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Effect of diphenyl diselenide on the development of experimental autoimmune encephalomyelitis

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated inflammatory and demyelinating disease of the central nervous system with clinical and pathological similarities with multiple sclerosis. The oxidative stress is one of the major mediators of demyelination and axonal damage in both, multiple sclerosis and EAE. Therefore, several studies are being performed to assess whether treatment with antioxidants prevents the progression of these diseases. Some organic forms of selenium that exhibit glutathione peroxidase-like activity have become good candidates for disease prevention and therapy since they catalytically remove oxidative stressors. Particularly, diphenyl diselenide ($(PhSe)_2$) exerts antioxidant activity and has neuroprotective effects in several systems. The aim of the present study was to prove the therapeutic activity of $(PhSe)_2$ on the development of EAE. Intraperitoneally administered $(PhSe)_2$ (1–25 μmoles/kg body weight/day) reduced the incidence of the disease but was also deleterious for the animals. Conversely, $(PhSe)_2$ given orally (80 μmoles/kg body weight/day) produced a significant inhibition of EAE without any toxic effect. In addition, there was a reduction of the characteristic histological alterations and a diminished *in vivo* and *in vitro* T-cell response against the encephalitogenic myelin basic protein. These results show an effective suppression of the autoimmune response that could be the base for future developments of successful antioxidants therapies in EAE as well as in multiple sclerosis.

1. Introduction

The human autoimmune demyelinating disease multiple sclerosis (MS) affects the central nervous system (CNS) and it is characterized by an intense inflammatory process with destruction of the myelin sheet (Centonze et al., 2010). CNS myelin is produced by oligodendrocytes and acts as an insulator for the nerve fibers, increasing the efficiency of the transmission of nerve impulse and protecting the axon from potentially harmful external factors. For this reason, the autoimmune attack on myelin leads to the damage of associated axons and oligodendrocytes, with atrophy of the CNS. The accumulation of these alterations in patients with MS results in progressive motor, sensory and cognitive deficiencies, including impaired vision, paralysis, ataxia, mental fatigue and incontinence. The pathogenesis of MS involves an immune response, characterized by the presence of inflammatory plaques of demyelination with accumulation of T cells, B cells, macrophages, and activated microglia in the lesions.

Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the CNS which, in several regards, mimics human MS and can hence be used to design or validate new strategies for treatment of this disease (Mix et al., 2008). Acute EAE can be induced in susceptible animals by immunization with whole myelin or with specific myelin proteins in an appropriate adjuvant. Wistar rats develop a monophasic course (acute stage, 11–13 days post-induction, dpi) characterized by weight loss and ascending progressive paralysis associated with fecal and urinary incontinence. Affected animals show a spontaneous neurological improvement 2–4 days after the onset of the disease regaining the full ability to walk by 17–18 dpi and without disease recurrence (Slavin et al., 1996). These stages are paralleled by an inflammatory response in the CNS. It has been convincingly shown that the onset of clinical symptoms is mediated by self-specific CD4 T cells, primarily against myelin basic protein (MBP). These cells are differentiated toward a Th1 profile and

Abbreviations: APCs, antigen-presenting cells; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CNS, central nervous system; Con A, concanavalin A; dpi, days post-induction; DTH, delayed type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; GPx, glutathione peroxidase; HRP, horseradish peroxidase; MBP, myelin basic protein; MNC, mononuclear cells; MS, multiple sclerosis; PBS, phosphate-buffered saline; $(PhSe)_2$, diphenyl diselenide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SI, stimulation index.

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secrete pro-inflammatory cytokines (Matsuki et al., 2006). Th1 cytokines may directly damage the oligodendrocytes or stimulate myeloid cells, which in turn secrete toxic factors. The resulting inflammation leads to the perivascular infiltration of other leukocyte types and demyelination. The self-limiting characteristic of this disease could be due to the change of the pattern of cytokines secreted by anti-MBP T cells that occurs after the acute period, a clear profile of a Th1 to Th2 type. In this way, the subset of Th2 lymphocytes secret suppressive cytokines, including IL-4, IL-10 and IL-13, which inhibit the development and the effect of inflammatory cells that secrete cytokines of Th1 type.

The mechanisms of pathogenesis of both MS and EAE are not yet fully understood. However, numerous studies point to the oxidative stress as a major contributor to the formation and persistence of lesions. Particularly, the oxidative stress generated by the excessive production of reactive oxygen and nitrogen species (ROS and RNS, respectively) by infiltrating mononuclear cells (MNC) and activated microglia is thought to be an important contributor to neuroinflammation, demyelination, axonal damage and disease progression (Mirshafiey and Mohsenzadegan, 2009). The tissue damage increases when meets weakened defense systems against oxidative damage in the CNS in MS patients and animals with EAE (Gilgun-Sherki et al., 2004; Carlson and Rose, 2006). Along with the oxidative stress, there is a diminution of several components of the natural antioxidant system in MS and EAE. Among them, the more important in removing ROS in the CNS is glutathione peroxidase (GPx). In MS and EAE there is an imbalance of GPx activity and glutathione levels (Mirshafiey and Mohsenzadegan, 2009). Indeed, depletion in the rat brain of glutathione can cause an oxidative stress capable of produce lipid peroxidation and protein carbonylation (Bizzozero et al., 2006). Thus, antioxidant therapies targeting the glutathione/GPx system might prevent or reduce the spread of tissue damage and neurological impairment in MS. On this regard, treatment with various antioxidant compounds showed a protective effect in animal models of MS (Gilgun-Sherki et al., 2004).

Selenium, initially known as a poison and even a potent carcinogen, was later found to be an essential micronutrient and a structural component of the active site of diverse antioxidant enzymes, in the form of selenocysteine (Flohé, 2009). From there, new interesting antioxidant reactions of selenium inorganic compounds were discovered. Two findings triggered the research of pharmacologically active organoselenium species, the significantly lower toxicity of organoselenium compounds with respect to inorganic forms and the mimicry of GPx enzyme (Nogueira et al., 2004). Several organoselenium species showed antioxidant properties in both *in vitro* and *in vivo* models, besides other properties like enzyme inhibition, neuroprotection, anti-tumor and anti-infectious activity, and immunomodulation. Among them, diphenyl diselenide ($(PhSe)_2$) has a more potent GPx-like and anti-inflammatory activity than Ebselen (Sausen et al., 2010) and has no toxic effects at doses at which expresses its anti-inflammatory activity (Savegnano et al., 2007; Straliotto et al., 2010). Therefore, considering that no evaluation of this molecule on EAE has been published and that this simple diorganochalcogenide has been reported as a promising pharmacological agent in inflammatory diseases, the aim of the present research was to assess its therapeutic effect on the development of EAE in rats.

2. Experimental procedures

2.1. Chemicals and reagents

Myelin and MBP were purified from bovine spinal cords as previously described (Degano and Roth, 2000). Complete Freund's adjuvant (CFA), bovine serum albumin (BSA), concanavalin A (Con A), RPMI 1640 medium, mouse monoclonal anti-rat IgG, IgG1 and IgG2a antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rat IgG, and HRP-conjugated anti-mouse IgG were from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Greiner Bio-One (Frickenhausen, Germany). [Methyl-^{3}H] thymidine ([^{3}H]TdR) was from NEN Life Science Products, Inc. (Boston, MA, USA). $(PhSe)_2$ was synthesized according to the literature methods. The obtained compound showed 99.9% purity and presented analytical and spectroscopic data in full agreement with its assigned structure (Straliotto et al., 2010). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Experimental animals and induction of EAE

All experiments were performed in accordance with international and institutional guidelines for animal care and the protocol was approved by the local institutional review committee for animal studies (Exp. No. 15-99-40426). Every effort was made to minimize both the number of animals used and their suffering. Albino rats from both sexes (originally Wistar strain) aged 43–45 days old and from a single inbred colony were used. Animals were anesthetized with a mixture of xylazine and ketamine (10 and 65 mg/kg respectively, i.p.) and the active disease was induced by intradermal inoculation in both hind feet with 8 mg whole myelin purified from bovine spinal cords in 0.5 ml of an emulsion consisting of 0.25 ml phosphate-buffered saline solution (PBS) and 0.25 ml CFA (Degano and Roth, 2000). Control animals received 0.5 ml of the same emulsion without any antigenic preparation (CFA group).

2.3. Treatment with $(PhSe)_2$

$(PhSe)_2$ was freshly dissolved in ethanol:water (70:30). Rats were i.p. or orally administered with different concentrations of $(PhSe)_2$ (groups CFA + $(PhSe)_2$ or EAE + $(PhSe)_2$) or with the vehicle alone (groups CFA or EAE). Animals were weighted, and assessed daily for clinical signs of EAE and scored as follows: 0, no evident clinical signs; 1, flaccid tail; 2, hind limb weakness; 3, definitive hindquarter paralysis and urinary incontinence; 4, tetraparalysis; 5, moribund state or dead. The mean clinical score was defined as the sum of the highest clinical score achieved by each rat during the acute phase of the disease divided by the number of rats that developed the disease signs in that group. The disease index was calculated as the sum of the daily clinical score for each animal throughout the experimental period divided by the day of onset of EAE clinical symptoms $\times$ 100 (Staykova et al., 2002). The animals were sacrificed at 11–12 dpi within 24 h since rats from the EAE group showed the first clinical signs of the disease. Rats were deeply anesthetized with xylazine and ketamine and bled to dead by cardiac puncture. Inguinal lymph nodes were aseptically removed and placed in ice-cold Dulbecco's-PBS with 2% FBS. After that, the animals were intracardially perfused with ice-cold PBS, pH 7.4 and spinal cords were dissected for histological studies.

2.4. Immunohistochemistry

The lumbar region of the spinal cords were dissected out and fixed for 48 h at 4 °C in 4% paraformaldehyde in PBS, pH 7.4. After that, they were placed consecutively in 15% and 30% sucrose for 48–72 h each time. Tissues were embedded in Cryoplast® and stored at −80 °C until they were processed. Transverse sections were cut into 20 μm slides with a cryostate (CM1510 S model, Leica Microsystems, Germany). They were dried for 2–4 h at 37 °C and a pretreatment of 30 min with 0.1% Triton X-100 and 1 M glycine in PBS preceded the blocking with 4% FBS and 0.1% Triton X-100

in PBS, pH 7.4 for 1 h. The tissues were incubated with anti-CD68 (monocytes/macrophages antigen) antibody diluted 1/300 or anti-rat IgG antibody diluted 1/500 over night at 4 °C, followed by anti-mouse IgG FITC-conjugated antibody diluted 1/100, 1 h at room temperature. Six to twelve step sections were examined for each animal on an Axiovert 200 microscope (Carl Zeiss, USA; 10× Olympus objective) and pictures were taken with an iXon camera (Andor Technology, Belfast, Northern Ireland). The histological findings were graded in a blind fashion according to the extent of leukocyte infiltrates in the white matter. The severity of inflammation was scored from 0 to 4 as follows: 0, no inflammatory cells; 1, inflammatory cells limited to meninges or submeninges; 2, inflammatory cells in meninges and perivascular spaces; 3, perivascular and parenchymal infiltrates limited to the white matter; 4, perivascular and parenchymal inflammatory infiltrates in both white and grey matter. Deposits of autoreactive IgG in the motoneurons of the grey matter was scored from 0 to 2: 0, no deposits; 1, low immunofluorescence intensity; 2, high immunofluorescence of IgG deposits.

2.5. Immunological T cell response determinations

For DTH reaction determination the animals were intradermally injected in the right ear with 60 μl of 3 mg/ml MBP in PBS, and 60 μl of PBS in the left ear (negative control), both sterile solutions. The ear thickness was determined in a blind fashion with a digital caliper. The results are expressed as the difference in mm between the thickness of the right ear and the left ear measured 24 h after the injection.

For the lymphocyte proliferation assay mononuclear cells (MNC) were isolated from inguinal and popliteal lymph nodes and cultured in triplicate in 96-well flat-bottomed plates, essentially as previously indicated (Degano and Roth, 2000). Briefly, 1.25×10^6 cells/ml were cultured in a total volume of 200 μl per well of RPMI 1640 medium complemented with 10% FBS, 50 μM 2-mercaptoethanol, 50 μM gentamicin and 0.03% glutamine, in the presence of 75 μg/ml MBP or 1 μg/ml Con A, during 72 h. Each well was pulsed with 20 μl of medium containing 1 μCi of [^{3}H]TdR during the last 18 h of culture. Then, the cells were harvested onto fiberglass filters and the radioactivity incorporated was counted by using standard liquid scintillation techniques. The results from the proliferation assays were expressed as stimulation index (SI), defined as the ratio between mean counts per minute of antigen stimulated culture and mean counts per minute of the unstimulated culture, considering an SI $\geqslant 2$ as a positive response (Degano and Roth, 2000).

To assess the effect of $(PhSe)_2$ on proliferation, MNC culture was performed as described above, and different concentrations of $(PhSe)_2$ dissolved in dimethyl sulfoxide and RPMI medium was added together with 0.5 μg/ml of Con A. Control cultures were performed by adding only vehicle and Con A 1 μCi of [^{3}H]TdR was incorporated the last 18 h of a 72 h lasting culture.

2.6. Antibody determinations

Titers of anti-MBP antibodies were measured in rat sera by ELISA as previously described (Rivero et al., 1999). Microtiter plates (Maxisorp, Nunc, Denmark) were coated overnight at 4 °C with 60 μl per well of 2 μg/ml bovine MBP in 0.05 M carbonate buffer, pH 9.6. After blocking with 1 % BSA in PBS for 1 h, each sample was assessed at six different dilutions (1/200–1/32,000 depending on the sample tested). Total IgG binding was detected by further incubation with HRP-conjugated goat anti-rat IgG (1/1000). To detect IgG subisotypes IgG1 and IgG2a, the plates were incubated with the corresponding monoclonal antibody (1/500), followed by the HRP-conjugated anti-mouse IgG (1/1000). Color reaction was carried out by adding 0.4 mg/ml of o-phenylenediamine and 0.5 μl/ml of 30% H_2O_2, and stopped after 2 min with 80 μl of 4 N H_2SO_4. The absorbance at 490 nm was measured with a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA). The endpoint titer was determined as the last dilution giving an absorbance value above the control serum mean plus 2 standard deviations at the same dilution.

2.7. Data analysis

The results were expressed as the mean ± SEM. Nonparametric comparison by two-tailed Mann–Whitney test was performed to examine clinical parameters and histological scores. Data from DTH and proliferation assay were first analyzed using Kruskal–Wallis test with a Dunn's multiple comparison post test, whenever there were significant differences ($p < 0.05$), groups were compared by pairs using two-tailed Mann–Whitney test. In all statistical analysis a $p \leqslant 0.05$ was considered to represent a significant difference between groups.

3. Results

3.1. Suppression of EAE by administration of $(PhSe)_2$

At an early stage, with the purpose of determining the optimal conditions of treatment, it was carried out a test of concentration, number of doses and route of administration of $(PhSe)_2$ in the different animal groups. There was also a vehicle test, in which ethanol:water mix was selected because of its lesser toxicity and absence of effects in disease development (data not shown). These experiments showed that $(PhSe)_2$ administered i.p. in concentrations greater than 5 mM and/or managed more than four times have toxic effects, since more than 50% of the treated rats showed deleterious effects although the remaining animals did not develop EAE. Nevertheless, $(PhSe)_2$ given orally showed no toxicity to rats in the concentrations tested (30–80 μmoles/kg body weight/day) and, compared with i.p. administration, it decreased considerably the stress applied to animals and augmented the suppressive effect (Table 1). Based on these results, all the following experiments were carried out with 80 μmoles $(PhSe)_2$/kg body weight administered orally on 5 and 7 dpi.

3.2. Clinical course of $(PhSe)_2$-treated EAE animals

The clinical analysis of the different groups of animals is summarized in Table 2. As previously shown in our laboratory (Scerbo et al., 2009), the incidence of the disease was about 80–85% in the EAE group which received the vehicle alone and was associated with a marked body weight loss during the acute phase (Fig. 1). The rest of the animals manifested a subclinical state of disease with immunoreactivity to MBP and histological alterations. Treatment with $(PhSe)_2$ of rats sensitized to induce EAE (EAE + $(PhSe)_2$ group) reduced the incidence of the disease to 38.5%, and partly prevents the loss of body weight, while this treatment had no effect on the CFA group. Furthermore, animals from the EAE + $(PhSe)_2$ group that got sick showed significantly less severe signs and tend to have a shorter length of the disease. After the acute period, all the animals began gradually to gain body weight.

3.3. Histopathological evaluation

In rats from the EAE group the most frequent mark is inflammation of the lumbar region of the spinal cord, in both symptomatic and non-symptomatic animals (Scerbo et al., 2009). Although MS and EAE are T-cell-mediated diseases, macrophages from the blood

Table 1
Suppression of EAE by administration of $(PhSe)_2$

Group	Route	$(PhSe)_2$ (μmoles/kg/day)	Days of treatment	Disease incidence (%)	Death incidence (%)
CFA	–	–	–	0/4 (0)	0/4 (0)
CFA + $(PhSe)_2$	I.p.	10	5, 7	0/2 (0)	1/2 (50)
CFA + $(PhSe)_2$	I.p.	0.5	5, 7	0/3 (0)	0/3 (0)
CFA + $(PhSe)_2$	Oral	80	5, 7	0/6 (0)	0/6 (0)
EAE	–	–	–	24/28 (85)	1/28 (3)
EAE + $(PhSe)_2$	I.p.	25	1, 2, 3, 4	0/3 (0)	2/3 (67)
EAE + $(PhSe)_2$	I.p.	25	1, 3, 5, 7	0/3 (0)	2/3 (67)
EAE + $(PhSe)_2$	I.p.	25	1, 3	0/3 (0)	2/3 (67)
EAE + $(PhSe)_2$	I.p.	25	5, 7	0/0 (0)	1/3 (33)
EAE + $(PhSe)_2$	I.p.	25	1	0/2 (0)	1/2 (50)
EAE + $(PhSe)_2$	I.p.	25	5	0/2 (0)	1/2 (50)
EAE + $(PhSe)_2$	I.p.	15	5, 7	0/4 (0)	1/4 (25)
EAE + $(PhSe)_2$	I.p.	10	5, 7	0/2 (0)	1/2 (50)
EAE + $(PhSe)_2$	I.p.	5	5, 7	0/4 (0)	2/4 (50)
EAE + $(PhSe)_2$	I.p.	1	5, 7	3/10 (30)	2/10 (20)
EAE + $(PhSe)_2$	I.p.	0.5	5, 7	7/10 (70)	1/10 (10)
EAE + $(PhSe)_2$	I.p.	0.1	5, 7	4/5 (80)	0/5 (0)
EAE + $(PhSe)_2$	Oral	80	5, 7	15/39 (39)	0/39 (0)
EAE + $(PhSe)_2$	Oral	30	5, 7	10/16 (63)	0/16 (0)

Table 2
Effect of $(PhSe)_2$ treatment on EAE clinical signs.

Group	Disease incidence (%)	M.M.C.S[a]	Day of onset (dpi)	Length of disease (days)	Body weight loss (g)	Disease index[b]
CFA + $(PhSe)_2$	0/10	0	0	0		0
EAE	24/28 (85.7)	2.56 ± 0.63	12.94 ± 1.85	5.14 ± 2.18	22.94 ± 14.14	81.69 ± 35.07
EAE + $(PhSe)_2$	15/39 (38.5)	1.90 ± 0.74*	13.82 ± 1.72	3.60 ± 1.43*	11.60 ± 9.51*	48.69 ± 24.29**

Treated animals were orally administrated with 80 μmoles $(PhSe)_2$/kg body weight at 5 and 7 days post-induction. The results are expressed as mean ± S.E.M. Significant differences are indicated: treated ($(PhSe)_2$) vs. vehicle (EAE) group.

* $p < 0.05$.

** $p < 0.01$.

[a] M.M.C.S., mean maximum clinical score is the sum of the highest clinical score (0–5) achieved by each rat during the acute phase of the disease divided by the number of rats that got sick in that group.

[b] The disease index was the sum of the daily clinical score for each animal throughout the experimental period divided by the day of onset of EAE clinical signs × 100.

are implicated in disease evolution. They can serve as antigen-presenting cells (APCs), phagocyte myelin debris and promote tissue damage by secreting proinflammatory cytokines, chemokines, nitric oxide and glutamate, supporting further leukocyte infiltration (Batoulis et al., 2010). Pronounced infiltration by $CD68^+$ cells from de monocyte/macrophage lineage were detected in spinal cords from the EAE group, not only in meninges and perivascular spaces but throughout the parenchyma, including grey matter in several cases (Fig. 2). In comparison, lumbar sections from EAE rats that received $(PhSe)_2$ showed a diminution in the extent of infiltrating macrophages, being absent or only restricted to meninges in non-symptomatic treated animals.

As previously described (Roth and Obata, 1991), deposits of immunoglobulins in the spinal cord motor neurons have also been found in positive control and treated animals, in a lesser extent in the latter, however no significant (Fig. 2C).

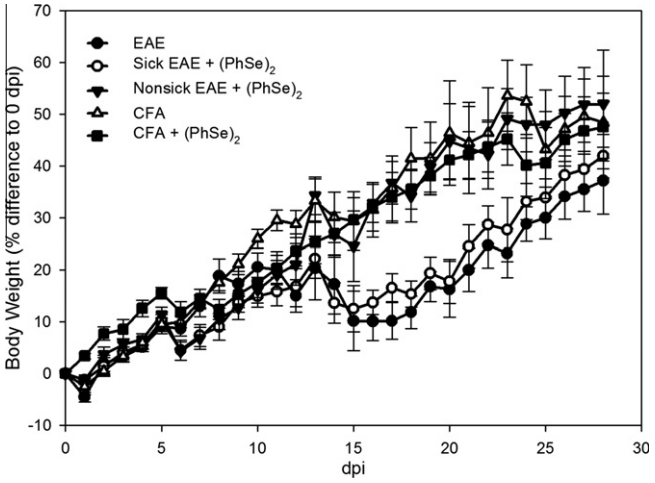

Fig. 1. Daily evolution of the body weight determined as the percentage difference with respect to day 0 of rats from the CFA and EAE groups orally treated with the vehicle alone or with 80 μmoles $(PhSe)_2$/kg body weight at 5 and 7 days post-induction. Values are expressed as the mean ± S.E.M.

3.4. Effect of $(PhSe)_2$ on MBP-specific T-cell responses

To assess whether there was a correlation between the observed clinical signs and the state of the immunological response, the T-cell response against the encephalitogenic MBP was tested *in vivo* and *in vitro*. Since both, DTH and EAE are mediated by the same antigen-specific $CD4^+$ T cells of the Th1 cytokine phenotype, the size of DTH response is linked to EAE susceptibility (Cua et al., 1995). DTH reaction to MBP challenged at 9 dpi was significantly lower in animals from the EAE + $(PhSe)_2$ group with respect to the vehicle-treated EAE group, although it did not reach the null value of the CFA group (Fig. 3A).

Activation and proliferation of encephalitogenic cells in peripheral lymph nodes is a key event in the early development of EAE (Pedotti et al., 2003). Thus, proliferation assay of MNC from lymph nodes was tested *in vitro* at the onset of the disease (11–12 dpi). Control EAE group showed a positive proliferation response upon MBP stimulation (SI < 2) while MNC from EAE + $(PhSe)_2$ group did

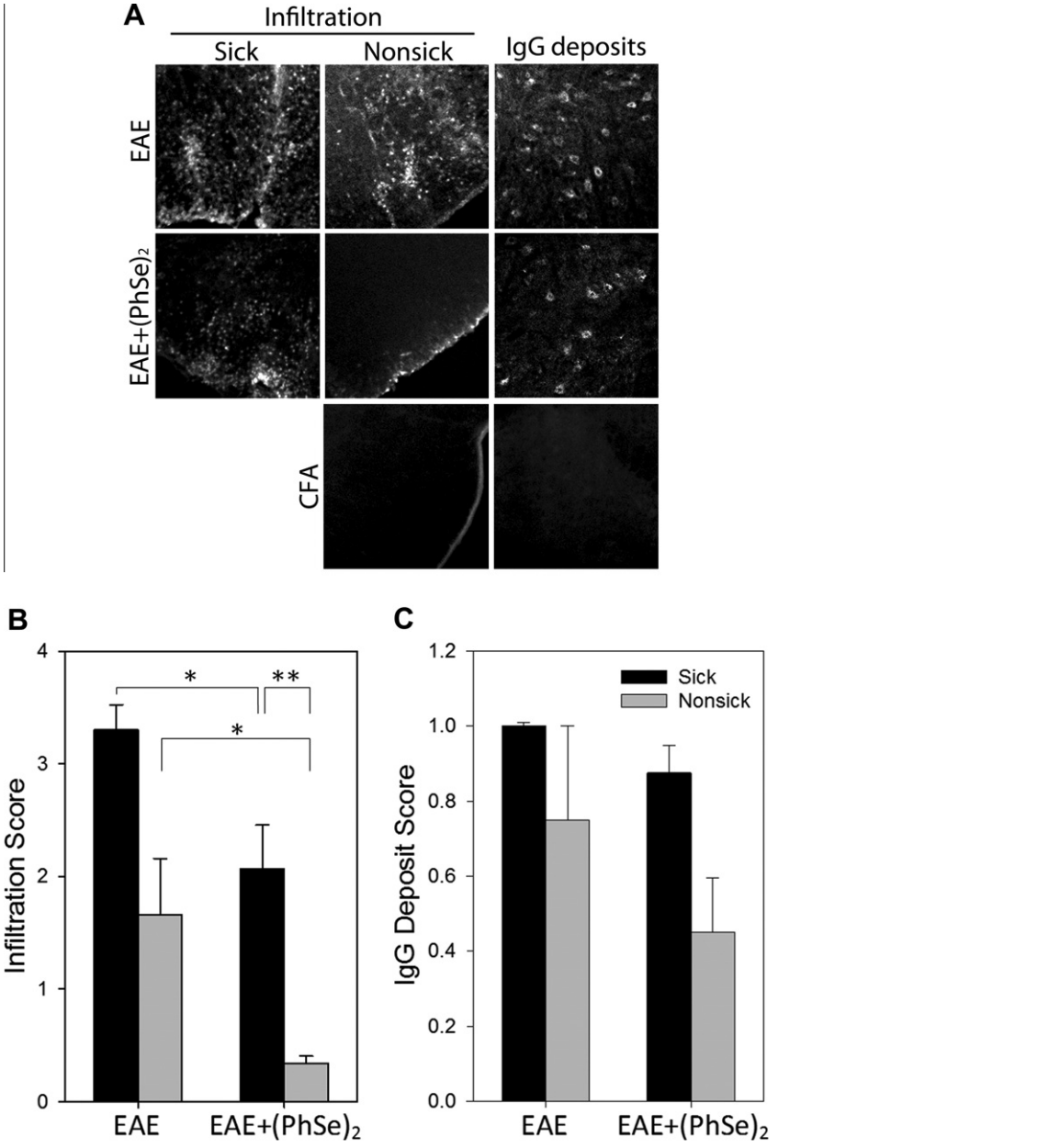

Fig. 2. Histology of spinal cord sections. CFA and EAE rats were treated with vehicle alone or with 80 μmoles $(PhSe)_2$/kg body weight at 5 and 7 days post-induction. Sections from the lumbar region (L1-3) of spinal cords obtained at the disease acute period were stained with anti-rat CD68 (monocyte/macrophage antigen) or anti-rat IgG antibodies. (A) Representative sections of CFA, and sick and nonsick animals from EAE (control) and EAE + $(PhSe)_2$ groups. (B) Inflammatory cell infiltration and (C) IgG deposits were scored as described in experimental procedures. Data are representative of four sick and non sick animals examined per group.

not proliferate (Fig. 3B). MNC from all groups were able to proliferate in the presence of the nonspecific mitogen Con A.

3.5. Effect of $(PhSe)_2$ on humoral response

Although B cells and antibodies have been shown to be incapable of inducing EAE, it is thought that they might facilitate CNS damage and promote disease progression in both MS and EAE (Weber et al., 2011). The presence of self-reactive antibodies also plays an important role in MS diagnosis. Measurement of serum antibodies is routinely used as an indirect measure of the immune response profile in models of autoimmune diseases involving the CNS, since the production of antibodies of the IgG1 isotype in rats is induced by Th2 type cytokines, while Th1 cytokines trigger the secretion of IgG2a and IgG2b isotypes (Mosmann and Sad, 1996). Therefore, the relationship IgG2a/IgG1 or IgG2b/IgG1 is an indirect indicator of the Th1/Th2 ratio. In the present work, no differences in total anti-MBP IgG titer and IgG2a/IgG1 ratio were found when comparing EAE and EAE + $(PhSe)_2$ groups (Fig. 4A–D). Sera from animals of the CFA group had no reactivity against MBP.

3.6. In vitro effect of $(PhSe)_2$ on MNC proliferation

To further understand the effect of $(PhSe)_2$ upon the immune response, this antioxidant compound was directly added in different concentrations to MNC cultures from lymph nodes of rats of the CFA and EAE groups stimulated with Con A (Fig. 5). $(PhSe)_2$ became sharply toxic at concentrations of 5 μM, reducing the proliferation to almost zero in both groups. At the nontoxic

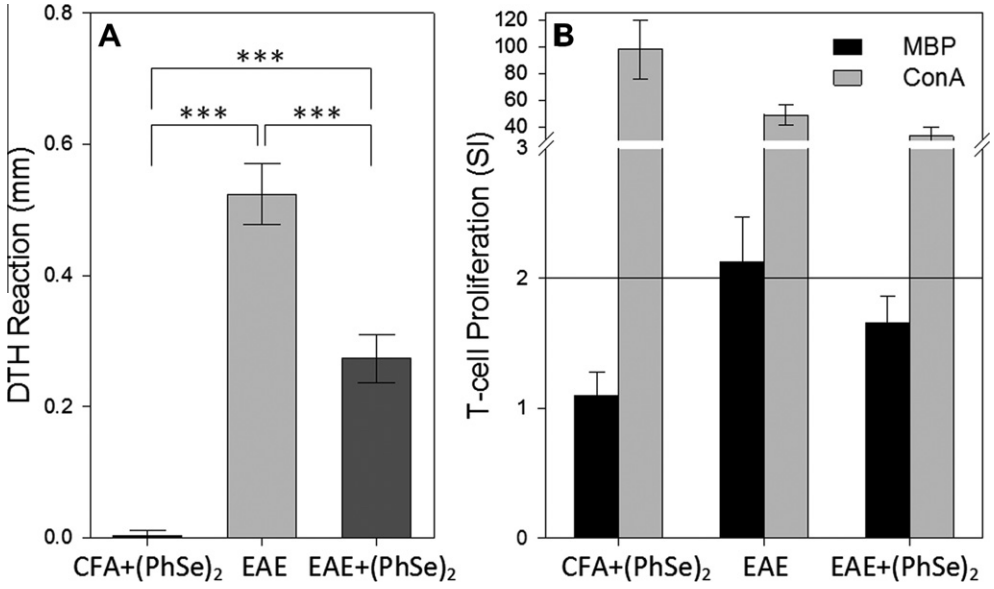

Fig. 3. (A) Effect of $(PhSe)_2$ on DTH response. Rats were treated as indicated and DTH was tested at 9 dpi. Animals were injected subcutaneously with MBP in the left ear and the vehicle in the right ear. Swelling was determined 24 h post-immunization and the difference of thickness (mm) between the left and right ear was calculated. Results are expressed as the average ± S.E.M. of three independent experiments with four animals per group each. (B) Effect of $(PhSe)_2$ on MBP-specific T-cell response. MNC were isolated from inguinal nodes of animals under different treatments at the beginning of the acute period (11–12 dpi). Specific MBP and Con A proliferative response are indicated as mean stimulation index (SI) ± S.E.M. of five different experiments with four animals per group each. Significant differences of treated group vs. vehicle are indicated by $^{***}p < 0.001$.

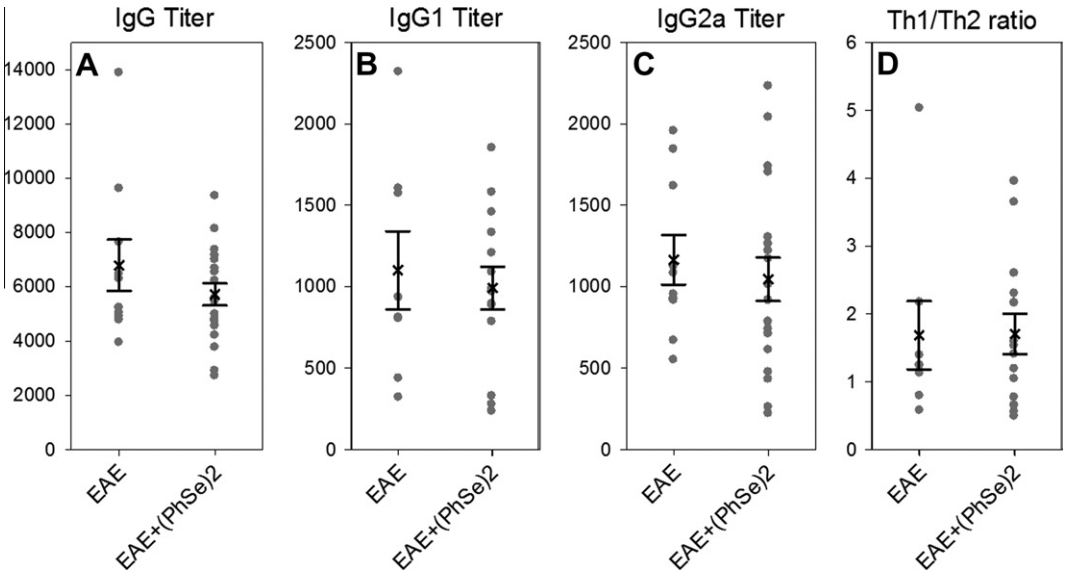

Fig. 4. Specific IgG subclasses of anti-MBP antibodies in sera from control and treated animals. Specific (A) IgG, (B) IgG1 and (C) IgG2a anti-MBP antibody titers (reciprocal) were determined by ELISA in sera from ten EAE and twelve EAE + $(PhSe)_2$ animals. Individual values and mean ± S.E.M. are represented. (D) Relationship of IgG2a/IgG1 antibodies (Th1/Th2).

concentrations tested, $(PhSe)_2$ had no significant impact on the proliferative capability of MNC cells.

4. Discussion

The present work shows the effect of an antioxidant compound in the development of EAE, a neuroinflammatory disease model. Numerous authors agree on the crucial role of oxidative stress and ROS in the formation and progression of the lesions in MS as well as in EAE (Gilgun-Sherki et al., 2004; Mirshafiey and Mohsenzadegan, 2009), and in consequence antioxidant agents arouse as promising therapies. Nevertheless, the findings about treatment with several antioxidants in MS as well as in EAE models have been controversial. $(PhSe)_2$ is a diorganochalcogenide with known antioxidant activity confirmed in several *in vitro* and *in vivo* systems, and thus has a protective effect against hepatic, renal and gastric injuries, in addition to its neuroprotective activity (Nogueira et al., 2004; Rosa et al., 2007), but it was never tested in models of neuroinflammation, like EAE. First, we found that the route of administration was an important determinant of the effectiveness of treatment with $(PhSe)_2$ because the rate of absorption, metabolism and bioavailability of chemicals is not the same when they penetrate through the digestive tract or are inoculated directly into the intraperitoneal cavity. We found differences in the clinical development of the disease among different groups. As can be seen comparing the different treatments dis-

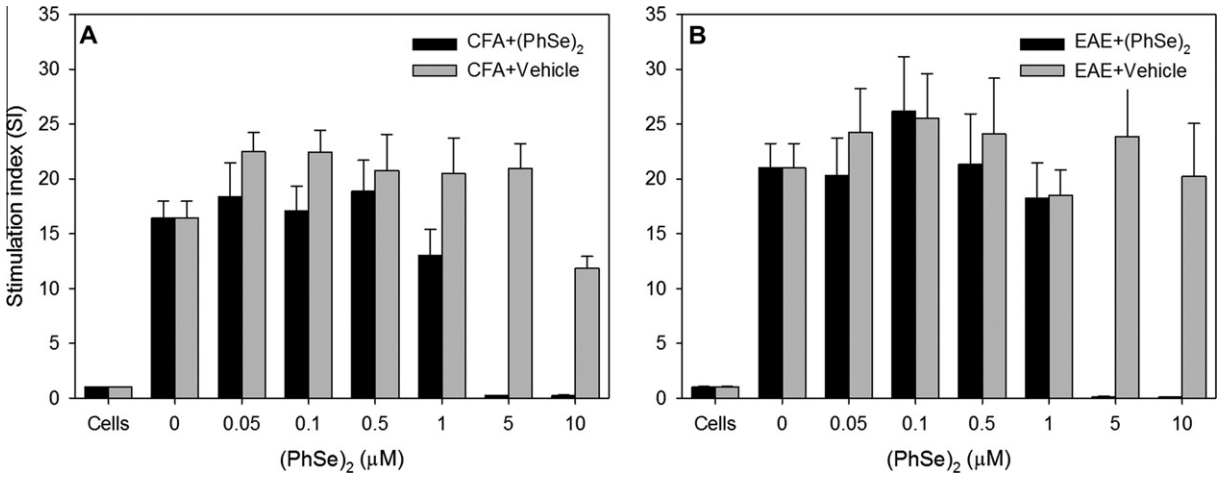

Fig. 5. Effect on cell proliferation of $(PhSe)_2$ added in vitro. MNC cultures from (A) CFA and (B) EAE rats were performed in the presence of different concentrations of $(PhSe)_2$ dissolved in dimethyl sulfoxide/RPMI added together with 0.5 μg/ml of Con A. Results are expressed as mean ± S.E.M. of tree independent experiments.

played in Table 1, the mortality caused by $(PhSe)_2$ is null when it is delivered orally, while it has some toxicity in i.p. administration, property that becomes more important with increasing concentration of the dose. Also, i.p. injection is a significant risk of damage to organs of the peritoneal cavity, which can cause infections and even lead to death. Conversely, oral administration is significantly more secure and administration through a gastric tube generates less stress. It was also found that the vehicle in which the drug is dissolved was of utmost importance, as their physical–chemical properties may improve or deteriorate the absorption and assimilation. Consequently, the present study was carried out with $(PhSe)_2$ delivered orally, since i.p. $(PhSe)_2$ must be used at lower concentrations to diminish toxicity, fact that also decreases its suppressive effect. Therefore, it was possible to work with higher concentrations and more efficiency in the case of gastric intubation.

Our clinical studies showed that $(PhSe)_2$, orally administered at 5 and 7 dpi, clearly reduced the incidence of EAE in rats and improve the symptomatology of treated animals that got sick (Table 2, Fig. 1). This could be due in part to the attenuation of macrophage infiltration in the CNS (Fig. 2B), which might lead to a local reduction in ROS and proinflammatory cytokines concentration preventing further leukocyte infiltration, myelin destruction and neurological damage. In line with this, our previous study using a mice model of atherosclerosis highlight the importance of $(PhSe)_2$ as an anti-inflammatory molecule by suppressing the monocyte recruitment into the vessel wall and decreasing the circulating levels of MCP-1 in hypercholesterolemic mice (Hort et al., 2011). In fact, infiltrating macrophages as well as resident microglia function as APCs during the first steps of the disease, reactivating T cells and favoring their subsequent infiltration (Almolda et al., 2011). Thus, in some way they could modulate lymphocyte response through the antigen presenting mechanism, controlling the initiation of the neuroinflammatory process. Both, the innate and the acquired immune systems and the inflammatory response are tightly regulated by the redox state and thus by antioxidant selenoproteins. In a previous study was reported that in murine macrophages exposed to an oxidant agent pretreated with low concentration of $(PhSe)_2$ efficiently decreased the secretion of both proinflammatory cytokines, TNF-α and MCP-1 (Hort et al., 2011). Consistent with our results, Shin et al. (2009) have recently reported that bis-(3-hydroxyphenyl) diselenide reduces the expression of proinflammatory cytokines, including TNF-α, through downregulation of NF-kB-binding activity in LPS stimulated macrophages. Besides holding ROS levels inside immune cells, selenoproteins are involved in macrophage migration across the extracellular matrix, T-cell activation and T-cell mediated antibody production (Carlson et al., 2010). Immune cells, and above all T lymphocytes, are the most sensitive to redox homeostasis deregulation and selenoproteins deprivation (Zeng, 2009). Interestingly GPx1 is the most expressed selenoprotein mRNA in T cells and macrophages (Carlson et al., 2010). Those findings together with our results indicate that $(PhSe)_2$, an antioxidant drug which mimics GPx activity, could modulate inflammation in EAE via avoidance of macrophage ROS production and migration, T-cell activation and proliferation and the consequent oligodendroglial and neuronal damage. In this respect, the different effect obtained when EAE was induced in guinea pigs and treated with Se in the form of selenite (Scelsi et al., 1983) could be explained taking into account that $(PhSe)_2$ seems to be a molecule safer than other organoselenium compounds or Se itself, that mimics GPx activity, together with the different animal species and form of administration used.

To complement the results of clinical analysis, we studied the state of the immune system at the cellular and humoral levels. Herein, treatment with $(PhSe)_2$ of rats induced to develop EAE reduced the proliferative response of MNC against the encephalitogenic MBP *in vitro* (Fig. 3B), and also significantly diminished *in vivo* the DTH reaction, a specific T-cell-mediated response which involves migration, activation, proliferation and infiltration (Fig. 3A). On this regard, as stated before it has been demonstrated by several authors that diverse organic and inorganic selenium compounds inhibit NF-κB and AP-1 nuclear translocation, DNA-binding and transcriptional activity in T lymphocytes, macrophages and other mammalian cancer cell lines, possibly because binding of these transcription factors to its target DNA depends on the redox state of specific cysteine residues (Shin et al., 2009; Tewari et al., 2009). Furthermore, these compounds lead, as consequence, to a decrease in nitric oxide and ROS production, iNOS and cyclooxygenase-2 expression, proinflammatory cytokines secretion (TNF-α, IL-1β, IL-6) and cell migration and invasive potential. However, in MNC cultures stimulated with the nonspecific mitogen Con A, $(PhSe)_2$ did not affect the proliferative capacity of immune cells, probably because these cultures consist mainly of T lymphocytes from lymph nodes, which lacks of the cellular and chemical environment of the inflamed area, namely the CNS. Since macrophage infiltration is the first event in EAE which afterwards leads to T cell recruitment and reactivation, it is probably that macrophages are the initial target for $(PhSe)_2$ action. Corroborating this, we have previously described a significant effect of $(PhSe)_2$ in decreasing the ROS generation in macrophage culture stimulated with oxidized LDL (Hort et al., 2011). This could indicate that the pharmacology

of $(PhSe)_2$ is complex and lays on the interaction between components of the immune system and the target tissue, instead of a direct effect upon a single isolated cell type. Notwithstanding, we do not discard the possibility that $(PhSe)_2$ could be being metabolized in the liver, and that a secondary product is responsible for the suppression of the disease, what could also explain why it has a very low efficiency when i.p. administered.

Regarding to the humoral component of the immune response, despite the slight decrease of self-reactive IgG deposits in the spinal cord (Fig. 2C), no significant changes in antibody serum levels or motor neuron reactivity were found (Fig. 4), which could indicate that in this EAE model the antioxidant activity of $(PhSe)_2$ might be modulating only the cellular component of the inflammatory response. Taking into account the significant decrease in disease incidence and the clinical parameters analyzed and the low cellular immune responses observed in the suppressed animals, lead us to conclude that $(PhSe)_2$ does not produce a change in the kind of response from a Th1 to a Th2 profile, but inhibits the development of the intense autoimmune attack characteristic of EAE.

We propose that the observed pharmacological effects of $(PhSe)_2$ are the result of several mechanisms. Firstly, its GPx-like and antioxidant activities might be involved in ROS clearance and protection of oligodendrocytes, since they are especially vulnerable to oxidative stress (Thorburne and Juurlink, 1996). Secondly, $(PhSe)_2$ may modulate NF-κB signal transduction in macrophages and T-cells preventing proliferation, migration, CNS infiltration and secretion of ROS and other proinflammatory mediators, avoiding disease progression. It may be the integration of these different mechanisms which lead to reduction of neurological damage and symptomatic improvement in our EAE model, and probably that is why other treatments with drugs with only antioxidant properties have been unsuccessful.

5. Conclusion

The results obtained in this study indicate a considerable $(PhSe)_2$ effect in suppressing the autoimmune demyelinating disease EAE despite that other more specific studies are needed. It also contributes to the importance that this kind of compound are receiving at present as potential drugs in the treatment of autoimmune diseases like MS and other disorders involving the immune and nervous systems.

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