criteria, in that only brains showing necrosis in ⩾ 50% of the slices were included in the analysis. The other brains were considered as 'non responders' to the ischaemic stimulus, and were excluded from measurements.

Immunohistochemistry

At various time points after MCA occlusion, mice were anaesthetized and transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed, postfixed for 12 h in PFA and cut on a vibratome (40-μm sections). Slices were processed free-floating following standard protocols. Primary antibody was a rat monoclonal antimurine TNFα (Upstate Biotechnology, Lake Placid, NY, USA) which recognizes the active form of the cytokine.

Cell cultures

Cerebellar granule cells were prepared according to a published procedure (Levi *et al.*, 1991). Mixed cortical cell cultures, containing both neuronal and glial elements, were prepared from IL-$10^{-/-}$ and control mice as follows. Whole cerebral neocortices were removed from fetal mice (14–16 days of gestation) taking care to discard hippocampus, striatum and most of the meninges. The tissue was then minced, incubated in 0.25% trypsin for 10 min and dissociated by trituration. The single-cell suspension obtained was plated on 35-mm dishes (750 000 cells per dish), previously coated with poly L-lysine 10 μg/mL, in minimum essential medium (MEM) with Earle's salts, supplemented with 10% horse serum, 10% fetal bovine serum, 2 mM glutamine, 21 mM glucose and 38 mM sodium bicarbonate. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. After 5 days *in vitro* (DIV), non-neuronal cell division was halted by 36 h exposure to 3 μM cytosine arabinoside and cultures were shifted to a maintenance medium identical to plating medium but lacking fetal bovine serum. Subsequent partial medium replacements were carried out once a week. Only mature cultures, after 12–14 DIV, were used for neurotoxicity experiments.

In vitro *neurotoxicity*

Excitotoxicity experiments on mature cortical or cerebellar cultures were carried out at room temperature as follows. Culture medium was removed and the cells were washed twice with Locke's solution (in mM: NaCl, 154; KCl, 5.6; $CaCl_2$, 2.3; $NaHCO_3$, 3.6; glucose, 5.6; HEPES, 5; Glycine, 10; pH 7.4) and then exposed to various concentrations of *N*-methyl-D-aspartate (NMDA) or glutamate for 15 min. Excitoxicity was blocked by washing the cells with Locke's solution supplemented with 1 mM $MgCl_2$. Finally, cells were placed in culture medium supplemented with 1% horse serum, 21 mM glucose, 38 mM $NaHCO_3$ and 2 mM glutamine, and further incubated at 37 °C for 24 h. In some experiments, the reference, noncompetitive, NMDA receptor antagonist, MK-801 (5 μM) was added to cell cultures 5 min before and during NMDA exposure. Recombinant murine IL-10 (0.1–100 ng/mL) was added to culture medium during glutamate or NMDA pulse and, after washes, added again for an additional 24 h, when cell viability was determined. Neurotoxicity-induced morphological changes were assessed by phase contrast microscopy. Under our experimental conditions, cell death occurs strictly by necrosis and under these circumstances lactate dehydrogenase (LDH) release, measured using a Cytotoxicity Detection Kit (Boehringer Mannheim, Monza, Milan, Italy) according to manufacturers' instructions, closely reflects neuronal cell death. In all experiments, cell death was expressed as percentage of total LDH release elicited by 500 μM NMDA, a concentration which results in maximal neuronal cell death without affecting glial cell viability. All values were corrected for basal LDH release in each group. In the cerebellar granule cell cultures, where cell count is a more feasable

and established procedure (glial cells being < 3–5% of total cell population), cell viability was quantified by cell counting.

For oxygen–glucose deprivation (OGD), the culture medium was completely exchanged with deoxygenated glucose-free MEM. Cultures were kept in an anaerobic chamber saturated with N_2 for 15–60 min, maintained at 37 °C. OGD was terminated by removal of the cultures from the chamber and replacement of the medium with oxygenated MEM supplemented with 1% horse serum, 21 mM glucose, 38 mM $NaHCO_3$ and 2 mM glutamine. The cultures were returned to a humidified incubator containing 5% CO_2 and atmospheric oxygen at 37 °C for 24 h. As control, sister cultures were placed for a similar time in the same medium, maintained in the incubator and then moved to MEM containing 1% horse serum for 24 h.

Data analysis

Data in text and figures are expressed as mean ± SEM. In *in vitro* experiments, data were analysed by factorial ANOVA design or Student's *t*-test. In *in vivo* studies, infarct size in the two groups was compared using Student's *t*-test for unpaired data. 'Non-responder' rate was compared using Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Effects of MCA occlusion on infarct volume in IL-10$^{-/-}$ mice

In vivo studies were undertaken to test vulnerability of IL-10$^{-/-}$ mice to focal cerebral ischaemia elicited by permanent MCA occlusion.

Thirty-nine IL-10$^{-/-}$ and 36 wild-type mice were considered for image analysis with brain lesion volume evaluated at 24 h postischaemia. Triphenyl tetrazolium chloride (TTC)-unstained area of the fixed sections was defined as infarcted. The infarct was localized to frontoparietal and temporal cortex, with no involvement of subcortical regions; there were no signs of swelling in the ipsilateral hemisphere compared with the contralateral one. The lesion volume in the IL-10$^{-/-}$ mice was 30% greater than that of the wild-type mice ($21.8 \pm 1.2\,mm^3$ vs. $16.9 \pm 1.0\,mm^3$, respectively, $P < 0.01$) (Fig. 1A). The difference between the two groups was also confirmed when necrosis was expressed as percentage of the ipsilateral hemispheric volume (14.9 ± 0.8 vs. 11.4 ± 0.6% in IL-10$^{-/-}$ and wild-type mice, respectively, $P < 0.01$). The area recruited into infarction involved the medial edge of the cortical lesion throughout the rostrocaudal extent of the infarct (Fig. 1B). Because altered physiological responses to the ischaemic event can contribute to changes of brain lesions in the mutant strain after 24 h, we have measured key physiological parameters. No difference was found between the wild-type and IL-10$^{-/-}$ mice as to the time course of the rectal temperature, which ranged between 36.8 °C and 37.4 °C during surgery and the subsequent recovery period from anaesthesia. Two groups of five IL-10$^{-/-}$ and wild-type mice, not subjected to ischaemia, were also compared for their arterial pH and blood gasses. In the control group, values were 95.4 ± 1.4 mmHg (PO_2), 33.3 ± 3.6 mmHg (PCO_2), and 7.34 ± 0.09 (pH). In the IL-10$^{-/-}$ group, values were 98.0 ± 4.2 mmHg, 35.7 ± 2.4 mmHg, and 7.32 ± 0.02, for PO_2, PCO_2 and pH, respectively. There was no difference between the two groups.

Vulnerability to excitotoxicity and OGD in cortical neuronal cultures from IL-10$^{-/-}$ mice

Primary cortical neurons were established from IL-10$^{-/-}$ and wild-type C57Bl/6 mice. Cultures at 12–14 DIV were utilized in all experiments. No difference between the two groups of cell cultures was reported in terms of morphological appearance and rate of *in vitro* maturation (data not shown). Exposure to either glutamate or NMDA and combined OGD-induced neurotoxicity are commonly used experimental paradigms for studying early mechanisms of ischaemic damage in primary neuronal cultures. The paradigm of fast excitotoxicity, namely a 15-min exposure to NMDA in the absence of magnesium, was initially utilized to compare neuronal vulnerability in cortical cultures from IL-10$^{-/-}$ and wild-type mice. Under these

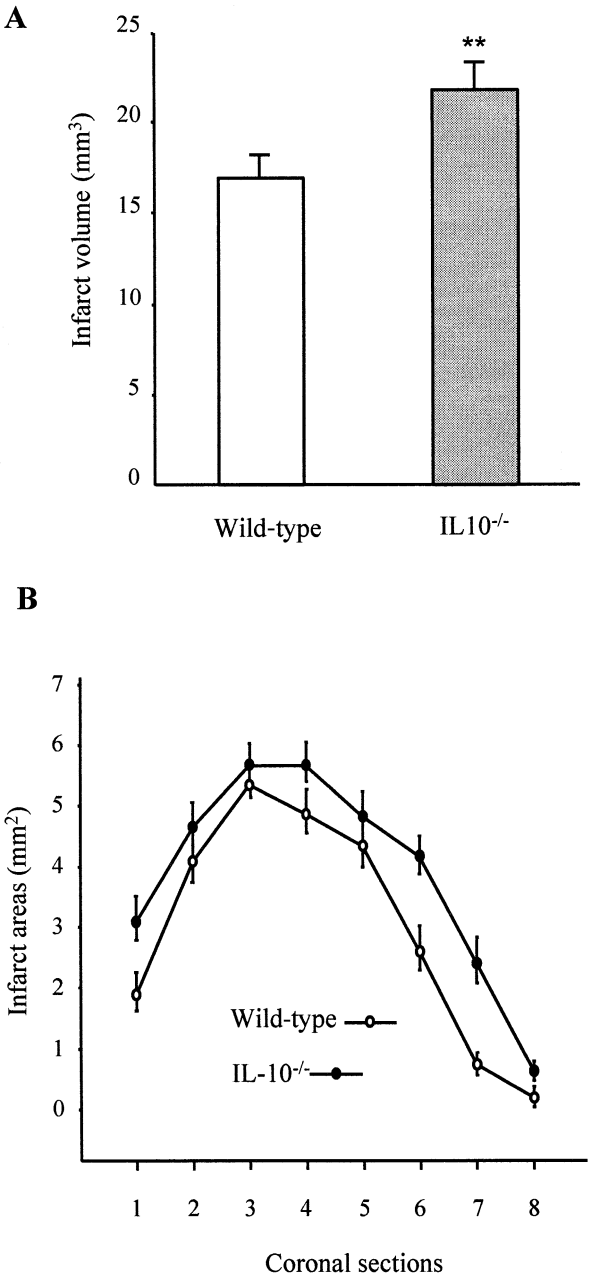

FIG. 1. Effects of permanent focal ischaemia in IL-10 $^{-/-}$ and wild-type mice evaluated as (A) infarct volume and (B) infarct areas 24 h after MCA occlusion. (A) MCA occlusion resulted in a larger infarct volume in IL-10$^{-/-}$ (grey bar) than in wild-type (open bar) mice. Values in the two groups were compared using Student's *t*-test; $^{**}P < 0.01$ vs. wild-type values. (B) Rostrocaudal distribution of the ischaemic lesion in wild-type (open circle) and IL-10$^{-/-}$ (filled circle) mice. A total of 92 animals were lesioned. Eleven mice in the control group (23%) and six mice in the IL-10$^{-/-}$ group (13%) were 'non responders' and were excluded from the analysis. Responders were 36 and 39 for wild-type and mutant mice, respectively.

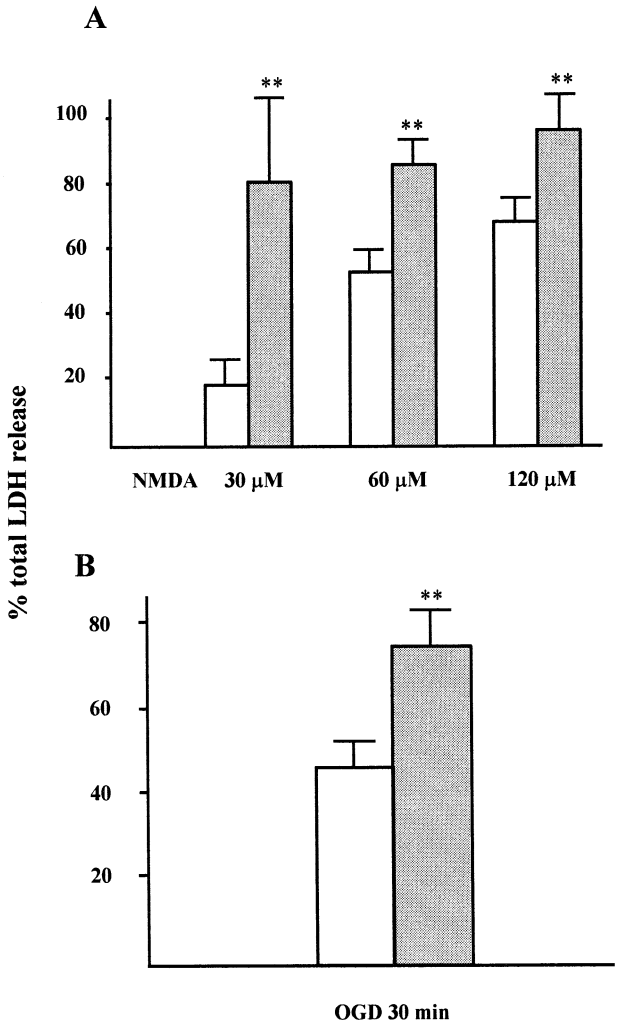

FIG. 2. Excitotoxicity and OGD-induced cell death in primary cortical cultures from IL-10$^{-/-}$ and wild-type mice. Cultures from IL-10$^{-/-}$ (grey bars) and wild-type (white bars) mice were exposed to (A) a 15-min NMDA pulse at the indicated concentrations or to (B) 30 min OGD. Neuronal cell death was expressed in terms of percentage of total LDH released by damaged cells 24 h after the neurotoxic treatment. Total LDH release, corresponding to complete neuronal death in absence of glial cell toxicity, was determined by exposing sister cultures to 500 μM NMDA for 24 h. Each data point is the mean ± SEM of three experiments run in triplicates/quadruplicates. Data were analysed by factorial ANOVA design. $^{**}P < 0.01$ vs. the corresponding values for wild-type mice.

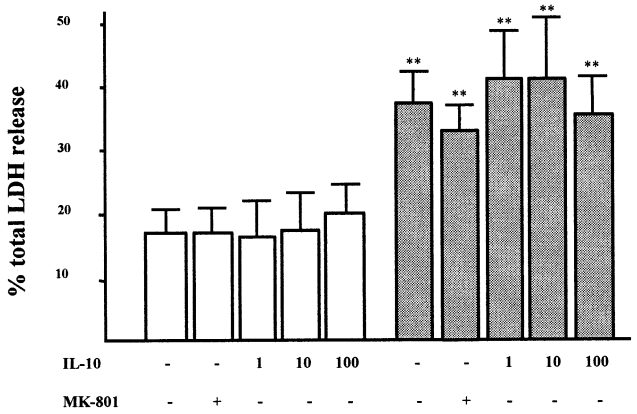

FIG. 3. Basal LDH release in primary cortical cultures from IL-10$^{-/-}$ (grey bars) and wild-type (open bars) mice. Neuronal death, expressed as percentage of total (500 μM NMDA-induced) LDH release, was estimated 24 h after growing medium was replaced with fresh 1% horse serum-containing medium. LDH release was significantly higher ($P < 0.01$) in cultures from IL-10$^{-/-}$ mice compared with wild-type cultures. Neither MK-801 (5 μM) nor recombinant murine IL-10 at the indicated concentrations (ng/mL) could modify basal LDH release either in IL-10$^{-/-}$ or in wild-type cultures. Values are mean ± SEM of three to six experiments run in triplicate. Data were analysed by factorial ANOVA design. $^{**}P < 0.01$ vs. values for wild-type mice.

experimental conditions, LDH release closely correlated with both the morphological assessment and cell counting for quantification of neuronal cell death (data not shown). In wild-type-derived cortical cultures, NMDA (30–500 μM) elicited a dose-dependent neurotoxic effect, with 60 μM NMDA producing ≈ 50% of total cell death. Both wild-type and IL-10$^{-/-}$-derived cultures were challenged with NMDA (30, 60 and 120 μM) and neuronal death was measured as a percentage of total LDH release. More extensive neuronal cell death was reported in cultures from IL-10$^{-/-}$ mice compared with control cultures at all NMDA concentrations (Fig. 2A). Exposure to 30, 60 or 120 μM NMDA for 15 min resulted in 18.6% ± 2.7, 52.2% ± 2.0 and 67% ± 2.9 cell death in wild-type cultures and 79.1% ± 19.6, 84.1% ± 2.8, 94.3% ± 3.4 cell death in IL-10$^{-/-}$-derived cultures, respectively. The neurotoxicity elicited by OGD in cortical cultures has been shown to involve NMDA receptor activation (Goldberg & Choi, 1990). Previous characterization of wild-type derived cell cultures identified 30 min OGD as the time required to kill ≈ 50% of cells (data not shown). IL-10$^{-/-}$-derived cultures subjected to OGD for 30 min showed a significantly increased neuronal cell death compared with wild-type cultures (75.8% ± 7.6 vs. 47.1% ± 2.2, respectively) (Fig. 2B). The reference neuroprotective agent, MK-801, at a concentration of 5 μM, completely counteracted both NMDA- and OGD-mediated toxicity in both wild-type and IL-10$^{-/-}$-derived cortical cultures (91% and 96% reduction, respectively). Interestingly, LDH basal release was also significantly increased in IL-10$^{-/-}$-derived cultures compared with control (Fig. 3), but MK-801 could never abolish the difference in basal LDH release consistently reported between the two experimental groups (Fig. 3). This observation enabled us to rule out the possibility that increased basal LDH release in IL-10$^{-/-}$-derived cell culture was dependent on NMDA receptor activation by endogenous glutamate release. Because the experimental paradigm required that culture medium was switched from 10% to 1% serum, this spontaneously occurring neuronal death is reminiscent of serum-deprivation-mediated cell injury.

Effect of recombinant murine IL-10 on excitotoxicity-mediated cell death

We sought to determine whether exogenously administered recombinant IL-10 could affect neuronal vulnerability to ischaemic damage *in vitro*. Recombinant murine IL-10 (1, 10 and 100 ng/mL) was tested in cortical cultures. IL-10 *per se* did not affect basal LDH release either in wild-type or in IL-10$^{-/-}$-derived cultures (Fig. 3). When the cytokine was added to cortical cultures subjected to a 120-μM NMDA pulse (15 min), a dose-dependent inhibition of excitotoxic damage was observed, which was maximally effective at a concentration of 100 ng/mL (Fig. 4A). Exogenous IL-10 was equieffective in both wild-type and IL-10$^{-/-}$-derived cells. Another well characterized *in vitro* model for excitotoxic damage is represented by cerebellar granule cells. A 50-μM glutamate pulse produced ≈ 60% cell death. Also in this experimental setting, IL-10 (0.1, 1 and 10 ng/mL) treatment resulted in a reduction of glutamate-induced neuronal death. In the presence of 0.1, 1 and 10 ng/mL IL-10, cell death was 42.2% ± 2.9, 32.5% ± 3.9 and 37.7% ± 4.9, respectively (Fig. 4B).

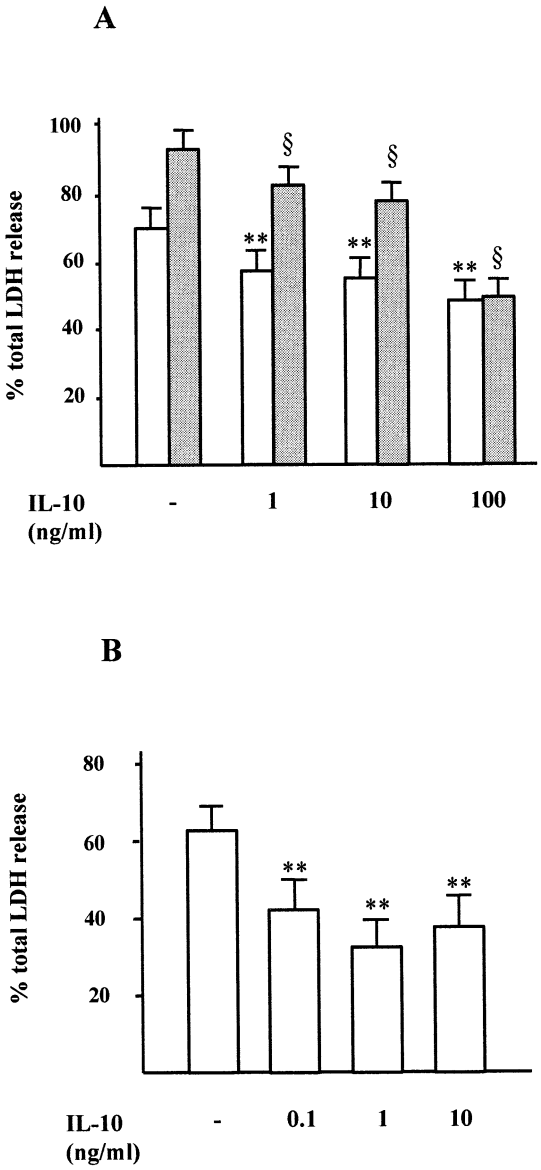

FIG. 4. Effects of IL-10 on excitatory amino acid-induced neurotoxicity in (A) cortical or (B) cerebellar granule cell cultures. (A) Primary cortical cultures from wild-type (white bars) and IL-$10^{-/-}$ (grey bars) mice were exposed to an NMDA pulse (15 min, 120 μM) in the absence (–) or presence of the indicated concentrations of recombinant murine IL-10. (B) Wild-type-derived cerebellar granule cell cultures were exposed to a glutamate pulse (15 min, 50 μM) in the absence (–) or presence of the indicated concentrations of recombinant murine IL-10. Neuronal cell death was estimated as a percentage of total LDH released for 24 h after NMDA or glutamate pulse. Values are mean ± SEM of three to four experiments run in triplicate. Data were analysed by factorial ANOVA design. (A) $^{**}P < 0.01$ vs. NMDA-alone values in wild-type cultures; $^{§}P < 0.01$ vs. NMDA-alone values in IL-$10^{-/-}$ derived cultures. (B) $^{**}P < 0.01$ vs. glutamate-alone values.

Focal ischaemia-induced TNF-α in IL-$10^{-/-}$ mice

The lack of IL-10 may profoundly alter the balance between pro-inflammatory and anti-inflammatory arms of cerebral ischaemia-induced inflammation because IL-10 is a powerful inhibitor of the synthesis of several pro-inflammatory molecules which are upregulated after cerebral ischaemia. Among the most attractive potential targets of IL-10 biological activity in the ischaemic cascade is certainly TNF-α, whose biological significance in the outcome of stroke, whether protective or deleterious, is still a matter of debate. Immunoreactivity for TNF-α was assayed in IL-$10^{-/-}$ mouse brain at different time points (1, 6, 24 and 48 h) after MCA occlusion. In particular, Fig. 5 describes the pattern of immunoreactivity for the cytokine at 6 and 24 h after focal ischaemia. Six hours from MCA occlusion, immunolabeled cells appeared within the ipsilateral cortex, in particular subsets of neuronal cells and endothelial cells were positively stained within the ischaemic core (Fig. 5a and b for C57Bl/6 and IL-$10^{-/-}$ mice, respectively). At 24 h, when most neurons had degenerated within the infarct area, numerous neuronal cells in the cortical regions surrounding the ischaemic area were strongly positive for TNF-α both in C57Bl/6 (Fig. 5c) and IL-$10^{-/-}$ (Fig. 5d) mouse brain. At any time point, we could not observe qualitative or quantitative differences in TNF-α immunoreactivity in IL-$10^{-/-}$ mice compared with wild-type mice. No TNF-α immunostaining was apparent in the contralateral cortical areas either at 6 h (Fig. 5e and f) or 24 h (g,h) in wild-type (e,g) and IL-$10^{-/-}$ (f,h) mice.

Discussion

IL-10 is an immunosuppressive cytokine identified in human, murine and other organisms, mainly produced by Th2 and Th0 subsets of $CD4^+$ T cells, B cells and macrophages (Mosmann, 1994). Expression of IL-10 has been demonstrated, both *in vitro* and *in vivo*, by resident brain cells, mainly microglia. Importantly, IL-10 expression is modulated in several acute and chronic neuropathological conditions in humans and in their animal model counterparts so as to suggest a role for this cytokine in immune-regulatory functions in CNS pathophysiology. Low IL-10 expression in the CNS has also been associated with protracted relapsing experimental autoimmune encephalomyelitis (Diab *et al*., 1997). IL-10 is increased in the CSF of children after severe traumatic brain injury (Bell *et al*., 1997), after birth asphyxia (Savman *et al*., 1998) and in stroke patients (Tarkowski *et al*., 1997). Moreover, IL-10 gene expression is induced early (within 6 h) in rats subjected to middle cerebral artery occlusion (Zhai *et al*., 1997).

The present study demonstrates that, in the absence of IL-10 production, mice brain tissues are more sensitive to ischaemic damage as a consequence of focal ischaemic stroke. Many physiological variables can markedly influence the effects of vascular stroke and in particular the extent of brain damage, even independently of specific gene targeting. Several observations argue against this possibility in the present study. IL-$10^{-/-}$ mice develop a chronic enterocolitis (Kühn *et al*., 1993) which might hamper a correct comparison with corresponding controls as to their response to various experimental stimuli. In the present study, caution was taken in order to minimize the influence of a concomitant intestinal pathology on the outcome of the *in vivo* work. IL-$10^{-/-}$ mice underwent 10 backcrosses with the C57Bl/6 strain to obtain a congenic strain, carrying the mutation on a definite genetic background which has been shown to confer resistance to the disease (Berg *et al*., 1996). Moreover, animals were kept in SPF conditions, which have been shown to attenuate the intestinal pathology (Kühn *et al*., 1993). In fact, the group of mutant mice did not show a higher mortality, either spontaneous or postoperative, than the control group. It has been shown that IL-$10^{-/-}$ mice affected by the intestinal pathology develop anaemia whose severity is clearly correlated with weight loss (Kühn *et al*., 1993). In the present study, body weight differences between the two groups were not detected. More importantly, no difference was demonstrated in arterial blood PO_2, PCO_2 and pH between IL-$10^{-/-}$ and control animals. It is therefore conceivable that the higher sensitivity to ischaemic injury displayed

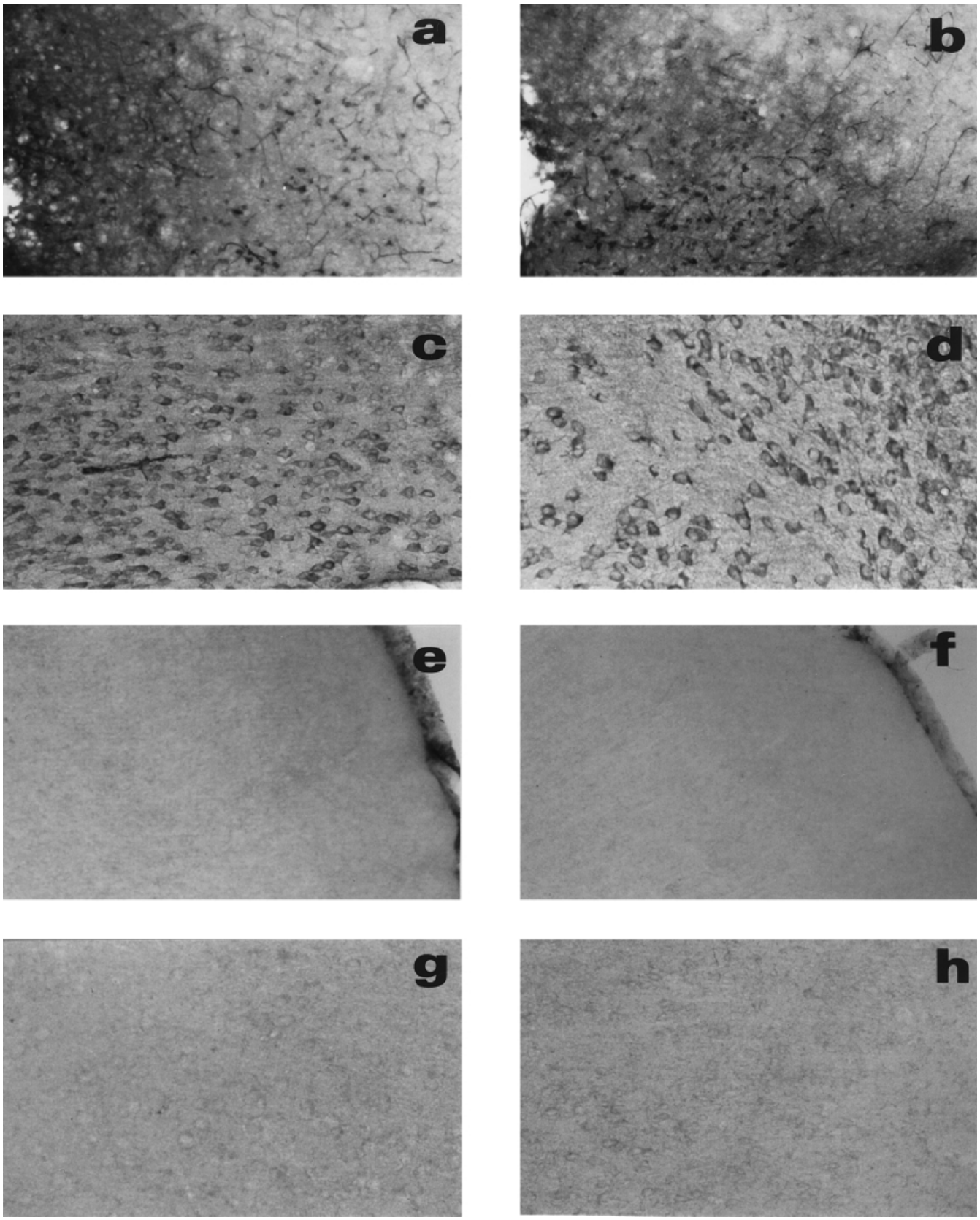

FIG. 5. TNF-α induction after focal cerebral ischaemia by MCA occlusion. Immunoreactivity for murine TNF-α in C57Bl/6 (a, c, e and g) and IL-$10^{-/-}$ (b, d, f and h) mouse brain at different time points (6 and 24 h) after MCA occlusion. At 6 h, the areas shown represent the ischaemic core (a and b) and the corresponding contralateral regions (e and f). At 24 h, an area at the periphery of the ischaemic core (c and d) is shown in comparison with the contralateral corresponding ones (g and h). No difference in TNF-α pattern of immunoreactivity was observed in the two experimental groups at either time point.

by mutant mice resulted from the lack of endogenous IL-10 in brain. This is strongly substantiated by the *in vitro* results. Primary embryonal cortical cultures from IL-10$^{-/-}$ mice appeared more vulnerable to conditions mimicking the sequelae of processes following brain ischaemia, such as either OGD or exposure to the excitotoxic action of micromolar concentrations of NMDA, compared with cultures from wild-type animals. We have also demonstrated that exogenous IL-10 could prevent, in a dose-dependent manner, neuronal death induced by excitotoxic stimuli in cultures from two different regions of the brain, with different developmental time frames, namely cortical neurons and cerebellar granule cells. Interestingly, IL-10-mediated neuroprotection was observed not only in wild-type but also in IL-10$^{-/-}$-derived cultures, suggesting that the expression of a functional IL-10 receptor is not influenced by lack of the ligand.

Specific studies should be undertaken to elucidate the mechanism by which the lack of IL-10 gene may influence *in vivo* neuronal survival to ischaemia. The *in vitro* studies demonstrating an augmented vulnerability to both excitototoxic and hypoxic/hypoglycaemic damage provided initial hints about the mechanistic bases of increased vulnerability of IL-10$^{-/-}$ mice to damage associated with focal cerebral ischaemia. The lack of IL-10 may profoundly alter the balance between pro-inflammatory and anti-inflammatory arms of cerebral ischaemia-induced inflammation because it is a powerful inhibitor of the synthesis of several pro-inflammatory molecules which are upregulated after cerebral ischaemia, including pro-inflammatory cytokines such as IL-1 β, TNFα and IL-6 (Wang *et al*., 1995), chemokines such as MIP-1α, RANTES and IL-8 (Berkman *et al*., 1995; Ehrlich *et al*., 1998) and adhesion molecules such as ICAM-1 (Shrikant *et al*., 1995). We decided to proceed further in understanding the role of IL-10 in brain damage and an immunohistochemical study was carried out to look at differences in the inflammatory response of IL-10$^{-/-}$ and wild-type mice to ischaemic damage. Among the most relevant potential targets of IL-10 biological activity in the ischaemic cascade is certainly TNFα. IL-10 inhibits TNFα release by astrocytes and microglial cells *in vitro* (Benveniste *et al*., 1995). IL-10-mediated protection in several *in vivo* models of tissue injury, i.e. traumatic brain injury (Knoblach & Faden, 1998), cardiac ischaemia-reperfusion (Hayward *et al*., 1997), hindlimb ischaemia-reperfusion (Engles *et al*., 1997) and traumatic spinal cord injury (Bethea *et al*., 1999) has been consistently associated with inhibition of TNFα synthesis. Moreover, ICV injection of human recombinant IL-10 reduces brain TNFα production stimulated by lipopolysaccaride administration in mice (Di Santo *et al*., 1997). Temporal profile of expression for TNFα and IL-10 in animal stroke models has been interpreted as if IL-10 results from TNFα activation (Sheng *et al*., 1995) and IL-10 may, subsequently, contribute to downmodulate TNFα synthesis (Zhai *et al*., 1997). In our experimental setting, we have been unable to observe any differences in TNF-α induction after cerebral ischaemia in IL-10 mutant mice when compared with wild-type animals. Our efforts to unravel a possible mechanism for increased susceptibility to ischaemic damage in IL-10 mutant mice have also been focused on nuclear factor-kappa B (NF-κB) activity, which is known to modulate expression of several genes crucial for both inflammatory and immune responses and whose activation has been correlated with excitotoxic and ischaemic-mediated neuronal cell death both *in vitro* (Grilli *et al*., 1996) and *in vivo* (Clemens *et al*., 1998). Although IL-10 is a potent inhibitor of NF-κB (Wang *et al*., 1995; Ehrlich *et al*., 1998), we have not observed differences in the modulation of NF-κB activities in ischaemic IL-10$^{-/-}$ mice compared with control mice (data not shown).

We cannot rule out that IL-10 may potentially play a beneficial role in ischaemic lesions not only as a strictly anti-inflammatory cytokine but also as an 'antidegenerative' agent. Some *in vitro* observations appear to substantiate this working hypothesis. In our study, IL-10 is neuroprotective against excitotoxicity-induced neuronal death not only in mixed glial–neuronal cultures but also, in agreement with the report by Sanna *et al*. (1998), in cerebellar granule cell cultures which are 95–98% pure neuronal cells. In this regard, it should be mentioned that IL-10 suppresses the production of reactive oxygen intermediates and NO, molecules which have been shown to exert toxic activities in neuronal cultures (Chao *et al*., 1992; Piani *et al*., 1992). Finally, it has also been suggested that IL-10, similarly to other Th-2-derived cytokines, may provide neurotrophic support to injured neurons, because it enhances nerve growth factor synthesis by astrocytes *in vitro* (Brodie, 1996). In this regard, it is intriguing that not only excitotoxicity- and OGD-mediated cell death is augmented in IL-10$^{-/-}$-derived cultures compared with wild-type cells. Indeed, spontaneously occurring cell death during experimental procedures requiring switch from high- to low-serum-containing medium, likely to be interpreted as trophic factor withdrawal-induced neuronal death, is also increased in IL-10$^{-/-}$-derived cultures compared with wild-type cells.

In conclusion, we have demonstrated that the lack of production of IL-10 in genetically modified mice resulted in increased vulnerability following cerebral ischaemia, therefore suggesting a protective role of endogenous IL-10 following stroke. Moreover, we have shown that exogenously administered IL-10 resulted in neuroprotection against excitotoxic cell death in *in vitro* models. Following this line, we are currently attempting to further characterize the mechanisms underlying the neuroprotective properties of IL-10.

Recently, Spera *et al*. (1998) have demonstrated that human IL-10, given either intracerebroventricularly or systemically resulted in reduction of infarct volume in spontaneously hypertensive rats subjected to permanent MCA occlusion. Altogether, ours and Spera's findings certainly stimulate to further investigate the potential therapeutical usefulness of this anti-inflammatory cytokine in the treatment of ischaemic damage.

Abbreviations

CSF, cerebrospinal fluid; DIV, days *in vitro*; IFNγ, interferon gamma; IL, interleukin; IL-10, interleukin-10; IL-1ra, interleukin-1 receptor antagonist; LDH, lactate dehydrogenase; MCA, middle cerebral artery; MEM, minimum essential medium; NF-κB, nuclear factor-kappa B; NMDA, *N*-methyl-D-aspartate; OGD, oxygen–glucose deprivation; PFA, paraformaldehyde; SPF, specific pathogen free; TNFα, tumor necrosis factor alpha; TTC, triphenyl tetrazolium chloride.

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