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Delayed administration of erythropoietin and its non-erythropoietic derivatives ameliorates chronic murine autoimmune encephalomyelitis

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Abstract

Erythropoietin (EPO) mediates a wide range of neuroprotective activities, including amelioration of disease and neuroinflammation in rat models of EAE. However, optimum dosing parameters are currently unknown. In the present study, we used a chronic EAE model induced in mice by immunization with the myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) to compare the effect of EPO given with different treatment schedules. EPO was administered intraperitoneally at 0.5, 5.0 or 50 μg/kg three times weekly starting from day 3 after immunization (preventive schedule), at the onset of clinical disease (therapeutic schedule) or 15 days after the onset of symptoms (late therapeutic schedule). The results show that EPO is effective even when given after the appearance of clinical signs of EAE, but with a reduced efficacy compared to the preventative schedule. To determine whether this effect requires the homodimeric EPO receptor ($EPOR_2$)-mediated hematopoietic effect of EPO, we studied the effect of carbamylated EPO (CEPO) that does not bind $EPOR_2$. CEPO, ameliorated EAE without changing the hemoglobin concentration. Another non-erythropoietic derivative, asialoEPO was also effective. Both EPO and CEPO equivalently decreased the EAE-associated production of TNF-α, IL-1β and IL-1Ra in the spinal cord, and IFN-γ by peripheral lymphocytes, indicating that their action involves targeting neuroinflammation. The lowest dosage tested appeared fully effective. The possibility to dissociate the anti-neuroinflammatory action of EPO from its hematopoietic action, which may cause undesired side effects in non-anemic patients, present new avenues to the therapy of multiple sclerosis.
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1. Introduction

In addition to its hemopoietic effects, erythropoietin (EPO) possesses neuroprotective and neurotrophic properties (Brines and Cerami, 2005; Ghezzi and Brines, 2004; Jelkmann and Wagner, 2004; Juul, 2004). Our observations (Brines et al., 2000) and those of others (Juul et al., 2004) have shown that EPO crosses the intact blood brain barrier to provide protection from injury. The systemic administration of EPO mediates a wide range of neuroprotective actions in animal models of brain traumatic injury (Brines et

Abbreviations: MOG_{35-55}, myelin oligodendrocyte glycoprotein peptide 35–55; EAE, experimental autoimmune encephalomyelitis; ConA, concanavalin A; EPO, erythropoietin; asialoEPO, desialylated EPO; CEPO, carbamylated EPO; EPOR, EPO receptor; IFN-γ, interferon-gamma; IL-, interleukin; IL-1Ra, IL-1 receptor antagonist; TNF, tumor necrosis factor; GFAP, glial fibrillary acidic protein; GM–CSF, granulocyte–macrophage colony-stimulating factor; VEGF, vascular endothelial growth factor; HIF-1, hypoxia inducible factor-1; BBB, blood–brain barrier; MS, multiple sclerosis.

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al., 2000), cerebral ischemia (Chang et al., 2005; Villa et al., 2003; Wang et al., 2004), of spinal cord injury and ischemia (Celik et al., 2002; Gorio et al., 2002), and diabetic neuropathy (Bianchi et al., 2004). Using a rat model of cerebral ischemia, we observed that the neuroprotective action is paralleled by a marked anti-neuroinflammatory effect, with inhibition of cytokine production and glial activation/proliferation (Villa et al., 2003).

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) associated with demyelination, inflammation and loss of axons. Several cytokines have shown some efficacy in reducing the severity of experimental autoimmune encephalomyelitis (EAE), an animal model for MS, including anti-inflammatory cytokines and inhibitors of IL-1, TNF, or IFN-γ (reviewed in Martino et al., 2002; Steinman, 2003; Steinman, 2004), but none have so far proven clinically effective.

The observation that EPO is effective in models of diseases possessing common neuroinflammatory components prompted us to study its effectiveness in an acute EAE model in Lewis rats, induced by immunization against myelin basic protein and we observed a reduction of inflammation and amelioration of clinical signs with EPO (Agnello et al., 2002; Brines et al., 2000). Others have confirmed these findings in different EAE models including optic neuritis (Diem et al., 2005; Li et al., 2004; Sattler et al., 2004). Since optimum dosing, particularly whether EPO is effective only when given before the development of clinical symptoms (i.e., a preventive schedule) is unknown, we evaluated the potential utility of administration after the development of clinical disease.

The present study examines the effects of EPO in a chronic model of EAE using C57BL/6 mice immunized with MOG_{35-55} (Amor et al., 1996; Furlan et al., 2001). This model, unlike the Lewis rat model where the disease is acute and lasts only a few days, allows a comparison of the effectiveness of EPO when it is given 3 days after immunization, before disease onset, as we have done in the acute model (Agnello et al., 2002; Brines et al., 2000), or beginning late after its onset, to mimic clinically relevant settings.

In this model, we also studied the effect of EPO on spinal cord inflammation by use of immunohistochemical markers such as CD11b, GFAP, and TNF-α. We also measured expression of TNF-α, IL-1, and IL-1Ra in the spinal cord, and studied the antigen-specific or concanavalin A-induced T cell proliferation, and production of IFN-γ by peripheral lymphocytes using ELISPOT and ELISA assays.

A major issue in the use of EPO as neuroprotective, anti-neuroinflammatory drug, is represented by its erythrodifferentiating action that represent a potential cause of several side effects, including vascular perfusion defects (Natali et al., 2005). In mice the increase in hematocrit induced by EPO causes vasoconstriction and cardiac dysfunction due to NO depletion and endothelin activation (Quaschning et al., 2003; Ruschitzka et al., 2000). In animals and humans, EPO can lead to hypertension (Group, 1991; Lim, 1991; Varet et al., 1990) or thrombosis (reviewed in Bokemeyer et al., 2004).

We have successfully identified derivatives of EPO, including asialoEPO (Erbayraktar et al., 2003) and carbamylated EPO (CEPO) (Leist et al., 2004), which do not increase the hematocrit but retain neuroprotective actions in animal models of cerebral ischemia, spinal cord injury, and diabetic neuropathy. We studied their effect in EAE not only because these could represent drugs that lack the undesired effects of EPO but also to investigate whether protection by EPO is associated with an increase in red blood cells.

Furthermore, since others have hypothesized that the neuroprotective effects of EPO are mediated through a mechanism implicating dimerization of the classical EPO receptor (EPOR) (Maiese et al., 2005), we have studied the effects on the clinical expression and CNS pathology of EAE of CEPO, a tissue-protective cytokine which we have previously shown does not bind the classical, dimeric EPOR (Leist et al., 2004).

Since both EPO and its non-erythropoietic derivatives are effective in ameliorating EAE when administered using either a preventive or a therapeutic treatment schedule, this effect cannot be associated with an increase in hematocrit. Decreased production of inflammatory cytokines in the spinal cord and lymphocytes suggest that inflammation is a primary target of tissue-protective cytokines.

2. Materials and methods

2.1. EAE induction

EAE was induced in C57BL/6 female mice (6–8 weeks of age). Mice were obtained from Charles River (Calco, Italy) and housed in specific pathogen-free conditions, allowing access to food and water ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with national (D.L. n. 116, G.U., suppl. 40, Feb. 18, 1992) and international laws and policies (EU Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). The protocols for the proposed investigation were reviewed and approved by the Animal Care and Use Committees (IACUC) of the Istituto di Ricerche Farmacologiche "Mario Negri". EAE was induced by subcutaneous immunization in the flanks with a total of 200 μg of MOG_{35-55} (Multiple Peptide Systems, San Diego, CA, USA) in incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) supplemented with 8 mg/ml of *Mycobacterium tuberculosis* (strain H37RA; Difco, Detroit, MI, USA). Mice received 500 ng of pertussin toxin (Sigma) i.v. at the time of immunization and 48 h later. Weight and clinical score were recorded daily (0 = healthy, 1 = flaccid tail, 2 = ataxia, and/or hind-limbs paresis, or slow righting reflex,

3 = paralysis of hind limb and/or paresis of forelimbs, 4 = paraparesis of fore limb, 5 = moribund or death). The food pellets and the drinking water were placed on Petri plates on the floor of the cage to enable sick mice to eat and drink.

2.2. Experimental design and treatments

EPO (recombinant human EPO) was obtained from Dragon Pharmaceuticals, Vancouver, BC, Canada. AsialoEPO and CEPO were prepared as described (Erbayraktar et al., 2003; Leist et al., 2004) and kindly supplied by Warren Pharmaceuticals and H. Lundbeck A/S, Copenhagen. For the preventive schedule, mice were treated intraperitoneally (i.p.) with EPO or its derivatives at the indicated dose, three times a week starting on day 3 after immunization. In the therapeutic treatment schedule, treatment was started at the exordium of EAE (normally between days 10 and 12 after immunization). In some experiments, we also administered the drug using a late therapeutic schedule beginning 15 days after the onset of disease. The experimental schedule is schematized in Fig. 1.

2.3. Hematocrit

Blood was collected in heparinized capillary tubes and centrifuged at 2500×*g* for 20 min at 4 °C and hematocrit was determined.

2.4. Immunohistochemistry

Mice were anesthesized with Equithesin (1% phenobarbitol/4% (vol./vol.) chloral hydrate, 30 μl/10 g, i.p.) and transcardially perfused with 100 ml saline followed by 250 ml of sodium phosphate buffered 4% paraformaldehyde solution. Spinal cords were rapidly removed, fixed in sodium phosphate-buffered 4% paraformaldehyde solution for 2 h, transferred to 20% sucrose solution in PBS overnight, then in 30% sucrose solution until they sank and finally frozen in 2-methylbutane at −45 °C. Sections (30 μm) were cut on a cryostat at −20 °C in the transverse plane through the lumbar spinal cord and every fifth section selected for histochemistry against different antigens or hematoxylin–eosin staining. Free floating sections were processed for immunoreactivity both with anti-GFAP mouse monoclonal antibody (1:250; Immunological Sciences) and with anti-CD11b (MRC OX-42) mouse monoclonal antibody (1:50; Serotec, UK), or anti-TNF (1:50; HyCult Biotechnology b.v, Uden, The Netherlands) according to the protocols described, respectively, by Houser et al. (1984) and the manufacturer's protocol. All sections were mounted for light microscopy in saline on coated slides, dehydrated through graded alcohol, fixed in xylene and cover-slipped using DPX mountant (BDH, Poole, UK). Adjacent sections were stained with hematoxylin and eosin as described (Gill et al., 1974). Slides were analyzed under light microscopy in a blinded fashion.

2.5. Cytokine expression in the spinal cord

Total RNA was isolated from tissue samples according to the acid guanidium–phenol–chloroform method (Chomczynski and Sacchi, 1987). The samples were digested by a denaturing solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, pH 7.0. The lysate was extracted with a mixture of 0.1 m sodium acetate, pH 4.0/phenol/chloroform (1:10:2) and isoamyl alcohol (49:1), and nucleic acids were precipitated with equal volumes of isopropyl alcohol. Total RNA was quantified by spectrophotometer and an aliquot was loaded onto 1% agarose gel to visually assess for RNA integrity. Before performing RNA reverse transcription to cDNA, an aliquot of each sample (1–2 μg) was treated with 1–2 U/μl of DNase I (Invitrogen, S. Giuliano Milanese, Italy) to eliminate genomic DNA contaminants. DNase-treated total RNA (1–2 μg) from each sample was used as a substrate for single-stranded cDNA synthesis using murine leukemia virus reverse transcriptase (MMLV-RT, 50 U/μl; Perkin-Elmer, Emeryville, CA, USA), random hexamers (2.5 μM), and deoxyNTP mix (1.25 mM each) in a final volume of 20 μl. The mixture was incubated at room temperature for 10 min, then incubation was performed in a thermocycler (Omn-E; Hybaid, Ashford, UK) at 42 °C for

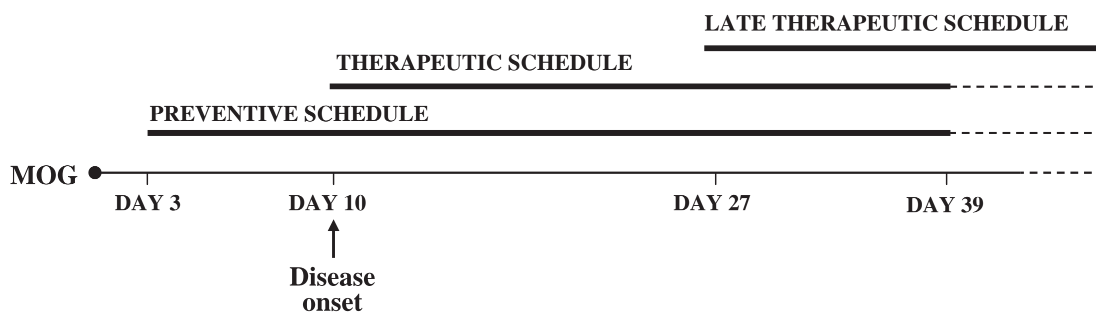

Fig. 1. Treatment schedules. EAE was induced by immunization with MOG on day 0. EPO or CEPO were administered intraperitoneally three times a week at the doses indicated within the text. Length of treatment is indicated by the thick, solid line. On day 39, treatment was discontinued in order to evaluate the persistence of the pharmacological activity.

15 min, at 99 °C for 5 min, and at 5 °C for 5 min. Real-time quantitative PCR was carried out using the 5700 SDS (Applied Biosystems, Monza, Italy), exploiting SYBR GREEN as fluorescent dye. The primers used were the following: IL-1β, forward primer, TAACCTGCTGGTGTGTGACGTT; IL-1β, reverse primer, CGGAGCCTGTAGTGCAGTTGT; IL-1Ra, forward primer, TGGGAAAAGACCCTGCAAGA; IL-1Ra, reverse primer, AAGGTCAATAGGCACCATGTCTATC; TNF-α, forward primer, ATGCTGGGACAGTGACCTGG; TNF-α, reverse primer, CCTTGATGGTGGTGCATGAG; β-actin, forward primer, TGTCCACCTTCCAGCAGATGT; β-actin, reverse primer, CGGACTCGTCATACTCCTGCTT.

2.6. Cytokine production from spleen cells

Mononuclear cell suspensions were prepared from spleens removed from mice treated with EPO, CEPO and controls, and cultured at 3.5×10^6 cells/ml in a 24-well plates with different concentration of MOG (1, 3, and 10 μM) or with 4 μg/ml of Concanavalin A (Sigma-Aldrich, Italy). Culture media consisted of RPMI-1640 containing 1% penicillin–streptomycin, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids (all from Euroclone Celbio, Milan, Italy), 2×10^{-5} M 2-Mercaptoethanol (2-ME, BDH, Milan Italy) and 10% FCS (Sigma-Aldrich, Milan, Italy). Supernatants were collected at different time points for measurements of cytokine levels: 24 h for IL-2, 48 h for IFN-γ, and 96 h for IL-4 and IL-10. Cytokine concentrations were determined in supernatants by using specific enzyme-linked immunosorbent assay (ELISA) with capture and detection antibodies for the specific cytokines according to the manufacturer's protocols (BD OptEIA ELISA Set, Pharmingen, San Diego, CA, USA). Standard curves for each cytokines were generated with recombinant mouse cytokines and cytokine levels in supernatants were determined by interpolation with the appropriate standard curve. Detection limits for the different cytokines were: 31.3 pg/ml for IFN-γ and IL-10, 7.8 pg/ml for IL-4 and 3.1 pg/ml for IL-2. Means and standard error mean (S.E.M.) were determined using data from individual animals tested in duplicates.

2.7. Cytokine production in spleen cells by ELISPOT

IFN-γ and IL-10 production in response to MOG_{35-55} peptide or Concanavalin A (ConA, positive control) was assessed by ELISPOT assays. Mononuclear cell suspension were prepared from spleens aseptically removed from vehicle and EPO- or CEPO-treated mice. IFN-γ and IL-10 ELISPOT kits (from R&D Systems, Space Import/Export, Milan, Italy) were used following manufacturer's instruction. Briefly, quadruplicate cultures of 3×10^5 cells were seeded with antigens (10 μM MOG_{35-55} and 4 μg/ml ConA) in RPMI-1640 medium containing 1% penicillin–streptomycin, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids (all from Euroclone Celbio, Milan, Italy), 2×10^{-5} M 2-mercaptoethanol (2-ME, BDH, Milan Italy) and 10% FCS (Sigma-Aldrich, Milan, Italy). Cells were incubated at 37 °C in 5% CO_2, and harvested after 24 h for IFN-γ detection and 48 h for IL-10 detection. After several washes, biotinylated secondary antibodies specific for mouse IFN-γ or IL-10 were added to the wells; after overnight incubation, alkaline-phosphatase conjugated to streptavidin was added for 2 h, and revealed by substrate solution (BCIP/NBT). The number of specific spots, representing an individual cluster of cytokine-secreting cells, was determined using an automated ELISPOT reader (AID, Strassberg, Germany) with set parameters for size, intensity and gradient. Background mean values (cells cultured in medium alone) were subtracted from ConA on MOG induced mean spot numbers for each mouse.

2.8. Statistical analysis

EAE weight and scores and PCR results were compared using the Mann–Witney nonparametric test or Student's *t* test for unpaired data or by covariance analysis according to the indications in table and figure legends.

3. Results

3.1. EPO treatment ameliorates EAE pathology

Mice were injected intraperitoneally with EPO (50 μg/kg bw) either at day 3 post-immunization (preventive schedule) or at the onset of clinical disease (therapeutic schedule) as outlined in Fig. 1. As shown in Fig. 2, EPO effectively prevented and/or ameliorated the disease both in terms of disease severity (Fig. 2A) and of reduced body weight loss (Fig. 2B). In addition, EPO administered according to the preventive schedule significantly delayed the disease onset (14.7 ± 1.5 vs. 11.7 ± 0.4 of vehicle-treated mice, $P < 0.05$). In the experiments shown in Fig. 2, we discontinued EPO treatment on day 39 and monitored the mice for a further period of 20 days. The cumulative scores at day 39 were 68.1 ± 6.0, 27.7 ± 6.2 and 53.4 ± 2.3 for vehicle treated, EPO preventive and EPO therapeutic groups, respectively and both schedule of treatment were statistically significantly different from vehicle-treated mice. On day 60, mice treated with EPO, both according to the preventive and the therapeutic schedule still showed a lower disease score when compared to vehicle-treated mice (vehicle $= 3.00 \pm 0.2$, EPO preventive $= 1.17 \pm 0.3$, and EPO therapeutic $= 1.56 \pm 0.06$). This indicates that protection persisted for at least 20 days after the discontinuation of EPO. The clinical parameters of EAE in these experiments are summarized in Table 1.

In untreated mice, on day 39 after immunization, the hematocrit was 47.3 ± 2.2% (range $= 42.2$–49.2), while in

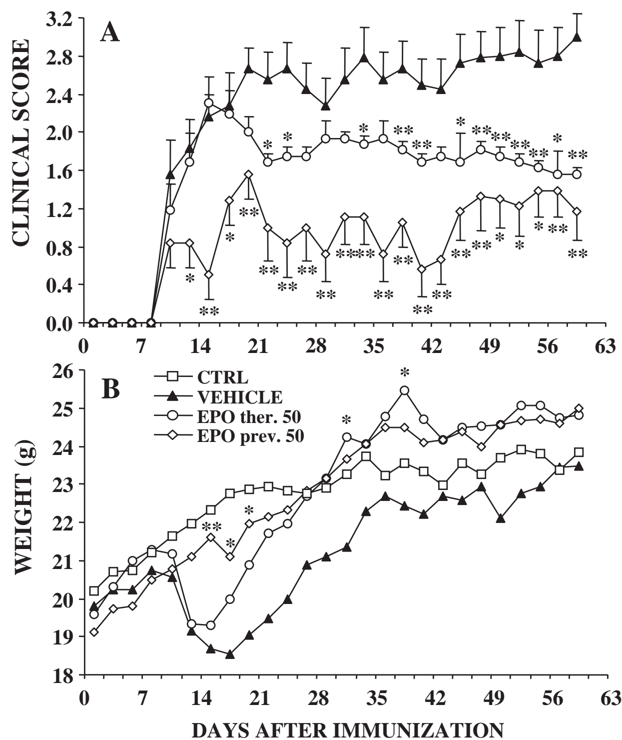

Fig. 2. Preventive or therapeutic EPO treatment ameliorates EAE. Mice were immunized with MOG and injected with vehicle or EPO (50 μg/kg i.p.) starting at day 3 following EAE induction (preventive schedule) or at symptom onset (therapeutic schedule). In panel A, disease score is reported only from day of onset. Data are the mean ± S.E.M., number of animals per group was: vehicle = 9; EPO preventive = 9; EPO therapeutic = 8. * $P < 0.05$, ** $P < 0.01$ by Mann–Whitney test.

EPO-treated mice it was 38.1 ± 11.4% (range = 21.5–52.6) and 60.0 ± 6.4% (range = 49.0–69.2) for preventive and therapeutic schedule, respectively. It must be noted that mice treated according to the preventive schedule received EPO for a period of 39 days, while in those treated using the therapeutic schedule EPO was given for a total of 29 days, and the more prolonged treatment resulted in a decrease in the hematocrit in some of the mice, due to the formation of neutralizing autoantibodies against human recombinant EPO. By day 60 the hematocrit in EPO-treated mice was 40.0 ± 10.3% (range = 23.8–50.4) and 48.1 ± 3.1% (range = 42.6–50.7) for preventive and therapeutic schedule, respectively. Again, it can be seen that the longer treatment in the preventive schedule resulted in a paradoxical decrease in the hematocrit. We did not, however, observe a significant correlation between the hematocrit and the EAE score (not shown).

Finally, we tested EPO using a late therapeutic treatment schedule, where EPO was administered from day 27 after immunization, i.e. about 15 days after disease onset. The results are shown in Fig. 3. Although the disease score did not significantly differ when comparing untreated and EPO-treated mice, using the Mann–Whitney test to compare the two curves, the overall status of the animals was improved in the EPO group, as it is evident from the body weight curves reported in Fig. 3B, where at the end of the experiment, on day 46, the body weight loss was significantly less in mice treated with 50.0 μg/kg of EPO (untreated EAE mice weighted 52% of healthy controls; EPO-treated EAE mice weighted 78% of controls).

Table 1
Clinical EAE parameters in mice treated with EPO starting at day 3 after immunization (preventive schedule) or EPO and CEPO starting at symptom onset (therapeutic schedule)

Treatment	Mean maximum score	AUC (days 11–60)	No. of mice with score ≥2	No. of mice with score ≥3
Vehicle	3.2 ± 0.3	123.1 ± 10.9	7/9	6/9
EPO 50.0 μg/kg preventive	1.8 ± 0.2[a]	50.9 ± 10.4[b]	3/9	0/9
EPO 50.0 μg/kg therapeutic	2.5 ± 0.2[c]	88.0 ± 2.8[d]	1/8	0/8
CEPO 5.0 μg/kg	2.3 ± 0.2[c]	75.3 ± 11.8[b]	3/9	0/9
CEPO 50.0 μg/kg	2.4 ± 0.2	76.0 ± 10.7[d]	3/7	0/7

Data are mean ± S.E.M. Values are calculated at day 60.
[a] $P < 0.01$ vs. EAE vehicle-treated mice, by Mann–Whitney test.
[b] $P < 0.01$ vs. vehicle by ANOVA.
[c] $P < 0.05$ vs. EAE vehicle-treated mice, by Mann–Whitney test.
[d] $P < 0.05$ vs. vehicle by ANOVA.

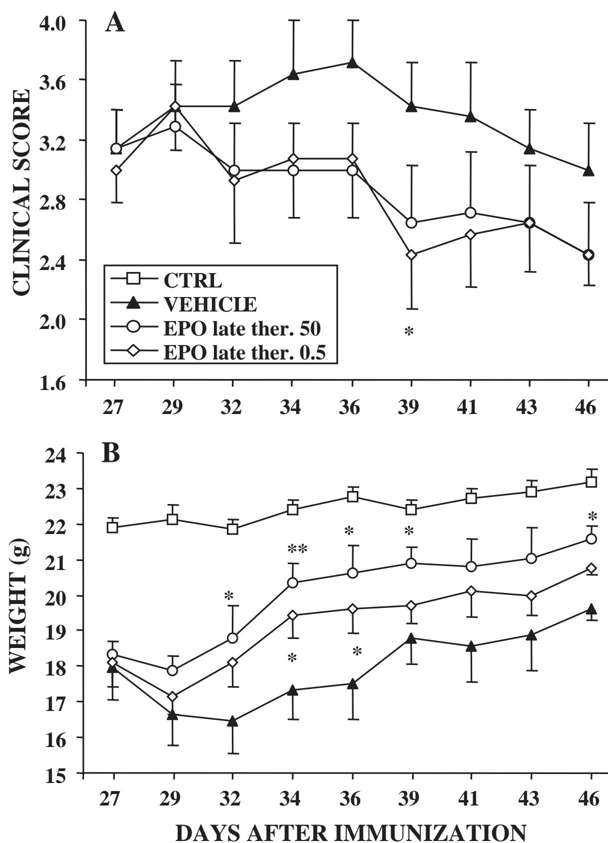

Fig. 3. Therapeutic efficacy of late EPO treatment on disease score (A) and body weight (B). Mice were immunized with MOG and injected with vehicle or EPO (50 μg/kg i.p.) starting at day 27 following EAE induction (late therapeutic schedule). Data are the mean ± S.E.M., $n = 7$ ($n = 5$ in the case of healthy mice). * $P < 0.05$ vs. respective vehicle-treated group by Student's t-test.

In vehicle-treated EAE mice, at sacrifice, the hematocrit was 53.4±3.5% (range=46.3–57.1), while it was 70.6±4.2% (range=65.6–74.4) and 53.5±4.0% (range=49.4–60.5) in EPO-treated mice for 50.0 and 0.5 μg/kg doses, respectively.

3.2. Non-erythropoietic EPO derivatives are effective in EAE

We first tested CEPO using the therapeutic schedule (administration starting at the onset of the disease) at two doses (50 and 5 μg/kg bw). As shown in Fig. 4A, CEPO, at both doses, significantly improved the course of the disease. As previously observed for EPO, the effect of CEPO was also evident on the body weight of the animals (Fig. 4B). Also in the case of CEPO, the therapeutic effect was maintained for at least 20 days after treatment was interrupted at day 39. Table 1 summarizes the clinical features of the disease in the experiments reported in Fig. 4.

In EAE-untreated mice, at day 39 the hematocrit was 47.3±2.2% (range=42.2–49.2), while it was 49.2±4.5% (range=44.4–53.2), and 44.9±2.6% (range=42.2–47.5) in CEPO-treated mice for 50.0 and 5.0 μg/kg, respectively. By day 60, after treatment discontinuation, the hematocrit was 48.7±4.7% (range=39.6–52.1) and 47.1±1.8% (range=43.9–49.6) in CEPO-treated mice for 50 and 5 μg/kg bw, respectively.

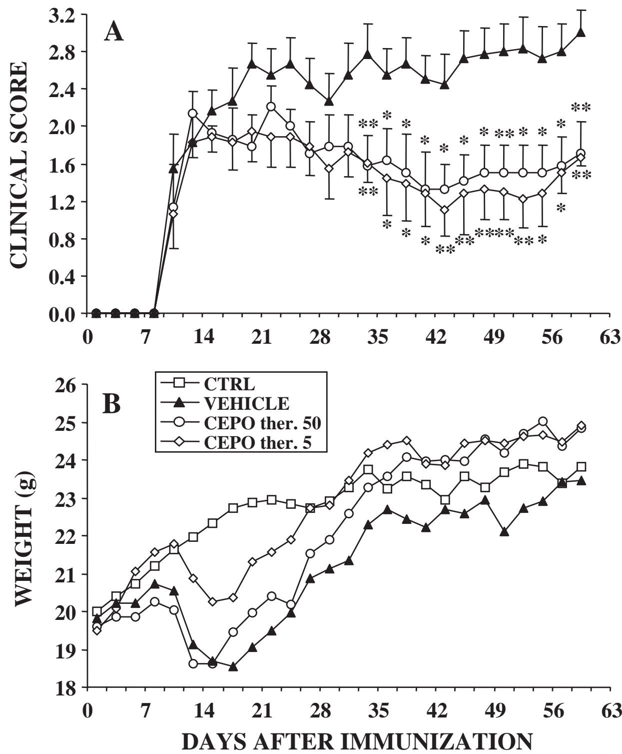

Fig. 4. Preventive or therapeutic CEPO treatment ameliorates EAE. Mice were immunized with MOG and injected with vehicle or CEPO (5.0 or 50.0 μg/kg i.p.) starting at symptoms onset (therapeutic schedule). In panel A, disease score is reported only from day of onset. Data are the mean±S.E.M., number of animals per group was: vehicle=9; CEPO 50.0 μg/kg=7; CEPO 5.0 μg/kg=9. $*P<0.05$, $**P<0.01$ by Mann–Whitney test.

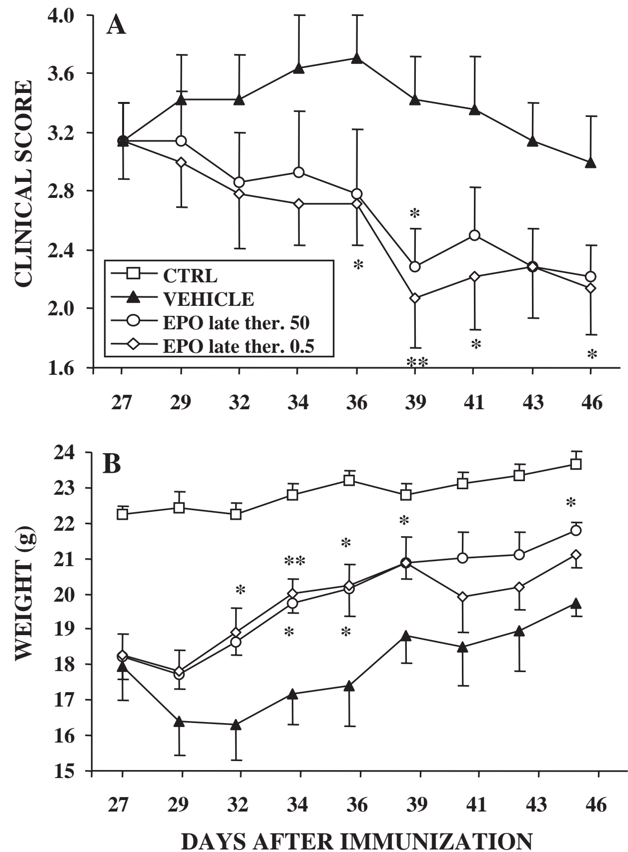

Fig. 5. Therapeutic efficacy of late CEPO treatment on disease score (A) and body weight (B). Mice were immunized with MOG_{35-55} and injected with vehicle or CEPO (50.0 or 0.5 μg/kg i.p.) starting at day 27 following EAE induction (late therapeutic schedule). Data are the mean±S.E.M., $n=7$ ($n=5$ in the case of healthy mice). $*P>0.05$ vs. respective vehicle-treated group by Student's t-test.

When CEPO treatment was started late after disease onset (day 27), the clinical score was improved (Fig. 5A), an effect which was reflected by a significantly reduced body weight loss (Fig. 5B). The clinical parameters of EAE mice treated with CEPO (Fig. 5) or EPO (Fig. 2) according to this late treatment schedule are reported in Table 2. It can be seen that, as observed in the experiments from Table 1, the effect of CEPO was more prominent in terms of reducing the number of mice with the most severe (>3) clinical score.

Table 2
Clinical EAE parameters in mice treated with EPO or CEPO starting 15 days after onset (late therapeutic schedule)

Treatment	Score at sacrifice (day 46)	AUC (days 27–46)	Day of maximal score	No. of mice with score ≥2	No. of mice with score ≥3
Vehicle	3.0±0.8	26.6±2.3	36	7/7	5/7
EPO 0.5 μg/kg	2.4±0.5	22.6±2.5	29	5/7	3/7
EPO 50.0 μg/kg	2.4±0.9	22.7±2.6	29	7/7	3/7
CEPO 0.5 μg/kg	2.1±0.8	19.9±2.4**	27	6/7	1/7
CEPO 50.0 μg/kg	2.2±0.6	21.0±2.4*	27	5/7	2/7

Data are mean±S.E.M. of the data shown in Figs. 3A and 5A.
* $P<0.05$ vs. vehicle by ANOVA.
** $P<0.01$ vs. vehicle by ANOVA.

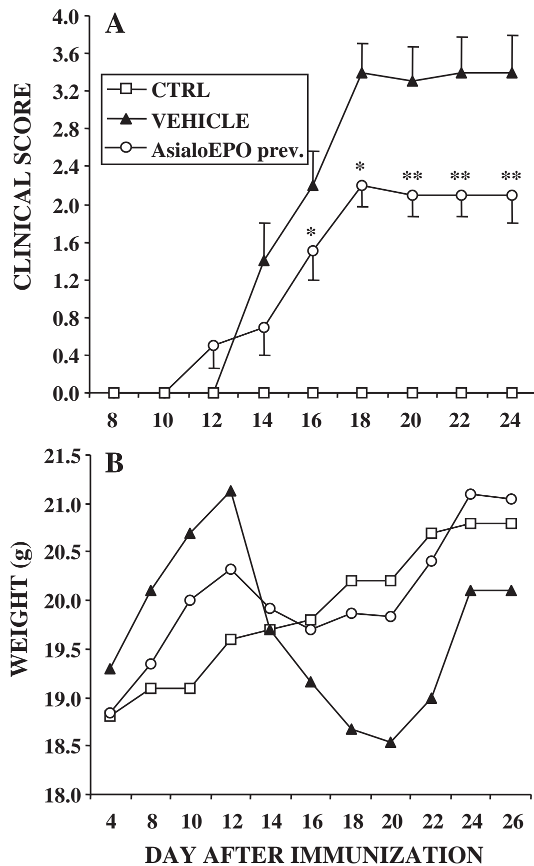

Fig. 6. Efficacy of asialoEPO on disease score (A) and body weight (B). Mice were immunized with MOG_{35-55} and injected with vehicle or asialoEPO (50 μg/kg bw i.p.) starting at day 3 following EAE induction and continuing three times weekly (preventive schedule). In panel A, disease score is reported only from day of onset. Data are the mean ± S.E.M., $n = 10$. *$P < 0.05$, **$P < 0.01$ by Mann–Whitney test.

In this experiment, in untreated EAE mice, the hematocrit at sacrifice was 53.4 ± 3.5% (range = 46.3–57.1), while it was 56.0 ± 2.5% (range = 52.6–59.5) and 53.1 ± 2.4% (range = 49.7–56.7) in CEPO-treated mice for 50.0 and 0.5 μg/kg, respectively.

We also tested the effect of asialoEPO using the preventive treatment schedule and the same dose used for most of the experiments described above (50 μg/kg bw, i.p., starting from day 3 after immunization). As shown in Fig. 6, asialoEPO significantly protected from EAE, reducing the disease severity, delaying its onset and reducing body weight loss, in a way comparable to EPO. In these experiments, the hematocrit was determined at sacrifice, on day 24, and found not to be altered by asialoEPO (vehicle: 43.2 ± 3.8, range = 37.4–48.8; asialoEPO: 41.8 ± 4.9, range = 37.2–50.9).

3.3. EPO and CEPO administration reduces IFN-γ production by MOG-stimulated spleen cells

For these experiments, mice were treated with EPO or CEPO at the doses of 0.5 or 50.0 μg/kg i.p. starting from day 27 after immunization (“late therapeutic” schedule), and treatment was continued until day 60, when mice (4 animals per group) were sacrificed. Splenocytes were prepared and stimulated with different concentrations of MOG_{35-55} or ConA and cytokines were measured in supernatants at the times indicated in Materials and methods. Although there was a trend towards a reduced IFN-γ production in splenocytes, this was not statistically significant.

We therefore evaluated IFN-γ and IL-10 production in the same cultures by ELISPOT. As shown in Table 3, using the late therapeutic schedule, all treatments of EPO and CEPO significantly reduced the number of IFN-γ-secreting cells, both in ConA- and MOG-stimulated cells. However the reduction was more evident in MOG-stimulated cells (mean change = −67.3%) than in ConA-stimulated cells (mean change = −19.3%). On the contrary, a statistically significant reduction in the number of IL-10-secreting cells was observed only in MOG_{35-55}-stimulated cells. The same table shows that healthy, non-EAE mice, had a normal response to ConA but did not respond to the antigen.

3.4. EPO diminishes the inflammatory response

Immunohistochemical analysis of the lumbar spinal cord sections were done in untreated healthy mice and in EAE mice treated with vehicle or EPO using the preventive treatment schedule. Anti-GFAP- and anti-CD11b-stained

Table 3
Cytokine production from spleen cells: ELISPOT measurement

Treatment	IFN-γ-secreting cells		IL-10-secreting cells	
	ConA	MOG	ConA	MOG
Vehicle	654.6 ± 12.1	162.1 ± 19.9	196.6 ± 16.2	117.1 ± 11.6
EPO 0.5 μg/kg	553.4 ± 36.2***	45.4 ± 11.7**	143.4 ± 24.8	53.1 ± 23.9**
EPO 50.0 μg/kg	533.8 ± 42.1*	61.4 ± 5.4**	145.1 ± 39.3	84.6 ± 24.4
CEPO 0.5 μg/kg	499.7 ± 27.3*	50.3 ± 4.8**	204.3 ± 21.9	36.8 ± 20.6*
CEPO 50.0μg/kg	526.1 ± 41.1*	54.8 ± 14.8**	159.6 ± 28.1	49.8 ± 18.4***
Non-EAE controls	519.2 ± 10.1	3.8 ± 1.1	162.5 ± 24.2	24.5 ± 8.5

Data are mean ± S.E.M.
* $P < 0.02$ by Student’s t-test vs. vehicle.
** $P < 0.0001$ by Student’s t-test vs. vehicle.
*** $P < 0.05$ by Student’s t-test vs. vehicle.

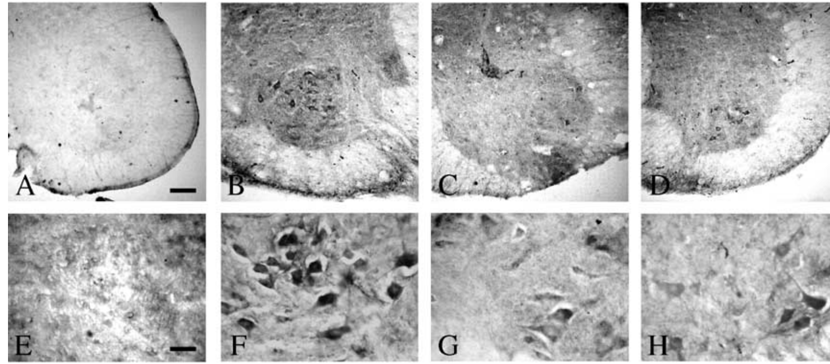

Fig. 7. EPO decreases EAE-induced TNF-α immunostaining in the spinal cord. Mice were treated with or without EPO (50 μg/kg i.p. three times weekly from day 3) and sacrificed on day 29. Representative sections obtained from the lumbar spinal cord from healthy mice (A, E), EAE mice treated with PBS (B, F), EAE mice treated with EPO (C, G) and EAE mice treated with CEPO (D, H). Upper panels (A–D), scale bar = 500 μm; lower panels (E–H), scale bar = 125 μm.

sections of lumbar spinal cord showed a weak increase in glial immunoreactivity that was not affected by EPO (not shown). A well-defined immunoreactivity for TNF-α can be seen in EAE mice, almost exclusively in the anterior horn neurons (Fig. 7). Treatment with EPO and, to a lesser extent, with CEPO reduced both the number of TNF-α-positive neurones, and their immunoreactivity.

We also measured the mRNA expression for IL-1β, IL-1Ra and TNF-α in the spinal cord of mice receiving EPO or CEPO according to the preventive schedule and at two different doses (50.0 and 0.5 μg/kg). As shown in Table 4, expression of all three cytokines was greatly induced by EAE, compared to naive mice. Both EPO and CEPO diminished cytokine expression, and this effect was greater at the highest doses tested.

4. Discussion

The present study shows that: 1) EPO is effective in a chronic model of EAE; 2) A protective effect, although diminished compared to the preventative schedule is observed also when the drug is given late after the induction of the disease and lasts for at least 3 weeks after interruption of treatment; 3) The EPO effect is independent of an increase in hematocrit, as non-erythropoietic variants asialoEPO and CEPO are also active; 4) this effect is also observed with CEPO that does not bind the classical EPOR; 5) Action of EPO and CEPO is associated with a decrease in the production of inflammatory cytokines in the spinal cord and peripheral lymphocytes.

Table 4
Effect of EPO or CEPO on spinal cord cytokine mRNA in EAE mice

EAE treatment group	IL-1β	IL-1Ra	TNF-α
Non-EAE controls	198 ± 75	98 ± 33	175 ± 44
Vehicle	797 ± 93	1097 ± 325	563 ± 125
EPO 0.5 μg/kg	626 ± 266	463 ± 220	350 ± 121
EPO 50.0 μg/kg	286 ± 112*	189 ± 78*	141 ± 37*
CEPO 0.5 μg/kg	600 ± 211	697 ± 421	541 ± 159
CEPO 50.0 μg/kg	344 ± 83*	220 ± 74*	340 ± 93

PCR data (arbitrary units normalized to housekeeping gene, as described in Materials and methods) ± S.E.M. ($n = 3–4$).
* $P < 0.05$ vs. vehicle.

It is important to note that in most experiments, treatment with EPO or CEPO for periods longer than one month induced anaemia in ~50% of the animals (data not shown) because administration of human proteins to mice induces formation of neutralizing antibodies recognizing also endogenous EPO, a well-known phenomenon with EPO (Casadevall et al., 2002). Moreover, this effect was more pronounced with mice treated according to the preventive schedule, since they received EPO for 24 days longer than with the late therapeutic schedule, and 10 days longer than with the therapeutic schedule. In spite of this, the preventative schedule provided superior protection from EAE.

A main finding of this study is that non-erythropoietic variants of EPO that do not increase the hematocrit are active in this model of EAE. The two EPO derivatives used are representative of two different strategies to dissociate erythropoietic and neuroprotective action. AsialoEPO, like EPO, binds dimeric EPOR and induces erythroid differentiation in vitro (Erbayraktar et al., 2003); its activity at the molecular level is therefore identical to EPO and it lacks an effect on the hematocrit in vivo due to its very short half-life (Erbayraktar et al., 2003; Fukuda et al., 1989; Imai et al., 1990).

On the other hand, CEPO lacks the ability to bind EPOR, both on soluble Fc-EPOR fusion constructs and on haematopoietic cells and does not activate classical EPOR-mediated signalling mechanisms (Leist et al., 2004). It can therefore be safely assumed that CEPO, and possibly EPO, act through different receptor systems. For instance, we have recently shown that CEPO requires the common β-receptor (CD131), the signalling subunit of the receptors for IL-3, IL-5 and GM-CSF to be effective in models of spinal cord injury (Brines et al., 2004).

In fact, EPOR-mediated neuroprotection, in terms of protection from neuronal apoptosis in vitro, was suggested to proceed though activation of NF-κB (Digicaylioglu and Lipton, 2001). This cannot be the case in EAE, as NF-κB induces transcription of inflammatory genes, including

TNF-α, rather than decreasing them as we observed in this and other (Agnello et al., 2002) EAE models, as well as in cerebral ischemia (Villa et al., 2003). In fact, inhibition of NF-κB, using knock-out mice (Hilliard et al., 1999, 2002) or using chemical (Pahan and Schmid, 2000) or peptide (Dasgupta et al., 2004) inhibitors of NF-κB activation, is protective in EAE.

Clearly, the complexity of EAE makes it difficult identify a single mechanism of action for CEPO. However, inflammation is clearly a common pathogenic component of the chronic (EAE, diabetic neuropathy) and acute (spinal cord injury, cerebral ischemia) models where CEPO has shown efficacy (Leist et al., 2004). As shown here, EPO reduced the spinal cord expression of TNF-α, a key molecule in the development of this disease (reviewed in Steinman, J Exp Med 2003). Expression of IL-1β was also reduced by EPO or CEPO treatments, and peripheral lymphocytes from mice treated with EPO or CEPO produced less IFN-γ and more IL-10, an important suppressor cytokine and a major regulatory agent in inflammatory response. On the other hand, unlike IFN-β (Nicoletti et al., 1996), EPO does not increase spinal cord expression of IL-1Ra whose induction, in this context, probably reflects a response to the inflammatory process. Taken together, these results indicate that EPO importantly reduces the inflammatory milieu in EAE.

Another possible mechanism of action of these compounds is via promotion of repair mechanisms. Neurorepair, including remyelination, is an important physiological process and a therapeutic strategy (Martino, 2004), as indicated by the effectiveness of stem cell therapy in EAE (Pluchino et al., 2003). EPO has been reported to promote angiogenesis, neurogenesis as well as differentiation of oligodendrocytes (Shingo et al., 2001; Sugawa et al., 2002; Wang et al., 2004). This could be an important effect of EPO in the chronic model of EAE used here. In fact, a recent report has indicated that EPO increases oligodendrocyte progenitor cell proliferation in a different model of EAE, induced in SJL/J mice by immunization with myelin proteolipid protein peptide 139–151 (Zhang et al., 2005) and augments BDNF expression in the CNS in this and other experimental conditions (Viviani et al., 2005; Zhang et al., 2005). On the other hand, it should be noted that, while an augmented oligodendrogenesis might be important in chronic EAE models, this can hardly contribute to the effect of EPO in acute EAE, as in Lewis rats the disease recovers very rapidly (Agnello et al., 2002). Since EPO has neurotrophic activity (Ghezzi and Brines, 2004), it is important to note that other growth factors, including nerve growth factor (Micera et al., 2000), ciliary neurotrophic factor (Linker et al., 2002), insulin-like growth factor-1 (Yao et al., 1995), and granulocyte colony-stimulating factor (Lock et al., 2002), are effective in models of EAE. In a previous work, we noted that CEPO lacks its tissue-protective activity in the absence of the common β-receptor, the signal-transducing subunit shared by the granulocyte–macrophage colony-stimulating factor (GM–CSF), and the IL-3 and IL-5 receptors. However, GM–CSF was reported to worsen, rather than protecting from, EAE (Marusic et al., 2002; McQualter et al., 2001), indicating that the mere engagement of this common β-chain does not explain the protective action of CEPO.

Finally, it is important to consider EPO in the context of the pathogenesis of MS. EPO transcription is regulated by the hypoxia inducible factor-1 (HIF-1) and a hypoxia-like metabolic injury occurs in MS (Lassmann, 2003). More recently it was shown that up-regulation of neuroprotective pathways against hypoxia are activated in MS brains (Graumann et al., 2003). (It should also be pointed out that cytokines are known to stimulate HIF directly in normoxic cells, which presumably plays a role in inflammatory injuries.) It is therefore interesting that a HIF-1 target gene product has protective effects in animal models of MS. The other major HIF-1 target gene, vascular endothelial growth factor (VEGF), which was also shown to be induced in MS patients (Graumann et al., 2003; Proescholdt et al., 2002) and in a model of EAE in rats and guinea pigs (Proescholdt et al., 2002; Kirk and Karlik, 2003), seems not to share the protective action of EPO and was suggested, on the contrary, to be implicated in the inflammatory component of EAE (Kirk and Karlik, 2003). In fact, intracerebral infusion of VEGF in a rat model of EAE exacerbated the inflammatory response by inducing BBB damage (Proescholdt et al., 2002). The different behaviour of EPO and VEGF might be related, among other things, to the fact that, while VEGF is known to reduce tight junction proteins allowing leakage from within the capillary into the brain parenchyma via increased permeability of the blood–brain barrier (BBB), EPO counteracts this effect (Martinez-Estrada et al., 2003). In support, BBB disruption takes place in EAE and EPO was shown to diminish BBB leakage in EAE (Li et al., 2004). Since leakage contributes to the inflammatory process and, vice versa, may be a consequence of inflammation (de Vries et al., 1997), this mechanism could be part of the overall anti-neuroinflammatory action of EPO in EAE.

Translation of effectiveness of EPO in animal models of cerebral ischemia to human disease has already been validated in a proof-of-concept clinical trial showing that administration of EPO in patients with cerebral ischemia is both safe and beneficial (Ehrenreich et al., 2002). Hopefully, a successful translation from EAE to multiple sclerosis or other demyelinating diseases will also occur. Antagonism of inflammation by CEPO and related tissue-protective cytokines with reduced EPO adverse effects may thus provide a new avenue for the therapy of MS.

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