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Prevention of clinical and histological signs of proteolipid protein (PLP)-induced experimental allergic encephalomyelitis (EAE) in mice by the water-soluble carbon monoxide-releasing molecule (CORM)-A1

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Summary

We have evaluated the effects of the carbon monoxide-releasing molecule CORM-A1 [$Na_2(BH_3CO_2)$; ALF421] on the development of relapsing–remitting experimental allergic encephalomyelitis (EAE) in SJL mice, an established model of multiple sclerosis (MS). The data show that the prolonged prophylactic administration of CORM-A1 improves the clinical and histopathological signs of EAE, as shown by a reduced cumulative score, shorter duration and a lower cumulative incidence of the disease as well as milder inflammatory infiltrations of the spinal cords. This study suggests that the use of CORM-A1 might represent a novel therapeutic strategy for the treatment of multiple sclerosis.

Keywords: animal models, carbon monoxide-releasing molecules, CORM-A1, experimental allergic encephalomyelitis, SJL mice

Introduction

During the past decade, numerous studies have shown that carbon monoxide (CO) exerts pleiotropic activities *in vivo* by eliciting various physiological responses that can have beneficial effects for several pathological conditions, such as inflammation, transplantation-related hypoxia/reperfusion damage and pulmonary hypertension (for a comprehensive review see [1]). CO is produced intracellularly through the catabolism of haem molecules by haem oxygenase (HO) enzymes, which are present in several mammalian tissues, including brain, liver, spleen, vascular endothelium and smooth muscle [2]. Two isoforms of HO are known, the inducible enzyme HO-1 and the constitutively expressed HO-2 [1]. Haem degradation by HO generates three products, CO biliverdin and Fe^{2+}; however, there is good evidence that CO represents the effector signalling molecule responsible for the anti-inflammatory activity of HO-1. The anti-inflammatory activity of HO is supported strongly by data from animal models of HO-1 deficiency [3] and the described cases of HO-1 deficiency in humans [4]. In a number of animal studies it has been shown that exogenous administration of controlled quantities of CO represents an effective strategy for the treatment of multiple conditions [1] characterized by inflammatory responses such as endotoxaemia [5–9], acute pancreatitis [10], lung injury [11–15] and arteriosclerotic lesions [16].

Recent evidence has shown that CO mitigates microglial activation in neuroinflammatory diseases [17] and reduces autoimmune inflammation in a model of relapsing–remitting experimental allergic encephalomyelitis (EAE) by inhibiting major histocompatibility complex (MHC) class II expression, T helper and CD8 T cell accumulation within the central nervous system (CNS) [18]. In addition, HO-1 activation limits neutrophil infiltration into the CNS by stabilizing the blood–spinal cord barrier, and decreases vascular dysfunction in cases of spinal cord injury [19]. Taken together, these findings open the possibility of using exogenous CO for the treatment of autoimmune neuroinflammatory diseases such as multiple sclerosis (MS). Unexpectedly, however, in a pilot study conducted on human volunteers [20], inhalation of CO gas [500 parts per million (ppm) CO for 1 h] upon infusion with lipopolysaccharide (LPS) did not exert an effect on cytokine production, as had been predicted from previous animal studies. This may imply that new and more accurate ways of CO administration are needed

for the clinical development of such therapeutic strategies. CO-releasing molecules (CORMs) are a class of compounds capable of liberating controlled quantities of CO in biological systems, thus overcoming the limitations of inhaled CO gas [1,21]. Several CORMs are currently under investigation in order to tailor potential therapeutic approaches for the prevention of inflammatory disorders, vascular dysfunction and organ rejection [1].

In this work we tested the efficacy of the water-soluble compound CORM-A1 on the development of clinical and histological features of murine relapsing–remitting EAE. CORM-A1 is a boranocarboxylate with a half-life of 21·4 min that is able to slowly liberate CO from its carboxyl group through hydrolysis under physiological conditions [22]. This molecule has already proved to have vasodilatory [22], renoprotective [23], anti-ischaemic [24] and anti-apoptotic [25] properties.

Materials and methods

Animals

Female Swiss Jackson Laboratory (SJL) mice ranging in age from 6 to 7 weeks were purchased from Charles River (Calco, Italy). The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Induction of EAE

Proteolipid protein (PLP) (139–151) was synthesized by Genemed Synthesis (San Francisco, CA, USA). EAE was induced as described previously [26]. Briefly, mice were immunized with 75 μg PLP emulsified in complete Freund's adjuvant (CFA) with 6 mg/ml *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA) to make a 1:1 emulsion. Each mouse received subcutaneous injections of 200 μl emulsion divided among four sites draining into the axillary and inguinal lymph nodes. Pertussis toxin (Calbiochem, Nottingham, UK) was used as a co-adjuvant, and was administered intraperitoneally (i.p.) at a dose of 200 ng/mouse on day 0 and again on day 2.

Drug

CORM-A1 (ALF421) was synthesized as described elsewhere [22]. It was dissolved in sterile phosphate-buffered saline (PBS) and administered i.p. to mice at a dose of 2 mg/kg in a final volume of 200 μl. Dexamethasone (Soldesan, Laboratorio Farmacologico Milanese, varese, Italy) was bought from a local pharmacy and used as the positive control drug. It was administered at a dose of 0·3 mg/kg body weight i.p. in a final volume of 200 μl.

CO determination in the blood

CD-1 female mice from Charles River (6–8 weeks old) were treated with 3, 10 or 40 mg/kg CORM-A1 (i.p.). After the respective times (10, 40 or 70 min) blood was collected and diluted 50 times in ice-cold MilliQ water (non-treated mice were used as control – time 0 min). For CO quantification, the protocol described by Vreman *et al.* [27] was followed. Briefly, CO was liberated as gas in a closed vial by adding 25 μl of water and 5 μl of sulphosalicylic acid [SSA, 30% (wt/vol)] to 30 μl of diluted blood sample. The vials were incubated on ice for at least 10 min before being analysed. The gas in the headspace of the vials was analysed quantitatively with a gas chromatograph (GC) equipped with a reducing-compound photometry detector (RCP detector) (Peak Laboratories, Mountain View, CA, USA), which allows quantification of CO in gas at concentrations as low as 1–2 parts per billion (ppb). The amount of CO was calculated using a calibration curve prepared from CO standards. The COHb was determined using a AVOXimeter 4000 (A-VOX Systems, Inc., San Antonio, TX, USA).

Experimental design

Different treatment regimens were used to evaluate the influence of CORM-A1 on the development of EAE. In one experiment, CORM-A1 was administered using an 'early prophylactic regimen' for 30 consecutive days starting from the day of immunization. The mice were observed for an additional 15 days to evaluate the possible rebound effects after termination of the treatment. In a second set of studies, the mice were treated with either CORM-A1 or its vehicle under a 'late prophylactic regimen' starting from day 10 post-immunization for 20 consecutive days. None of the mice had developed clinical signs of EAE at initiation of the treatment (see Table 1). Finally, another study was performed where the mice were treated for only 16 consecutive days from immunization in order to verify whether this short-term treatment was sufficient to prevent EAE development. These mice were observed until day 30 post-immunization. Control animals were treated with sterile PBS under the same experimental conditions. For only the early prophylactic regimen, an additional group of mice was included that were treated with the positive control drug dexamethasone (Soldesan) administered daily at the dose of 0·3 mg/kg from day 0 to day 30. We and others have shown previously that dexamethasone can prevent clinical and histological signs of PLP-induced EAE in SJL mice under this prophylactic regimen [28].

Clinical scoring

Animals were observed daily by measuring their body weight and assessing clinical signs of EAE. They were assigned one of the following clinical grades by an observer who was

Table 1. Clinical features of proteolipid protein (PLP)-induced experimental allergic encephalomyelitis (EAE) in Swiss Jackson Laboratory (SJL) mice upon treatment with carbon monoxide-releasing molecule (CORM)-A1, dexamethasone or vehicle.

Experimental groups	Cumulative score 0–30	Cumulative score 31–45	Onset	Duration	Incidence %	Incidence after interr. (%)
CORM-A1 early prophylaxis (0–30)	4·3 ± 7·3	2·8 ± 7·8	16·7 ± 1·5	2 ± 3·2	37·5	37·5
CORM-A1 prophylaxis (0–16)	15·3 ± 11·2		22·9 ± 4·6	10·9 ± 6·5	88·9	
CORM-A1 late prophylaxis (10–30)	4·8 ± 9·4		20·7 ± 5·5	2·2 ± 4·2	30·0	
Dexamethasone	6 ± 6·9	11·4 ± 11·6	18·4 ± 4·3	3·3 ± 3·2	62·5	75·0
Vehicle	19·6 ± 16	17·4 ± 14·1	17·5 ± 3·9	8·3 ± 6·0	85·7	85·7
Mann–Whitney						
CORM-A1 early prophylaxis (0–30)	0·04	0·03	0·90	0·03		
CORM-A1 prophylaxis (0–16)	0·94		0·71	0·31		
CORM-A1 late prophylaxis (10–30)	0·05		0·36	0·05		
Dexamethasone	0·07	0·34	0·65	0·06		

blinded to the treatment: 0, no illness; 1, flaccid tail; 2, moderate paraparesis; 3, severe paraparesis; 4, moribund state; and 5, death.

A cumulative clinical score was calculated for each mouse by adding the daily scores from the day of onset (score disease ≥ 1) until the end of the experiment. The duration of disease was calculated each day by assigning the animal a score of 0 for a clinical score of 0 and 1 for any higher clinical score.

Histological analyses of brain and spinal cord

At the end of the study (day 30), animals were anaesthetized with tri-bromo-ethanol (300 mg/kg) and then perfused through the left ventricle with cold PBS (4°C) for 3–5 min followed by 4% paraformaldehyde (Sigma-Aldrich, Milan, Italy) for 10 min. The brain and spinal cord were resected and stored in 10% paraformaldehyde at 4°C. Serial 5-μm thick cross-sections were prepared and were stained with haematoxylin and eosin to assess inflammation [29–31]. In each group, at least 10 sections per mouse distributed over the whole length of the spinal cord were examined histologically and quantified. Inflammation was scored as described previously [30] using a semi-quantitative scale: 0 = no inflammatory cells; 1 = a few cells; 2 = moderate, perivascular cuffing; 3 = dense inflammatory cell infiltrates, parenchymal necrosis.

Statistical analysis

The clinical results are shown as mean ± standard deviation, which was calculated based on data from two independent experiments. As the experiments were highly reproducible and with variability lower than 5%, the data were merged and are shown as a single study. Statistical analysis for significant differences on clinical scores was performed with the non-parametric Mann–Whitney test for the clinical course of EAE and the histopathological parameters.

Results

CORM-A1 releases CO *in vivo* in a controlled manner

The amount of CO released by CORM-A1 *in vivo* was monitored in blood samples obtained after i.p. administration in CD-1 mice and measured by the GC-RCP method [28]. As seen in Fig. 1, for the non-toxic doses of 3 and 10 mg/kg the values of CO measured in the blood have their maximum around 10 min post-administration and correspond roughly to COHb levels of 3% and 6%, respectively. A slow decay of the CO in circulation is then observed, and after 70 min post-administration the value of COHb is still slightly above

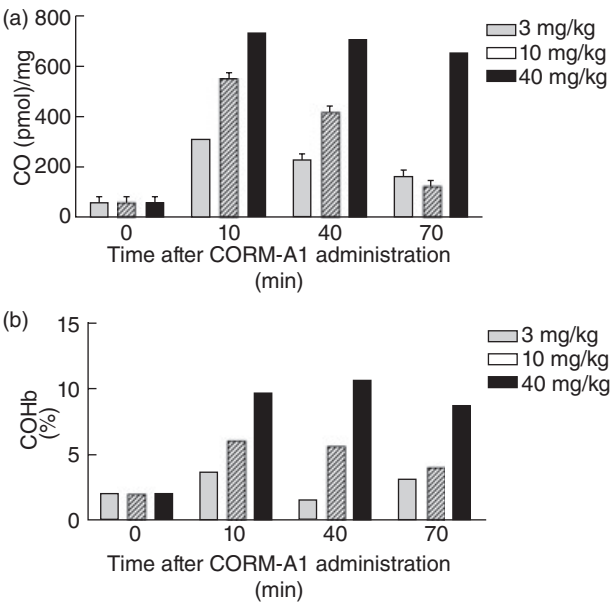

Fig. 1. Carbon monoxide levels in blood of mice treated with carbon monoxide-releasing molecule (CORM)-A1 (a) measured by gas chromatograph-reducing compound photometry detector (GC-RCP); (b) measured by oximetry. The GC-RCP results are expressed as mean ± standard deviation of two independent measurements.

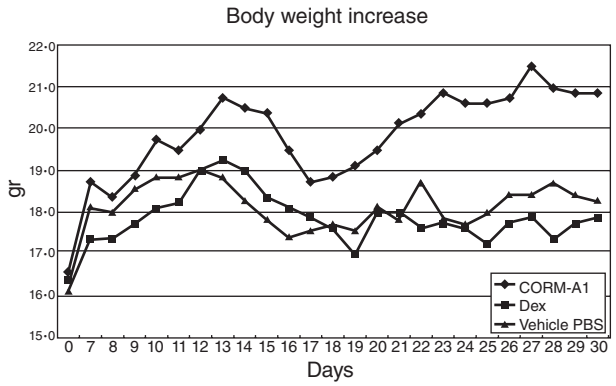

Fig. 2. Body weight variations of the mice treated with carbon monoxide-releasing molecule (CORM)-A1 (days 0–30) along with mice treated with dexamethasone or vehicle only starting on day 0.

the basal level. In the case of the higher dose (40 mg/kg), a similar slow decay of CO levels in the blood (or COHb levels) is also recorded.

CORM-A1 protects from EAE in both early and late prophylactic regimens

The treatment with CORM-A1 was well tolerated as judged by the clinical status of mice and by the steady increase in their body weight (Fig. 2). In contrast to the mice treated with CORM-A1, a significant reduction of body weight gain was observed both in the control mice treated with the vehicle and in those treated with the positive control drug dexamethasone. Reduced body weight gain during EAE or upon prolonged dexamethasone treatment are well-documented events [28,32]. In agreement with the established model of EAE in the SJL mouse strain, clinical signs of disease in the vehicle-treated mice appeared between 13 and 23 days post-immunization and the first attack was followed by variable relapses, with some mice exhibiting up to three relapses (Table 1, Fig. 3a–c). The early prophylactic treatment with CORM-A1 significantly reduced both the cumulative score and the duration of the disease compared to vehicle-treated controls (cumulative score $P = 0{\cdot}036$; duration $P = 0{\cdot}027$). CORM-A1-treated mice also exhibited a lower cumulative incidence of EAE than controls (38% *versus* 86%) (Table 1, Fig. 3a and b). Furthermore, the incidence of the disease in the mice treated with CORM-A1 was lower than in the vehicle-treated mice (Table 1, Fig. 3a and b). These results were rendered even more significant by the observation that these protective effects were maintained for a significant period of time after termination of the treatment; i.e. during the following 15 days the CORM-A1-treated mice showed a significantly lower cumulative disease score ($P = 0{\cdot}026$) and maintained a lower incidence of disease compared to the vehicle-treated mice (3% vs. 17%) (Table 1, Fig. 3a). In contrast, when the treatment was interrupted on day 16 (6 days after the beginning of the disease in the controls) the mice developed the disease and showed a clinical course similar to that of the vehicle-treated mice (Fig. 3c). Of relevance is the observation that the degree of protection exerted by prolonged prophylactic treatment with CORM-A1 was superior to that afforded by the reference compound dexamethasone (Table 1, Fig. 3).

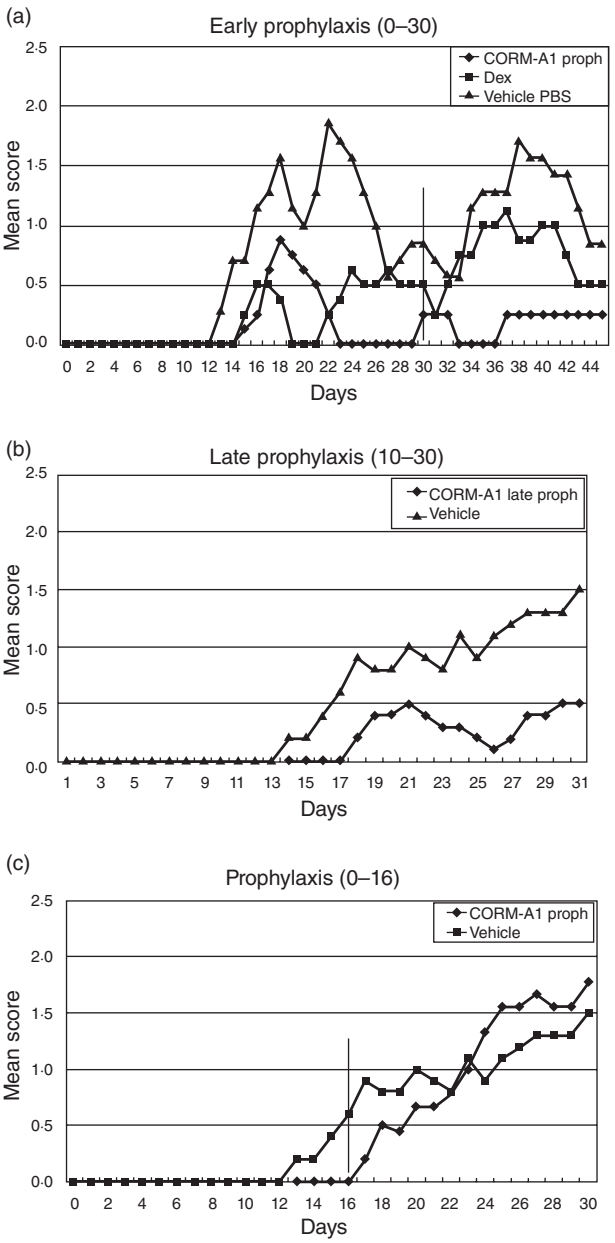

Fig. 3. Carbon monoxide-releasing molecule (CORM)-A1 treatment improves the clinical course of proteolipid protein (PLP)-induced experimental allergic encephalomyelitis (EAE) in Swiss Jackson Laboratory (SJL) mice in different treatment regimens: (a) early prophylaxis (days 0–30); (b) late prophylaxis (days 10–30); (c) prophylaxis (days 0–16).

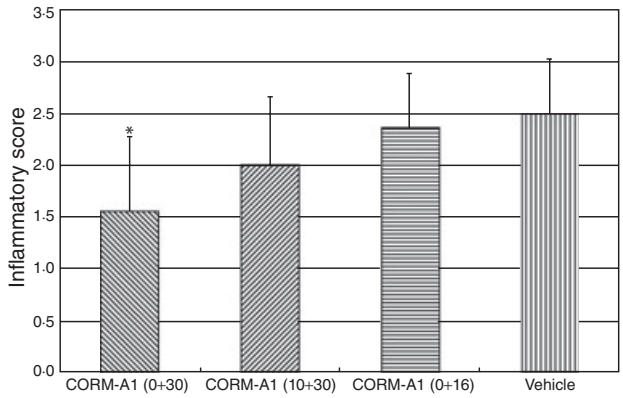

Fig. 4. Inflammation of the spinal cord assessed on haematoxylin and eosin-stained paraffin sections using a semi-quantitative scale: 0, no inflammatory cells; 1, a few cells; 2, moderate, perivascular cuffing; 3, dense inflammatory cell infiltrates, parenchymal necrosis. Values presented are the means ± standard deviation from eight mice assessed in the group.

A trend towards reduced cumulative scores, shorter duration of the disease and a reduction in the cumulative incidence (30% *versus* 86%) was also observed in the mice treated with CORM-A1 under the late prophylactic regimen (Table 1, Fig. 3a and b).

CORM-A1 improves histopathological changes in the spinal cord

Histopathological features were detectable only in the spinal cords of the mice that showed clinical symptoms, while few or no signs of inflammation were evident in the mice treated with the reference compound dexamethasone.

Histopathological examination of the spinal cords from the mice treated with the vehicle revealed an evident infiltration of polymorphonucleated cells (PMNC). This PMNC infiltration was clearly reduced in the mice prophylactically (days 0–30) treated with CORM-A1 (Fig. 4). Improved histological signs were also evident in the group treated with the 'late prophylactic' regimen (days 10–30), although these differences did not reach statistical significance in comparison to the vehicle group. No differences with the vehicle group were observed in the group of mice treated with the short prophylactic course (days 0–16), thus confirming the clinical course of the disease (Fig. 4).

Discussion

Unlike the first prototypic molecule CORM-3, CORM-A1 is a water-soluble CO-releasing molecule that does not contain a transition metal. CORM-A1 liberates CO to deoxymyoglobin in a pH- and temperature-dependent manner with a half-life of approximately 21 min at 37°C and pH 7·4, which corresponds to a slower rate of CO release under physiological conditions than that published for CORM-3 [22]. In isolated aortic rings CORM-A1 provoked a gradual but profound vasorelaxation over time. When administered *in vivo* intravenously, CORM-A1 exerted a mild decrease in mean arterial pressure over time. These pharmacological features, as well as the values of COHb obtained after administration *in vivo*, characterize CORM-A1 as a slow releaser of CO, which may be useful for the treatment of conditions that may benefit from controlled, slow CO release such as chronic immunoinflammatory diseases. Systemic treatment with CORM-A1 has been shown to exert cerebroprotective effects against seizure-induced neonatal vascular injury in pigs by delivering CO to the brain, eliciting the vasodilator and cytoprotective effects of CO in the cerebral circulation [33], but the compound has not been tested so far in preclinical models of immunoinflammation and autoimmune diseases. However, CORM-3 was shown to have beneficial effects in immunoinflammatory conditions such as murine arthritis [34,35]. We have therefore demonstrated for the first time that CORM-A1 exerts powerful immunomodulatory effects in PLP-induced EAE in SJL mice. The prolonged prophylactic treatment with CORM-A1 powerfully counteracted clinical and histological signs of relapsing–remitting EAE. This entailed reduced cumulative scores, shorter duration and lower cumulative incidence of the disease and milder inflammatory infiltration of the spinal cords compared to vehicle-treated controls. The duration of treatment with CORM-A1 appeared to be important for its beneficial action, as statistical significance was achieved only in the group of mice that were treated with CORM-A1 for 30 consecutive days from the day of immunization. Twenty consecutive days of treatment from day 10 after immunization only demonstrated a trend toward a milder course of EAE and a lower cumulative incidence compared to controls. Finally, mice treated with CORM-A1 for 16 consecutive days starting from the day of immunization developed a form of EAE indistinguishable from that of controls.

Although the mechanistic mode of action(s) by which CORM-A1 exerted its beneficial effects in this EAE model have not been currently ascertained, we attribute the action to the released CO and its pharmacological mode of action. The persistent protection against EAE development after drug withdrawal may hint at immunological effects, such as the induction of long-lasting regulatory T cells (T_{reg}) cells and/or elimination of autoreactive encephalytogenic T cells. Furthermore, a modulation of T cell responses in the treated mice towards anti-inflammatory type 2–type 3 cytokine profiles may also be considered based on the previously described capacity of CO therapy to modulate dendritic cell function [36]. In addition, by releasing CO, CORM-A1 may have down-regulated immunoinflammatory pathways contributing to EAE development, such as lymphocyte proliferation [18,37], the production of proinflammatory cytokines from peripheral immune cells [10,34,38,39], dendritic cell activation [18,36,40], the expression of adhesion molecules [34,41]

and the suppression of leucocyte infiltration [6]. Studies are in progress to dismantle the precise mode of action of CORM-A1 in this and possibly other rodent models of EAE.

The value of COHb attained at the dose of 2 mg/kg used in the present EAE study never exceeded 3% COHb levels, which are relatively safe and only slightly above basal values, indicating that CORM-A1 can exert its therapeutic activity within a range of COHb levels far lower from those considered to be toxic to humans [42].

Comparisons of the protective effects of CORM-A1 with those of the positive control drug dexamethasone in the model of EAE in SJL mice allows appreciation of the potency of its effects, as well as the different impacts of CORM-A1 and dexamethasone on the development of EAE. CORM-A1 treatment under the 'early prophylactic' regimen was superior to dexamethasone in the clinical disease score, the histopathological score, the time and degree of relapse after cessation of the treatment and the treatment-associated toxicity. CORM-A1 treatment seemed not to be associated with any significant toxicity, as indicated by the steady increase in the body weights of the animals. In fact, the transient decrease in the body weight by CORM-A1 between days 15 and 23 correlates with the transient increase in the disease score. In contrast, dexamethasone inhibited body weight gain or caused a drop in body weights during the entire treatment period.

Perhaps most remarkable was the protection afforded by CORM-A1 after cessation of treatment, whereas dexamethasone withdrawal led to rapid relapse of EAE. This may indicate that CORM-A1 and dexamethasone prevent EAE by interfering at different places or stages in the immunopathogenic pathway and that they possess distinct immunopharmacological modes of action in this model. If these differences can also be confirmed in other *in vivo* settings, then it might be hypothesized that CORMs and steroids could display additive or synergistic effects in combination treatments. Our present study supports previous findings by Chora *et al.*, which demonstrated an important role for CO in counteracting immunoinflamatory demyelinating pathways *in vivo* [18]. In that study the genetic deficiency of HO-1 was associated with more severe forms of EAE compared to wild-type mice and the induction of HO-1 by cobalt protoporphyrin after EAE onset reversed paralysis in both C57BL/6 and SJL mice affected with EAE [18]. Exogenous CO mimicked these effects, suggesting its important role in the protective effects of HO-1.

Recent and important progress has been achieved in the treatment of different forms of MS by using disease-modifying agents such as interferon (IFN)-β, glatiramer acetate, natalizumab and mitoxantrone, and the introduction of new therapies such as fingolimod is expected to further the management of the disease. None the less, many of these drugs have limitations, such as the high cost, the association with severe adverse events and the development of neutralizing antibodies [43].

Building upon the previously reported beneficial effects of CO exposure in murine EAE [18], in this study we observed the ability of a water-soluble CO releaser to prevent the development of clinical and histological signs of disease in a well-known preclinical model of MS. Indeed, the PLP-induced EAE in SJL mice represents an important proof of concept, indicating the feasibility and conceptual validity of developing CORMs for the treatment of MS. In contrast to CO gas exposure, such molecules, once pharmacologically improved, could provide a safe mode for the delivery of therapeutic doses of CO.

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Disclosures

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References

1 Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. Nat Rev Drug Discov 2010; **9**:728–43.
2 Ryter SW, Choi AM. Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy. Am J Respir Cell Mol Biol 2009; **41**:251–60.
3 Poss KD, Tonegawa S. Heme oxygenase 1 is required for mammalian iron reutilization. Proc Natl Acad Sci USA 1997; **94**:10919–24.
4 Yachie A, Niida Y, Wada T *et al.* Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 1999; **103**:129–35.
5 Sarady JK, Zuckerbraun BS, Bilban M *et al.* Carbon monoxide protection against endotoxic shock involves reciprocal effects on iNOS in the lung and liver. FASEB J 2004; **18**:854–6.
6 Mizuguchi S, Stephen J, Bihari R *et al.* CORM-3-derived CO modulates polymorphonuclear leukocyte migration across the vascular endothelium by reducing levels of cell surface-bound elastase. Am J Physiol Heart Circ Physiol 2009; **297**:H920–9.
7 Tsoyi K, Lee TY, Lee YS *et al.* Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high-mobility group box 1 release in vitro and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model *in vivo*. Mol Pharmacol 2009; **76**:173–82.
8 Lancel S, Hassoun SM, Favory R, Decoster B, Motterlini R, Neviere R. Carbon monoxide rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis. J Pharmacol Exp Ther 2009; **329**:641–8.
9 Desmard M, Davidge KS, Bouvet O *et al.* J. A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against *Pseudomonas aeruginosa* and improves survival in an animal model of bacteraemia. FASEB J 2009; **23**:1023–31.

10 Chen P, Sun B, Chen H *et al*. Effects of carbon monoxide releasing molecule-liberated CO on severe acute pancreatitis in rats. Cytokine 2010; **49**:15–23.
11 Goebel U, Mecklenburg A, Siepe M *et al*. Protective effects of inhaled carbon monoxide in pig lungs during cardiopulmonary bypass are mediated via an induction of the heat shock response. Br J Anaesth 2009; **103**:173–84.
12 Hoetzel A, Dolinay T, Vallbracht S *et al*. Carbon monoxide protects against ventilator-induced lung injury via PPAR-gamma and inhibition of Egr-1. Am J Respir Crit Care Med 2008; **177**:1223–32.
13 Hoetzel A, Schmidt R, Vallbracht S *et al*. Carbon monoxide prevents ventilator-induced lung injury via caveolin-1. Crit Care Med 2009; **37**:1708–15.
14 Sun B, Sun H, Liu C, Shen J, Chen Z, Chen X. Role of CO-releasing molecules liberated CO in attenuating leukocytes sequestration and inflammatory responses in the lung of thermally injured mice. J Surg Res 2007; **139**:128–35.
15 Nemzek JA, Fry C, Abatan O. Low-dose carbon monoxide treatment attenuates early pulmonary neutrophil recruitment after acid aspiration. Am J Physiol Lung Cell Mol Physiol 2008; **294**:L644–53.
16 Otterbein LE, Zuckerbraun BS, Haga M *et al*. Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. Nat Med 2003; **9**:183–90.
17 Bani-Hani MG, Greenstein D, Mann BE, Green CJ, Motterlini R. A carbon monoxide-releasing molecule (CORM-3) attenuates lipopolysaccharide- and interferon-gamma-induced inflammation in microglia. Pharmacol Rep 2006; **58** (Suppl.):132–44.
18 Chora AA, Fontoura P, Cunha A *et al*. Heme oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation. J Clin Invest 2007; **117**:438–47.
19 Yamauchi T, Lin Y, Sharp FR, Noble-Haeusslein LJ. Hemin induces heme oxygenase-1 in spinal cord vasculature and attenuates barrier disruption and neutrophil infiltration in the injured murine spinal cord. J Neurotrauma 2004; **21**:1017–30.
20 Mayr FB, Spiel A, Leitner J *et al*. Effects of carbon monoxide inhalation during experimental endotoxemia in humans. Am J Respir Crit Care Med 2005; **171**:354–60.
21 Motterlini R, Mann BE, Foresti R. Therapeutic applications of carbon monoxide-releasing molecules. Exp Opin Invest Drugs 2005; **14**:1305–18.
22 Motterlini R, Sawle P, Hammad J *et al*. CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. FASEB J 2005; **19**:284–6.
23 Sandouka A, Fuller BJ, Mann BE, Green CJ, Foresti R, Motterlini R. Treatment with CO-RMs during cold storage improves renal function at reperfusion. Kidney Int 2006; **69**:239–47.
24 Ryan MJ, Jernigan NL, Drummond HA *et al*. Renal vascular responses to CORM-A1 in the mouse. Pharmacol Res 2006; **54**:24–9.
25 Parfenova H, Basuroy S, Bhattacharya S *et al*. Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial cells: contributions of HO-1 and HO-2 to cytoprotection. Am J Physiol Cell Physiol 2006; **290**:C1399–410.
26 St Louis J, Uniyal S, Xu L *et al*. Tolerance induction by acylated peptides: suppression of EAE in the mouse with palmitoylated PLP peptides. J Neuroimmunol 2001; **115**:79–90.
27 Vreman HJ, Wong RJ, Kadotani T, Stevenson DK. Determination of carbon monoxide (CO) in rodent tissue: effect of heme administration and environmental CO exposure. Anal Biochem 2005; **341**:280–9.
28 Donia M, Mangano K, Quattrocchi C *et al*. Specific and strain-independent effects of dexamethasone in the prevention and treatment of experimental autoimmune encephalomyelitis in rodents. Scand J Immunol 2010; **72**:396–407.
29 Furlan R, Poliani PL, Galbiati F *et al*. Central nervous system delivery of interleukin 4 by a nonreplicative herpes simplex type 1 viral vector ameliorates autoimmune demyelination. Hum Gene Ther 1998; **9**:2605–17.
30 Sun X, Minohara M, Kikuchi H *et al*. The selective Rho-kinase inhibitor Fasudil is protective and therapeutic in experimental autoimmune encephalomyelitis. J Neuroimmunol 2006; **180**:126–34.
31 Soilu-Hanninen M, Epa R, Shipham K *et al*. Treatment of experimental autoimmune encephalomyelitis with antisense oligonucleotides against the low affinity neurotrophin receptor. J Neurosci Res 2000; **59**:712–21.
32 Rooman R, Koster G, Bloemen R, Gresnigt R, van Buul-Offers SC. The effect of dexamethasone on body and organ growth of normal and IGF-II-transgenic mice. J Endocrinol 1999; **163**:543–52.
33 Zimmermann A, Leffler CW, Tcheranova D, Fedinec AL, Parfenova H. Cerebroprotective effects of the CO-releasing molecule CORM-A1 against seizure-induced neonatal vascular injury. Am J Physiol Heart Circ Physiol 2007; **293**:H2501–7.
34 Ferrandiz ML, Maicas N, Garcia-Arnandis I *et al*. Treatment with a CO-releasing molecule (CORM-3) reduces joint inflammation and erosion in murine collagen-induced arthritis. Ann Rheum Dis 2008; **67**:1211–17.
35 Maicas N, Ferrandiz ML, Devesa I *et al*. The CO-releasing molecule CORM-3 protects against articular degradation in the K/BxN serum transfer arthritis model. Eur J Pharmacol 2010; **634**:184–91.
36 Remy S, Blancou P, Tesson L *et al*. Carbon monoxide inhibits TLR-induced dendritic cell immunogenicity. J Immunol 2009; **182**:1877–84.
37 Pae HO, Oh GS, Choi BM *et al*. Carbon monoxide produced by heme oxygenase-1 suppresses T cell proliferation via inhibition of IL-2 production. J Immunol 2004; **172**:4744–51.
38 Otterbein LE, Bach FH, Alam J *et al*. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000; **6**:422–8.
39 Takagi T, Naito Y, Inoue M *et al*. Inhalation of carbon monoxide ameliorates collagen-induced arthritis in mice and regulates the articular expression of IL-1beta and MCP-1. Inflammation 2009; **32**:83–8.
40 George JF, Braun A, Brusko TM *et al*. Suppression by CD4+CD25+ regulatory T cells is dependent on expression of heme oxygenase-1 in antigen-presenting cells. Am J Pathol 2008; **173**:154–60.
41 Lee IT, Luo SF, Lee CW *et al*. Overexpression of HO-1 protects against TNF-alpha-mediated airway inflammation by down-regulation of TNFR1-dependent oxidative stress. Am J Pathol 2009; **175**:519–32.
42 Foresti R, Bani-Hani MG, Motterlini R. Use of carbon monoxide as a therapeutic agent: promises and challenges. Intens Care Med 2008; **34**:649–58.
43 Niino M, Sasaki H. Update on the treatment options for multiple sclerosis. Exp Rev Clin Immunol 2010; **6**:77–88.