Trabajos experimentales

Trabajo experimental 1 — Neuroscience 2001; 105: 1055-1065

Differential expression of metallothionein in the CNS of mice with experimental autoimmune encephalomyelitis.

Expresión diferencial de metalotioneínas en el SNC de ratones con encefalomielitis autoinmune experimental

En este trabajo se analizó la expresión de las MT-I III en el SNC de ratones con EAE. Además, se estudió la posible función del IFN-, una citocina proinflamatoria, en el control de la expresión de MT durante la EAE en dos cepas diferentes de ratones deficientes para el receptor del IFN- (IFN-RKO): 129/Sv y C57BL/6x129/Sv.

Los ratones con EAE presentaron una inducción significativa de MT-I y MT-II en la sustancia blanca de la médula espinal y un poco menos en el cerebro. Los ratones IFN-RKO sufrieron una EAE más grave y, curiosamente, tuvieron mayor inducción de la expresión de MT-I y MT-II tanto en la sustancia blanca como en la sustancia gris de la médula espinal y del cerebro. Sin embargo, la expresión de MT-III permaneció inalterable durante la EAE. En los ratones IFN-RKO se observó una ligera inducción de esta isoforma, en la sustancia blanca de la médula espinal, aunque únicamente en la cepa C57BL/6x129/Sv. La expresión de MT-I y MT-II era prominente en las áreas de infiltración celular, siendo los astrocitos reactivos y la microglía/macrófagos las células que las expresban.

A raíz de estos resultados, sugerimos que la MT-I y MT-II pero no la MT-III pueden tener una función importante durante la EAE y que la citocina proinflamatoria IFN- no es un factor limitante en esta respuesta.

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DIFFERENTIAL EXPRESSION OF METALLOTHIONEINS IN THE CNS OF MICE WITH EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Abstract—Multiple sclerosis is an inflammatory, demyelinating disease of the CNS. Metallothioneins-I+II are antioxidant proteins induced in the CNS by immobilisation stress, trauma or degenerative diseases which have been postulated to play a neuroprotective role, while the CNS isoform metallothionein-III has been related to Alzheimer's disease. We have analysed metallothioneins-I–III expression in the CNS of mice with experimental autoimmune encephalomyelitis. Moreover, we have examined the putative role of interferon-γ, a pro-inflammatory cytokine, in the control of metallothioneins expression during experimental autoimmune encephalomyelitis in interferon-γ receptor knockout mice with two different genetic backgrounds: 129/Sv and C57BL/6x129/Sv.

Mice with experimental autoimmune encephalomyelitis showed a significant induction of metallothioneins-I+II in the spinal cord white matter, and to a lower extent in the brain. Interferon-γ receptor knockout mice suffered from a more severe experimental autoimmune encephalomyelitis, and interestingly showed a higher metallothioneins-I+II induction in both white and grey matter of the spinal cord and in the brain. In contrast to the metallothioneins-I+II isoforms, metallothionein-III expression remained essentially unaltered during experimental autoimmune encephalomyelitis; interferon-γ receptor knockout mice showed an altered metallothionein-III expression (a slight increase in the spinal cord white matter) only in the C57BL/6x129/Sv background. Metallothioneins-I+II proteins were prominent in areas of induced cellular infiltrates. Reactive astrocytes and activated monocytes/macrophages were the sources of metallothioneins-I+II proteins.

From these results we suggest that metallothioneins-I+II but not metallothionein-III may play an important role during experimental autoimmune encephalomyelitis, and indicate that the pro-inflammatory cytokine interferon-γ is unlikely an important factor in this response.

Key words: interferon γ, oxidative stress, antioxidant proteins, neuroprotein, multiple sclerosis.

Multiple sclerosis (MS) is a chronic demyelinating disease of the CNS and is the most common cause of non-traumatic neurological dysfunction in young adults. Lesions in MS are characterised by cellular infiltration, mostly of lymphocytes and macrophages, and various degrees of demyelination and axonal loss (Lassmann et al., 1998). Experimental autoimmune encephalomyelitis (EAE) is a recognised animal model for MS with clinical signs and lesions closely resembling those observed in MS (Martin et al., 1992; Raine, 1992).

Although the actual pathogenic mechanisms involved in MS induction are not well understood, there is much evidence pointing to a major role of oxidative stress in lesion development (Lin et al., 1993; Hooper et al., 1995; Cross et al., 1996; De Groot et al., 1997; Vladimirova et al., 1998)

Little is known about the role of antioxidants in MS. It has been shown that MS patients have significantly lower levels of serum uric acid (Hooper et al., 1998), plasma vitamin E, plasma ubiquinone, lymphocyte ubiquinone and erythrocyte glutathione peroxidase than controls (Syburra and Passi, 1999). Recently uric acid, a strong peroxynitrite scavenger, has been used successfully in treating EAE (Hooper et al., 1998).

Metallothioneins (MTs) are a family of low molecular

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Abbreviations: ABC, avidin–biotin–peroxidase complex; DAB, 3,3′-diaminobenzidine; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; IFN-γ-R, interferon-γ receptor; IL, interleukin; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; MTs, metallothioneins; NMDA, *N*-methyl-D-aspartate; NOS-α, nitric oxide synthase-α; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

weight, heavy metal-binding, cysteine-rich proteins. It has been demonstrated that MTs accumulate under conditions where oxidative stress has taken place (Shiraga et al., 1993) and they may provide protection against oxygen radicals and oxidative damage caused by inflammation, tissue injury and stress (Ebadi et al. 1995). Four isoforms of these proteins have been identified in the mouse (MT-I to MT-IV). The MT-I and MT-II isoforms are found in the brain and peripheral tissues, the MT-III isoform, possessing seven additional amino acids, is expressed mostly in the brain and to a limited extent elsewhere (Palmiter et al. 1992). MT-IV is not expressed in the CNS but it is found in the squamous epithelium (Quaife et al., 1994).

Although the physiological role of the different MT isoforms in the CNS still remains to be defined, MT-I and MT-II have been related to antioxidant functions and zinc metabolism (Sato and Bremner, 1993; Kelly et al., 1996; Aschner et al., 1997a; Hidalgo et al., 1997). The intracerebral expression of MT-I and MT-II in mice and rats is clearly upregulated during pathological conditions induced by immobilisation stress (Hidalgo et al., 1990; Belloso et al., 1996), trauma (Penkowa and Moos, 1995), kainic acid-induced seizures (Dalton et al., 1995; Zheng et al., 1995), excitotoxic *N*-methyl-D-aspartate (NMDA) cortex damage (Hidalgo et al., 1997; Acarín et al., 1999a) and administration of 6-aminonicotinamide (Penkowa et al., 1997). MT-I+II expression is increased in the myelin-deficient jimpy mouse (Vela et al., 1997) and in several human neurodegenerative disorders such as Alzheimer's disease (Duguid et al., 1989), Pick's disease (Duguid et al., 1989), amyotrophic lateral sclerosis (Sillevis Smitt et al., 1992), and after brain ischemia (Van Lookeren Campagne et al., 1999). Furthermore, the exogenous administration of pro-inflammatory cytokines increases the levels of MT-I+II in the liver (De et al., 1990) and CNS (Hernández et al., 1997; Carrasco et al., 1998; Hernández and Hidalgo, 1998; Hidalgo et al., 1998).

All these data suggest that both MT-I and MT-II may have an important role in the protection against oxidative damage in MS. MT-III was discovered unexpectedly as a decreased factor in Alzheimer's disease (Uchida et al., 1991), a result however not confirmed by other studies (Palmiter et al., 1992; Carrasco et al., 1999). MT-III expression is also significantly altered in a number of models of experimental CNS damage, including NMDA excitotoxicity (Hidalgo et al., 1997; Acarín et al., 1999b). A number of *in vitro* (Erickson et al; 1994; Palmiter, 1995; Sewell et al., 1995) and *in vivo* (Quaife et al., 1998) studies suggest that MT-III has functions that differ from those of MT-I and MT-II.

In this work we aim to analyse the expression of MTs in the CNS of mice with EAE and how interferon-γ (IFN-γ), a pro-inflammatory cytokine, regulates the expression of this antioxidant proteins in this inflammatory condition.

EXPERIMENTAL PROCEDURES

Mice

129/Sv, H-2^b mice of either sex, homozygous for IFN-γ receptor disrupted gene (IFN-γ-$R^{-/-}$) (Huang et al., 1993) and wild-type mice were purchased from BK Universal (UK). C57Bl/6x129Sv, H-2^b mice of either sex, with a disruption in the cytoplasmic domain of the IFN-γ-R (Arbonés et al., 1994) were generously provided by Cell Genesys Inc. (Foster City, CA, USA). Mice were 8–14 weeks old, were fed with standard chow and water *ad libitum*. Anaesthesia was induced by i.p. injection of 37 mg/kg of ketamine (Ketalar®, Parke and Davis, USA) and 5.5 mg/kg of xylazine (Rompun®, Bayer, Germany). Animal welfare was observed in compliance with Spanish legislation on 'Protection of Animals Used for Experimental and other Scientific Purposes', and in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) on this subject. All efforts were made to minimise animal suffering and to use only the number of animals necessary to attain reliable scientific data.

Immunisation

Animals were immunised with a s.c. injection of 0.9% saline containing 100 μg rat myelin oligodendrocyte glycoprotein (MOG) peptide 40–55 (MOG_{40-55}) emulsified in Freund's adjuvant (Sigma Chemical CO, St. Louis, MO, USA) (9:11 v/v) containing 4 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA). Rat MOG_{40-55} was synthesised by Dr Andreu (Departament de Síntesi de Pèptids, Facultat de Química, Universitat de Barcelona, Barcelona, Spain). Control animals were immunised in the same way but without the MOG peptide. Mice received 0.05 ml of emulsion s.c. in the four limb flanks. At days 0 and 2 post-immunisation each mouse received 0.2 ml (2 IU/ml) of inactivated *Bordetella pertussis* (Vaxicoq®, Pasteur Merieux, France) intravenously.

Clinical assessment

All animals were weighed and examined daily for neurological signs according to the following criteria: 0, no clinical signs; 0.5, partial loss of tail tonus for two consecutive days; 1, paralysis of whole tail; 2, mild paraparesis of one or both hind limbs; 3, paraplegia; 4, tetraparesis; 5, tetraplegia; 6, death (Brocke et al., 1994). All experiments were performed in a blinded manner. The blind was established and preserved throughout the entire experimental process in such a way that the investigator examining and clinically evaluating the animal was kept unaware of the strain of the mice.

Histopathological study

Animals were killed with CO_2 at day 28 post-immunisation and the brain and spinal cord were resected. Tissues were fixed overnight in 4% paraformaldehyde/phosphate-buffered saline (PBS). After fixation, the samples were cryoprotected in 30% sucrose/PBS and frozen in 2-methylbutane cooled in liquid nitrogen. They were then stored at −80°C until use.

For the histological analysis 8-μm-thick sections were cut on a cryostat and collected onto gelatinised slides. Sections were stained with hematoxylin and eosin for visualisation of inflammatory infiltrates and Luxol Fast Blue staining for visualisation of demyelination.

Determination of brain MT-I–III expression by in situ hybridisation

Brain MT-I and MT-III expression was evaluated by *in situ* hybridisation essentially as described elsewhere (Hernández et al., 1997). We did not analyse MT-II as the distribution of

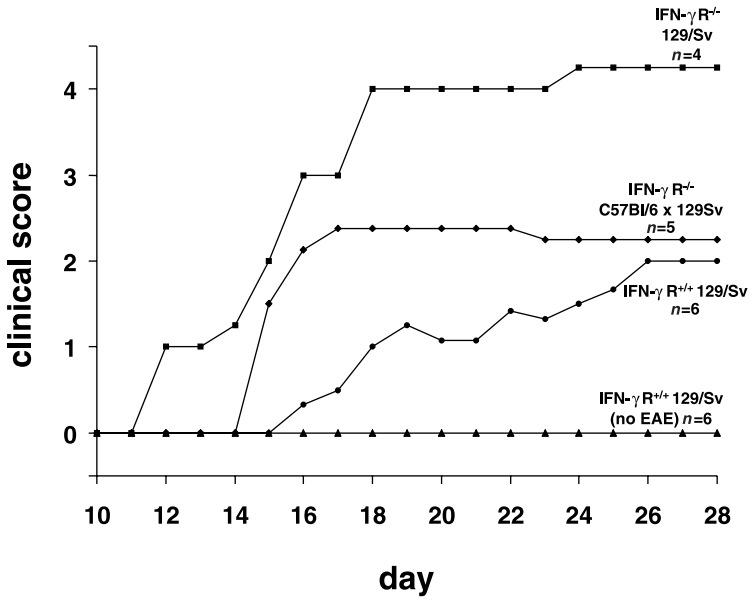

Fig. 1. Clinical course of EAE in 129/Sv wild-type, 129/Sv IFN-γ-R-deficient and C57Bl/6x129Sv IFN-γ-R-deficient mice. Mice were immunised as indicated in Experimental procedures. Daily scoring was performed from day 7 until day 28 post-immunisation (day of killing). All animals with clinical signs of EAE developed a chronic non-remitting disease.

MT-II mRNA is assumed to be identical to the distribution of MT-I mRNA since their genes are expressed coordinately (Yagle and Palmiter, 1985). Brains and spinal cords were frozen directly in methyl-butane cooled in liquid nitrogen. Serial longitudinal sections (10-µm-thick) were cut with a cryostat and mounted on slides coated with poly-L-lysine and preserved at −80°C until the day of analysis. MT-I and MT-III mRNA expression were analysed in the white and grey matter. Autoradiography was performed exposing the sections to autoradiographic film (hyperfilm-MP, Amersham, UK) for several days. MT-I and MT-III mRNA were semi-quantitated in the areas mentioned above determining the optical densities and the number of pixels with a Leica Q 500 MC system. Each measure was obtained from the average of three sections of each spinal cord. The investigator evaluating the sections was kept unaware of the mouse strain.

Immunohistochemistry: glial fibrillary acidic protein (GFAP) staining.

Spinal cord cryostat sections (8 µm) from IFN-γ-$R^{-/-}$ and wild-type mice were processed for the visualisation of GFAP of astrocytes. Sections were air-dried and fixed in reagent-grade acetone:methanol (1:1 v/v) for 30 min. Slides were treated with 10% H_2O_2 in PBS for 10 min and then twice washed in PBS for 5 min and incubated in PBS/10% foetal calf serum for 30 min prior to incubation with rabbit anti-GFAP polyclonal antibody (DAKO, Dakopatts, Denmark; code Z334) diluted 1:10 in PBS containing 10% foetal calf serum and 1% Triton X-100 at room temperature for 1 h. After washing with PBS twice for 5 min, sections were incubated for 30 min at room temperature with rabbit DAKO EnVision+System peroxidase. After two 10-min rinses in PBS, the peroxidase reaction product was visualised using 3,3′-diaminobenzidine (DAB). Sections were finally rinsed in PBS (twice for 5 min), counterstained with hematoxylin, dehydrated, and coverslipped in DPX.

As negative controls for the immunohistochemical procedure, sections were incubated in either media lacking primary antibody or by substituting the primary antibody with control antisera or isotype control antibodies.

Double immunohistochemical techniques

Sections were incubated in 3% H_2O_2 in Tris-buffered saline (TBS)/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl; with 0.01% Nonidet P-40, Sigma) for 15 min at room temperature to quench endogenous peroxidase. The sections were preincubated with pronase E (protease type XIV, Sigma No. P5147, 0.025 g dissolved in 50 ml TBS for 10 min, pH 7.4, at 37^0 C) and 10% goat serum in TBS/Nonidet (TBS with 0.01% Nonidet P-40, Sigma) for 15 min at room temperature, and incubated overnight at 4°C with polyclonal rabbit anti-rat MT-I+II diluted 1:500 (Penkowa et al., 1997). The primary antibody was detected using biotinylated monoclonal goat anti-rabbit IgG absorbed with rat serum proteins 1:400 (Sigma, code B3275). The secondary antibody was detected by using streptavidin–biotin–peroxidase complex (StreptABC/HRP, Dakopatts; code K377) prepared by manufacturer's recommended dilutions for 30 min at room temperature. The immunoreaction was visualised using 0.015% H_2O_2 in DAB/TBS (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl), for 10 min at room temperature.

To evaluate the extent of non-specific binding in the immunohistochemical experiments, control sections of normal and MT-I+II mice were incubated in (1) the DAB medium alone (to examine endogenous peroxidase activity); (2) the DAB medium and the ABC prepared at manufacturers recommended dilutions (to examine endogenous biotin activity); (3) the absence of primary antibody (to examine cross-reaction among IgG of the different species); (4) either normal goat, rat, donkey or mouse serum instead of the primary antibody. Results were considered only if these controls were negative.

To determine which cells contain MT-I+II during EAE, a double immunofluorescence staining for Texas Red-linked tomato lectin from *Lycopersicon esculentum* 1:50 (Sigma, code L9139) and polyclonal rabbit anti-rat MT-I+II (as mentioned above) was performed. Also, monoclonal mouse anti-horse MT-I+II, 1:50 (Dakopatts, code M0639) was incubated simultaneously with polyclonal rabbit anti-cow GFAP, (as a marker for astrocytes) diluted 1:250 (Dakopatts, code Z334).

The primary antibodies were detected by using goat anti-rabbit IgG (H+L) linked with fluorescein 1:50 (Southern Biotechnology Ass., USA, code 4050–02) and goat anti-mouse IgG (γ chain-specific) linked with Texas Red 1:50 (Southern Biotechnology Ass. Inc, code 1030-07) for 30 min at room temperature. The sections were embedded in 20 ml Fluorescent mounting (Dakopatts, code S3023) and kept in darkness. To evaluate the extent of non-specific binding of the antisera in the immunohistochemical experiments, the primary antibody was either omitted or sections thereof were incubated in normal goat, rat,

Table 1. Clinical course of IFN-γ-R$^{-/-}$) and wild-type mice after EAE induction with MOG_{40-55}

Strain	Mice	Day of onset	Daily score	Maximum score	Chronicity[a]
129/Sv	IFN-γ-R$^{+/+}$ ($n=6$)	20.50 ± 3.67	1.14. ± 0.79	2.33 ± 0.41	17.25 ± 8.83
	IFN-γ-R$^{-/-}$ ($n=4$)	15.00 ± 2.58[b]	3.06 ± 0.68[b]	4.25 ± 0.50[b,d]	56.38 ± 9.81[b,d]
C57Bl/6x129Sv	IFN-γ-R$^{-/-}$ ($n=5$)	16.20 ± 2.68[c]	2.57 ± 1.08[c]	2.88 ± 0.85	34.00 ± 18.29

Results are mean ± standard deviation.
[a]Measured by the adding up of clinical scores from each animal during the clinical follow-up.
[b]$P<0.05$, 129/Sv IFN-γ-R$^{-/-}$ vs 129/Sv IFN-γ-R$^{+/+}$.
[c]$P<0.05$, C57Bl/6x129Sv IFN-γ-R$^{-/-}$ vs 129/Sv IFN-γ-R$^{+/+}$.
[d]$P<0.05$, 129Sv IFN-γ-R$^{-/-}$ vs C57Bl/6x129Sv IFN-γ-R$^{-/-}$.

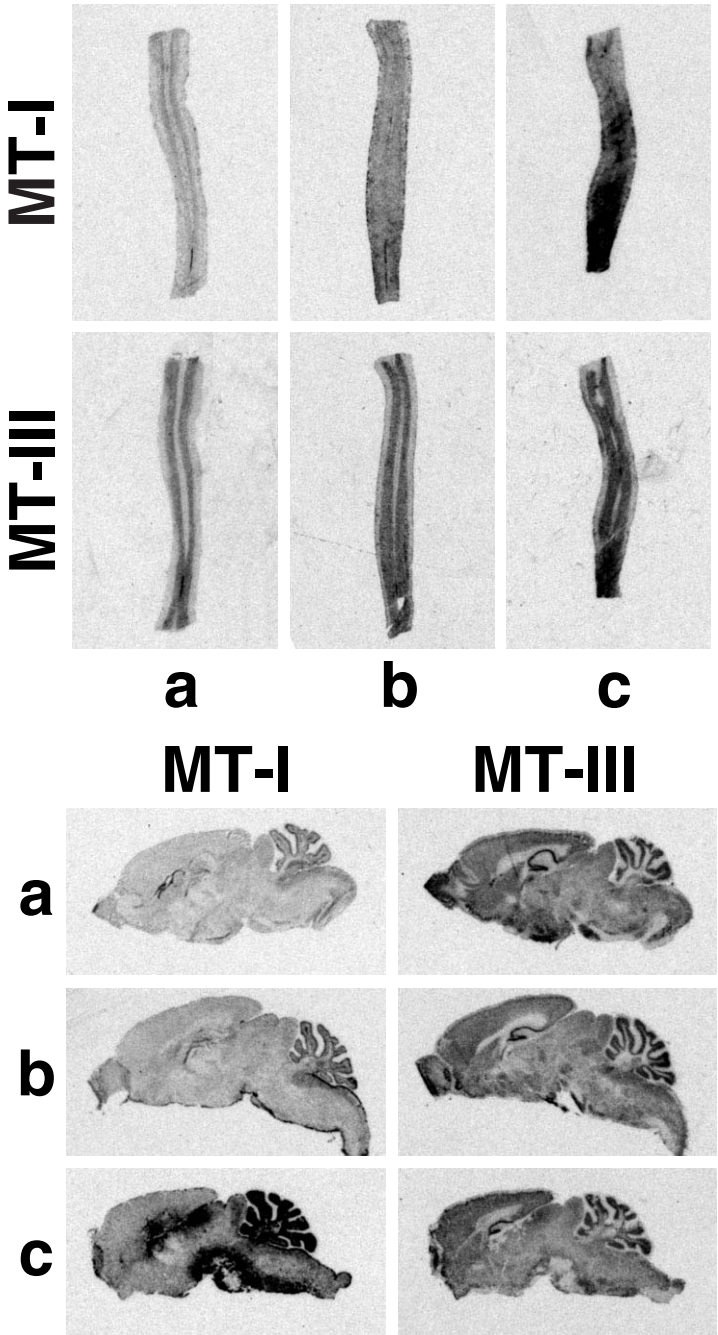

Fig. 2. MT induction in the CNS of mice with EAE. *In situ* hybridisation analysis of spinal cord (top) and brain (bottom) sections of (a) 129/Sv wild-type mouse without EAE, (b) with EAE or (c) 129/Sv IFN-γ R$^{-/-}$ with EAE. Sections were obtained from mice on day 28 post-immunisation and hybridised as specified in Experimental procedures. An increase in MT-I induction is observed related to the severity of CNS inflammation.

Table 2. MT-I induction in spinal cord of mice immunised with MOG_{40-55}

Strain	Mice	EAE	White matter MT-I	Grey matter MT-I
129/Sv	IFN-γ-$R^{+/+}$ ($n=6$)[a]	–	529 ± 315	1307 ± 917
	IFN-γ-$R^{+/+}$ ($n=6$)[a]	+	1436 ± 786[c]	2207 ± 970
	IFN-γ-$R^{-/-}$ ($n=4$)[a]	+	3007 ± 697[c,d]	3862 ± 428[c,d]
C57Bl/6x129Sv	IFN-γ-$R^{-/-}$ ($n=6$)[b]	–	702 ± 457	1979 ± 860
	IFN-γ-$R^{-/-}$ ($n=4$ or 5)[a]	+	1777 ± 606[e]	3822 ± 1044[e]

Results are the mean of arbitrary units (number of pixels × optical density) ± standard deviation.
[a]Immunisation with MOG_{40-55}.
[b]Immunisation with saline.
[c]$P<0.05$, 129/Sv with EAE vs no EAE.
[d]$P<0.05$, 129/Sv IFN-γ-$R^{-/-}$ vs IFN-γ-$R^{+/+}$.
[e]$P<0.05$, C57Bl/6x129Sv, IFN-γ-$R^{-/-}$ immunised with MOG_{40-55} vs saline.

donkey or mouse serum instead of the primary antibody. Results were considered only if the controls were negative.

Statistical analysis

Results were analysed with the Student's *t*-test or one-way analysis of variance and the Student–Newman–Keuls procedure, depending on the number of groups analysed. To determine the association between MT induction and disease severity, we used Pearson correlation. A significant difference was considered when $P<0.05$.

RESULTS

Induction of EAE in 129/Sv wild-type and IFN-γ-R-deficient mice with either 129/Sv or C57Bl/6x129Sv genetic background

Twelve 129/Sv wild-type mice and 12 129/Sv IFN-γ-$R^{-/-}$ were immunised as described above in two different experiments. All the 129/Sv IFN-γ-$R^{-/-}$ mice developed EAE, and only four animals were alive at the scheduled day of killing, whereas the incidence of the disease in the control group was 50%. In a third experiment, 12 C57Bl/6x129Sv IFN-γ-$R^{-/-}$ mice were immunised, six with MOG_{40-55} peptide and six with saline (basal control) emulsified in complete Freund's adjuvant. Incidence was also 100% in the six C57Bl/6x129Sv IFN-γ-$R^{-/-}$ mice immunised with the encephalitogenic peptide, five were alive at the end of the experiment. All animals, both IFN-γ-$R^{-/-}$ and wild-type mice with clinical signs of the disease developed chronic non-remitting EAE (Fig. 1). No gender differences were observed in disease incidence or severity (data not shown). Both strains of IFN-γ-$R^{-/-}$ mice presented a more severe course than the 129/Sv wild-type mice according to the day of onset, daily score, maximum score and chronicity (as measured by the adding up of clinical scores from each animal during the clinical follow-up) (Table 1). All animals with clinical signs of the disease showed inflammatory infiltrates with demyelination and astrogliosis in comparison with animals without EAE (data not shown).

MT induction in the CNS of mice with EAE

It has been postulated that MTs are induced in situations where inflammation is taking place. We therefore analysed the induction of MT-I and MT-III by *in situ* hybridisation in the groups of mice in which we had induced EAE in comparison with saline-injected animals and/or animals not developing the disease. We observed a strong induction of MT-I in 129/Sv mice with clinical signs of EAE compared to animals that did not develop the disease, whose levels did not differ from non-immunised control animals, either wild-type or IFN-γ-$R^{-/-}$ (data not shown). Thus, MT-I upregulation was clearly observed in both the grey and white matter of the spinal cord. This upregulation was more dramatic for the IFN-γ-$R^{-/-}$ than for the wild-type mice (Fig. 2, top a–c). Quantification of the optical densities of MT-I in the different groups is shown in Table 2.

In wild-type mice, MT-I expression was increased in brainstem and cerebellum. In IFN-γ-$R^{-/-}$ mice, MT-I

Table 3. MT-III induction in spinal cord of mice immunised with MOG_{40-55}

Strain	Mice	EAE	White matter MT-III	Grey matter MT-III
129/Sv	IFN-γ-$R^{+/+}$ ($n=6$)[a]	–	345 ± 113	2211 ± 654
	IFN-γ-$R^{+/+}$ ($n=6$)[a]	+	590 ± 270	2078 ± 552
	IFN-γ-$R^{-/-}$ ($n=4$)[a]	+	606 ± 245	2388 ± 505
C57Bl/6x129Sv	IFN-γ-$R^{-/-}$ ($n=6$)[b]	–	469 ± 90	2281 ± 363
	IFN-γ-$R^{-/-}$ ($n=5$)[a]	+	755 ± 211[c]	2757 ± 423

Results are mean of arbitrary units (number of pixels × optical density) ± standard deviation.
[a]Immunisation with MOG_{40-55}.
[b]Immunisation with saline.
[c]$P<0.05$, C57Bl/6x129Sv, IFN-γ-$R^{-/-}$ immunised with MOG_{40-55} vs saline.

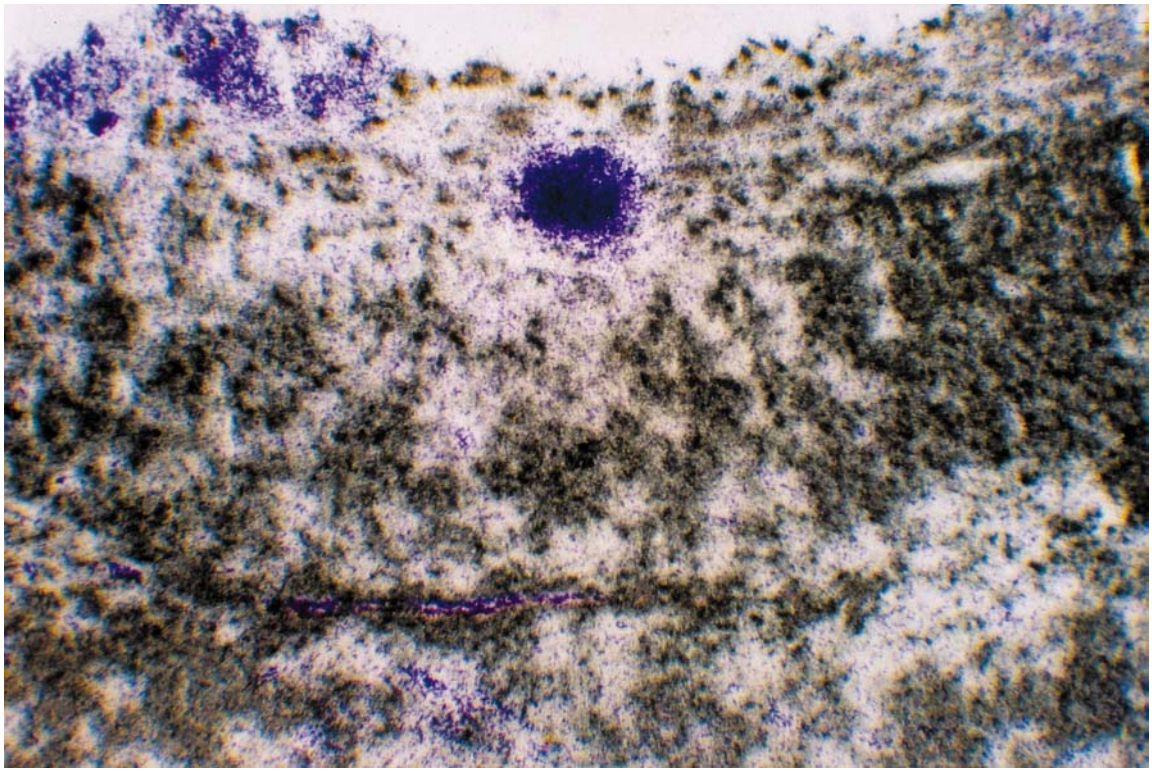

Fig. 3. Distribution of MT-I+II in the spinal cord of 129/Sv mice with EAE. Sections were obtained from mice on day 28 post-immunisation and hybridised as specified in Experimental procedures. Sections were counterstained with Toluidine Blue to visualise the inflammatory infiltrates. This picture shows the distribution of MT-I+II surrounding the inflammatory infiltrates in a representative 129/Sv mouse with EAE (250×magnification).

was dramatically upregulated in the cerebellum and some parts of the basal brainstem, diencephalon and mesencephalon (Fig. 2, bottom).

MT-III was constitutively expressed in the grey matter of the spinal cord of control mice, while that of the white matter was clearly low. In contrast to MT-I, the MT-III isoform was only modestly increased by EAE, an effect statistically significant only in the white matter of the IFN-γ-$R^{-/-}$ mice with C57Bl/6x129Sv genetic background (Table 3). No significant effects were observed in the brain either (Fig. 2).

MT-I induction was very prominent in areas surrounding EAE-induced cellular infiltrates, although certain reactivity was found in the infiltrates (Fig. 3). A significant correlation (Pearson) was found between mean chronicity (0.727, $P=0.05$) and mean daily score (0.678, $P=0.01$) and MT-I induction in white matter. In the same way, a correlation between disease duration and MT-I expression in white matter was also observed (0.627, $P=0.02$).

MT-I+II proteins are expressed by reactive astrocytes and microglialmacrophages in the CNS of mice with EAE

In 129/Sv control mice without EAE, MT-I+II was only seen in meninges, choroid plexus, and ependyma, while intraparenchymal cells showed a very low expression of these proteins (see Fig. 4B,D,F for MT-I+II stainings in the brainstem, spinal cord and cerebellum, respectively). In mice with EAE, MT-I+II staining was increased throughout the brainstem (Fig. 4A), spinal cord (Fig. 4C), and cerebellum (Fig. 4E).

To identify the cell populations expressing MT-I+II we performed a double immunohistochemical staining with anti-MT-I+II antibodies and lectin for microglia (Fig. 5A) or anti-GFAP for astrocyte staining (Fig. 5C) as specified in Experimental procedures. MT-I+II was increased in round or amoeboid macrophages (Fig. 5B) and in reactive astrocytes (Fig. 5D). No significant immunohistochemical staining product was observed in negative control sections.

DISCUSSION

It is widely accepted that oxidative stress plays a role in the pathogenesis of EAE although the results are controversial (Cross et al., 1994; Okuda et al., 1995; Ruuls et al. 1996; Fenyk-Melody et al., 1998; Sahrbacher et al., 1998). Treatment with selective inhibitors of nitric oxide synthase 2 (NOS-2) (Cross et al., 1994) or blocking of NOS-2 by antisense sequences (Ding et al., 1998), prevents the clinical development of EAE with a reduction in inflammation and demyelination. Furthermore, treatment with the antioxidant uric acid, a natural compound that selectively binds and inactivates peroxynitrite, inhibits clinical signs in an acute, aggressive form of mouse EAE (Hooper et al., 1998). MTs show cytoprotective effects that appear to be related to their ability to act as scavengers of hydroxyl and superoxide radicals

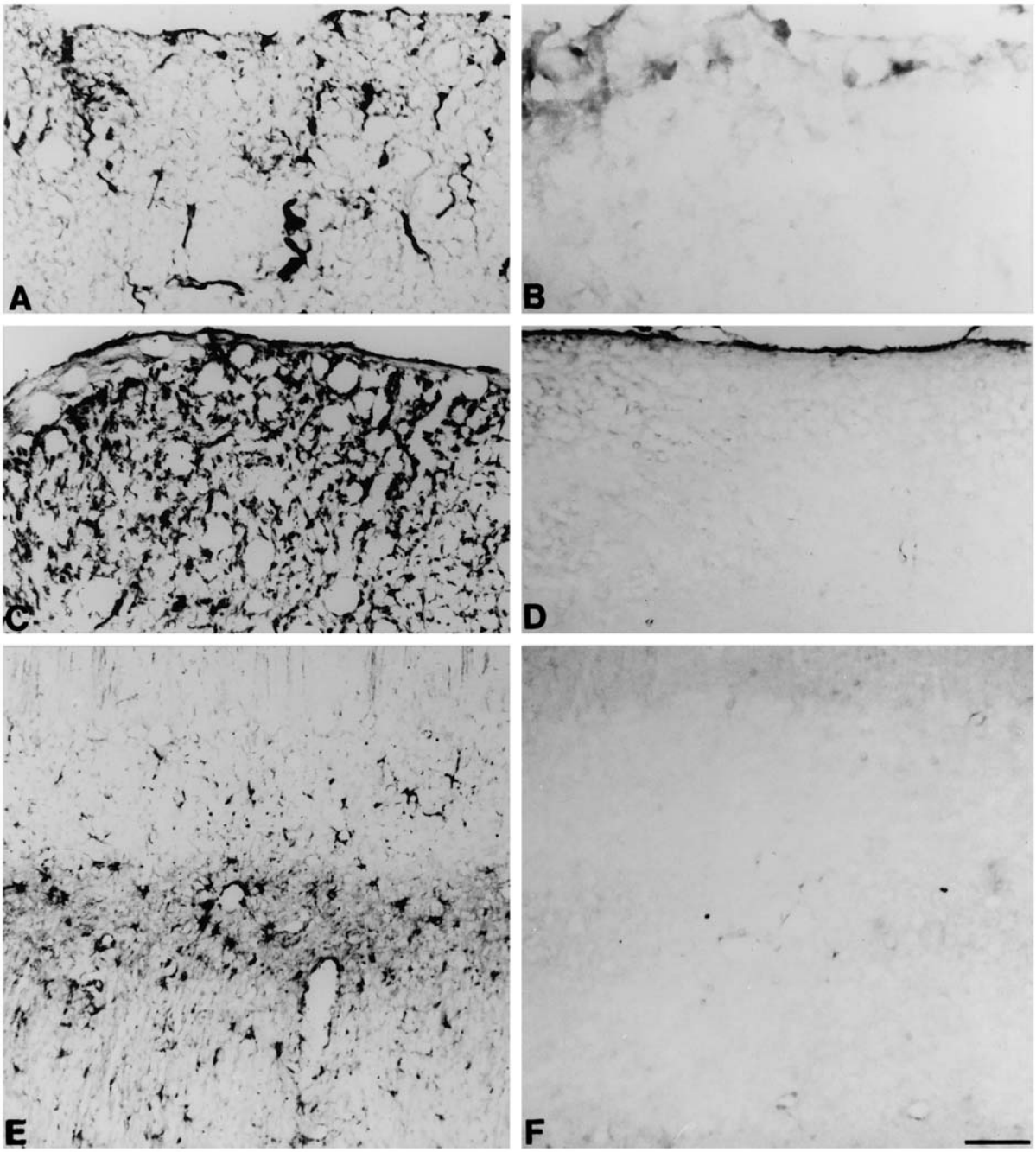

Fig. 4. MT-I+II immunohistochemistry in 129/Sv mice with (A,C,E) and without (B,D,F) EAE. (A) In the brainstem, many reactive astrocytes and macrophages situated around vessels show increased MT-I+II expression during EAE. (B) In control mice without EAE, MT-I+II are only seen in meninges and ependyma, while intraparenchymal cells show a very low expression level of MT-I+II. (C) In the spinal cord, several reactive astrocytes and macrophages of EAE plaques in white matter show increased MT-I+II expression. (D) In control mice without EAE, MT-I+II are only seen in meninges and ependyma of the spinal cord. (E) In the cerebellum grey and white matter, reactive astrocytes and macrophages increase their MT-I+II expression during EAE. (F) In control mice without EAE, intraparenchymal cells of the cerebellum show a very low expression level of MT-I+II. Scale bar = 50 μm.

(Thornalley and Vasak, 1985; Lazo et al., 1995). *In vitro* studies have shown that MTs protect against the cytotoxic and DNA-damaging effects of NO (Schwarz et al., 1995; Tsangaris and Tzortzatou-Stathopoulou, 1998). On the other hand, NO has been reported to mediate MT induction by lipopolysaccharides (Arizono et al., 1995) and *in vivo* inhibition of NO synthesis decreases the induction of MT-I in response to stress (Molinero et al., 1998). In this work we observed that EAE induction in mice significantly increased the levels of MT-I+II in the CNS and that the level of MT expression is related to the severity of the disease. This suggests that induction of these antioxidant proteins may be part of a defense mechanism triggered during EAE induction.

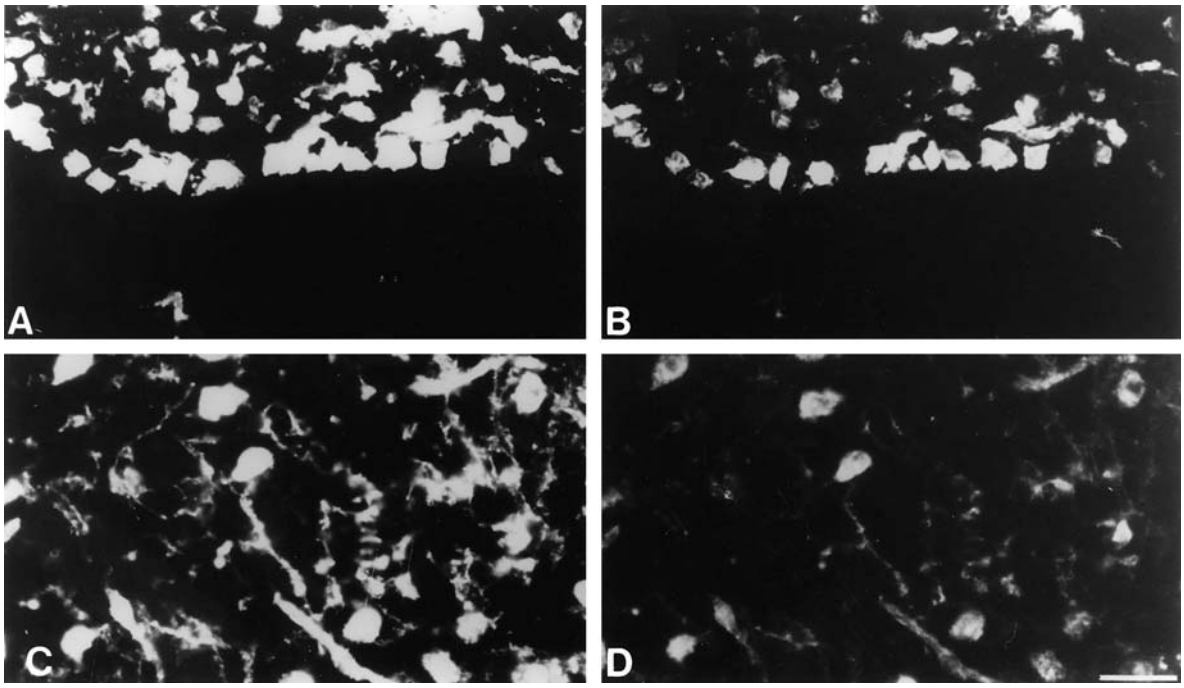

Fig. 5. Double immunofluorescence labeling for lectin or GFAP and simultaneously MT-I+II. Many of the lectin+macrophages (A) are expressing MT-I+II (B) during EAE. Some of the GFAP+astrocytes (C) are co-expressing MT-I+II (D). Scale bar = 25 μm.

To better understand the physiological functions of MTs in the CNS, it is essential to characterise the factors that control their expression. Members of the nuclear hormone receptor family, interferons, inducers of the acute phase response and metallo-regulatory proteins have been shown to induce them (De et al., 1990; Hernández et al., 1997; Carrasco et al., 1998; Hidalgo et al., 1998). It has been described that when inflammatory injury occurs in the CNS, astrocyte-derived interleukin (IL-1) induces the synthesis of MTs, protecting against free radicals and oxidative stress (Yan et al., 1992). MT-I is strongly induced in astrocytes and microglia of transgenic mice which have the cytokine IL-6 under control of the GFAP promoter (Carrasco et al., 1998). This induction correlates with the inflammatory response caused by IL-6. i.c.v. injection of IL-1 and IL-6 induces rat brain MT-I+II (Hernández and Hidalgo, 1998). Given that pro-inflammatory cytokines play an important role in the induction of EAE and are at the same time able to induce MTs, we analysed the induction of MTs in two different strains of IFN-γ-$R^{-/-}$ mice: 129/Sv and C57Bl/6x129Sv. We observed that IFN-γ-$R^{-/-}$ mice with a 129/Sv background have a more severe disease than control littermates. C57Bl/6x129Sv IFN-γ-$R^{-/-}$ mice also developed a more severe EAE than 129/Sv wild-type, but less severe than 129/Sv IFN-γ-$R^{-/-}$, probably because of their different genetic background, C57Bl/6 mice being less susceptible to EAE induction (Mendel et al., 1995). When MT-I+II induction in the different groups of mice was analysed, we observed that it paralleled the severity of the clinical signs, irrespective of the presence of the IFN-γ-R. In fact, C57Bl/6x129Sv IFN-γ-$R^{-/-}$ mice showed only slightly higher values than the 129/Sv wild-type animals, whereas 129/Sv IFN-γ-$R^{-/-}$ mice, with more severe EAE, had a higher induction of MT-I+II. Furthermore, these results demonstrate that IFN-γ is not necessary for MT-I+II induction in this experimental model. The fact that IFN-γ is one of the most powerful inducers of inducible NOS, and hence of NO, would suggest that in the IFN-γ-$R^{-/-}$ mice (probably with very low levels of NO) MT induction is regulated rather via some other stimulus than in response to increased levels of NO.

In contrast to MT-I+II, MT-III induction occurred differently. High levels of MT-III were constitutively expressed in the CNS of mice at basal levels (either wild-type or IFN-γ-$R^{-/-}$ mice). Moreover, no significant induction of MT-III was observed adjacent to the inflammatory infiltrates in mice with EAE. Only a slight though statistically significant increase of MT-III was observed in CNS white matter of C57Bl/6x129Sv IFN-γ-$R^{-/-}$ mice with EAE when compared to the same strain of mice immunised with saline (basal control). It is difficult to account for these results, as no differences were observed in 129/Sv IFN-γ-$R^{-/-}$ mice with EAE compared to wild-type controls without EAE, which suggests that the different genetic background might account for the distinct capability to induce MT-III during the disease. The different expression patterns between MT-I+II and MT-III, either basal or post-EAE induction support a different function of these proteins in the brain, as recently postulated (Carrasco et al., 1999).

In EAE, immunoreactivity to MT-I+II was found in the areas surrounding the inflammatory infiltrates and also perivascularly, where reactive astrocytes were localised. An increase in MT-I+II induction was also found in the grey matter of IFN-γ-$R^{-/-}$ mice with EAE (Table 2).

A general hypothesis invokes astrocytic MTs as a mechanism for the removal and sequestration of cytotoxic stimuli such as metal ions and free radicals from the extracellular space (either from damaged cells or from the plasma due to blood–brain barrier leakage) because of their proximity to neurones, the capillaries from the blood–brain barrier and other elements of the CNS (Aschner, 1997b). In fact, recent studies have demonstrated that MT-I+II are important in controlling the apoptosis of neurones induced by CNS trauma (Penkowa et al., 1999). Enhanced MT-I+II induction could thus be a response to an environmental pressure to protect neurones from destruction. This in part could account for the increased MT-I+II expression found in the grey matter of mice with EAE.

Immunohistochemical analyses of MT-I+II also showed positive reaction for these proteins in macrophages/microglia of the inflammatory infiltrates. In contrast, no positive reaction was found for infiltrating lymphocytes. This could explain the lower signal found for mRNA MT-I in the inflammatory infiltrates by *in situ* hybridisation in comparison to the areas surrounding the infiltrates, where high MT-I upregulation was observed. These results suggest that macrophages may contain these antioxidant proteins in their cytoplasm and support the hypothesis of the protective role of inflammatory cells in the resolution of inflammation. According to this, it has been recently demonstrated that activated peripheral monocytes produce *in vitro* brain-derived neurotrophic factor (Kerschensteiner et al., 1999) and that the implantation of activated macrophages into spinal cord lesions leads to partial functional recovery of paraplegic rats (Rapalino et al., 1998).

In summary, we show here that MT-I+II are induced in the CNS of mice with EAE, and that IFN-γ is unlikely a factor responsible for this response. More extensive knowledge of the mechanisms responsible for MT induction will be of great importance to clarify their role during the inflammatory process. Therapies addressed to inducing MT expression at the CNS might be promising candidates in the treatment of MS.

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