Reduction of free radicals in multiple sclerosis: effect of glatiramer acetate (Copaxone®)

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Free radicals have been found in high concentrations within inflammatory multiple sclerosis (MS) lesions. The superoxide anion (O_2^-) reacts rapidly with nitric oxide (NO), producing peroxynitrite ($ONOO^-$). Glatiramer acetate (GA) is a specific MS immunomodulator that induces the synthesis of Th2 cytokines, and reduces the frequency of relapses and the formation of active brain lesions. Proinflammatory cytokines could play a role in free radicals production in the peripheral immune system as well as in the central nervous system (CNS). The effect of GA on iNOS, superoxide radicals (O_2^-) and 3-nitrotyrosine production by peripheral blood adherent mononuclear cells (PBAMs) was assessed. Our findings demonstrate that *in vitro* GA reduced spontaneous and LPS-induced iNOS, 3-nitrotyrosine, NO and O_2^- production, and that similar inhibition can be demonstrated *ex vivo* in mononuclear cells obtained from GA-treated patients. The inhibition of the production of free radicals in PBAMs may represent a new therapeutic mechanism against inflammation during MS. *Multiple Sclerosis* 2008; **xxx:** 1–10. http://msj.sagepub.com

Introduction

Reactive nitrogen species can be generated by biochemical reactions of nitric oxide (NO) or by enzymatic catalysis of NO metabolism. The production of peroxynitrite ($ONOO^-$) represents a reaction between reactive nitrogen and reactive oxygen species (ROS) and its presence is an indirect measure of the production of both NO and O_2^-. In mammalian tissues there are three distinct isoforms of nitric oxide synthase (NOS): the neuronal form (nNOS); the inducible nitric oxide synthase (iNOS) form, present in various cell types upon inflammatory stimulation (e.g. macrophages); and the constitutive enzyme form primarily discovered in the endothelium (eNOS). All three isoforms have a similar molecular structure and require multiple cofactors [1].

The expression of iNOS and NO production in various cell types is activated by a combination of proinflammatory signals such as ligands that activate toll-like receptors and/or cytokines such as IL-1, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). In macrophages, monocytes, and other cells, both the induction of iNOS and the presence of L-arginine are sufficient to initiate the generation of NO [2]. The toxicity of NO is also greatly enhanced when it combines with O_2^- to generate $ONOO^-$, an oxidant capable of damaging lipids, proteins, and DNA.

The etiology of multiple sclerosis (MS) has not yet been fully elucidated, but it is believed that immunological mechanisms are the most important in disease initiation and progression [3]. The inflammatory environment in demyelinating lesions may lead to the generation of oxygen and

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nitrogen free radicals as well as proinflammatory cytokines. iNOS is up-regulated in the central nervous system (CNS) during MS and its rodent model experimental autoimmune encephalomyelitis (EAE) [4–6]. Recent clinical and animal studies indicate that NO and ONOO⁻ contribute to the pathogenesis of MS, and it is a matter of debate whether NO play a pathogenic or protective role in EAE and MS [7]. Tissue obtained post mortem from the CNS of MS patients demonstrated the presence of iNOS mRNA and nitrotyrosine. In cerebrospinal fluid (CSF) and plasma nitrite and nitrate levels are elevated in patients with MS [8,9]. Evidence suggests that NO contributes to tissue injury, blood–brain barrier (BBB) breakdown, axonal degeneration, and plaque formation [10–13]. On the other hand, NO may help to control the immune response via several immunoregulatory processes.

Glatiramer acetate (GA; Copaxone®), a synthetic random polymer of the amino acids L-alanine (A), L-glutamate (E), L-lysine (K), and L-tyrosine (Y), and an average length of 40–100 amino acids, is an approved agent for the immunomodulatory treatment of MS. When injected subcutaneously each day, GA reduces the frequency of relapses and the formation of active brain lesions [14–16]. Biochemical studies revealed that GA binds directly and with high affinity to purified HLA-DR1, HLA-DR2, and HLA-DR4 [17], suggesting that GA contains multiple epitopes enabling it to bind promiscuously to MHC class II molecules, where it could potentially be recognized by CD4 T cells. GA inhibits monocyte reactivity *in vitro* and *in vivo* [18] and is a potent inducer of Th2/3 cells that secrete high levels of anti-inflammatory cytokines, cross the BBB, accumulate in the CNS [19], and express *in situ* IL-10, TGF-β, and BDNF [20]. Therefore, to elucidate further the therapeutic effect of GA in MS, we have analyzed peripheral blood adherent mononuclear cells (PBAMs) from patients with RR-MS for evidence of iNOS, NO, O_2^-, and ONOO⁻ levels.

In the current combined *in vitro* and *in vivo* study, we confirm that iNOS and both NO and O_2^- levels and the derived ONOO⁻ play a role in the pathogenesis of MS. The most striking observation in these investigations was the ability of GA to inhibit the free radicals in non-GA-primed PBAM populations. Our data suggest that GA may be useful in a variety of disorders in which immune reduction of NO-mediated damage is desirable.

Materials and methods

Cells sources and cultures

Venipuncture was performed in the morning between 08:00 and 10:00. Peripheral blood samples were collected into 4 ml endotoxin-free EDTA tubes (Vacutainer, Becton Dickinson, NJ, USA). Tubes were kept at room temperature and transported to the laboratory for processing within 1 h of collection. PBAMs were isolated by Ficoll-Hypaque density gradient centrifugation. Isolation and purification of PBAMs were carried out taking advantage of their adherence to culture plates. After 1 h at 37°C, non-adherent cells were removed with the supernatant followed by three washes with phosphate buffer solution (PBS). Adherent cells (approximately more than 90% of the cells were CD14⁺), were then carefully detached from the surface of the culture plates and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 25 mM Hepes buffer, 50 U/ml penicillin, and 50 mg/ml streptomycin (all media and components were purchased from SIGMA, Italy). Cells (10⁶/ml) were immediately plated and incubated at 37°C in a 95% humidified 5% CO_2 cell culture incubator, with and without a mitogenic stimulation with 10 µg/ml of bacterial LPS (Sigma). At the end of incubation culture supernatants were removed, and wells were washed twice with HBSS to remove non-adherent cells. Adherent cells were detached and counted with a hemocytometer. Similar results were obtained in triplicate assay from each subject among the different groups. Cell viability in each culture was assessed by Trypan blue die exclusion.

GA (Copaxone®) obtained from Teva-Sanofi-Aventis Italy, and all compounds used in this study were dissolved in culture medium and added to cell cultures. An equal volume of media was added to controls to make normalized volumes. All reagents used were tested before use for endotoxin (<10 pg/ml; Associates of Cape Cod, Inc., Woods Hole, MA, USA) and mycoplasma contamination (General-probe II; General-probe Inc., San Diego, CA, USA) and found negative. The same batch of serum and medium were used in all experiments.

Detection of iNOS and 3-nitrotyrosine

Determination of iNOS protein and 3-nitrotyrosine (a stable end product of the ONOO⁻ decomposition) was performed in protein extracts by western blotting (WB). Equal amounts of protein (50 µg), extracted from human peripheral adherent mononuclear cells and quantified by spectrophotometric assay (HP 8452A, CA, USA) using the Lowry method, were separated by electrophoresis in a 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE, BIO-RAD, Hercules, CA, USA) and transferred to nitrocellulose membranes (BIORAD, Hercules, CA, USA) at 4°C in glycine-methanol buffer. Nitrocellulose was then blocked in Tris-

buffered saline [TBS]-milk (10% w/v) and incubated overnight, with a monoclonal anti-NOS$_2$ antibody (Transduction laboratories, USA) at a dilution of 1/2.500 and monoclonal anti-3-nitrotyrosine antibody (Alexis Biochemicals) at a dilution of 1/5.000. Nitrocellulose sheets were then washed in TBS, incubated for 1 h with a secondary antibody conjugated with alkaline phosphatase, washed again, and developed in an alkaline buffer with nitroblue-tetrazolium (NBT) as substrate (Alkaline Phosphatase Conjugate Substrate Kit, BIO-RAD, Hercules, CA, USA). β–actin (Sigma, 1/10 000) was used as an internal standard. The resulting blot image files were imported and analyzed with a commercially available gel analysis software package (Gel Doc 1000 Bio-Rad Milan, Italy). Data are expressed as mean IOD ± SD.

Determination of O$_2^-$

Production of O$_2^-$ was determined spectrophotometrically (Hewlett Packard 8452 A) by monitoring the reduction of ferricytochrome c (Type VI, Sigma) at 550 nm, as described by Pritchard [21]. Briefly, Ferricytochrome c (final concentration, 50 μmol/l) was added directly to the cuvette containing the cells and Dulbecco's phosphate buffered saline (DPBS) (final volume 1 ml), either in the presence or absence of superoxide dismutase [(SOD), 350 U/ml], and subsequent changes in absorbance were followed for 10 min. Rates of O$_2^-$ production were calculated on the basis of the molar extinction coefficient of reduced ferricytochrome c [$\varepsilon = 21\ 000$ cm^{-1} (mol/l)$^{-1}$]. Cell counts were used to calculate results as nanomoles O$_2^-$ per 10^6 cells per minute.

Measurement of nitrite levels

The transient and volatile nature of NO makes it unsuitable for most convenient detection methods; two stable breakdown products, nitrate (NO$_3$) and nitrite (NO$_2$) can be easily detected by photometric means. The presence of NO in plasma and in cells culture supernatants (cells were cultured in nitrite-free medium) was evaluated by measuring the amount of nitrite by a colorimetric assay based on Griess reactions, using a commercial kit (Assay Designs, USA).

Briefly, 5 μl of nitrate reductase enzyme solution, 10 μl of 2 mM NADPH and 100 μl of Griess Reagent (sulphanilamide and naphthalene–ethylene diamine dihydrochloride), were added to 100 μl of supernatant, after the supernatant had been kept at room temperature for 1 h to convert nitrate to nitrite. Absorbance of the wells was measured at 540 nm in an ELISA reader (Bio Rad 680) and amounts of nitrite were estimated by a standard curve. The intra- and inter-assay reproducibilities were over 90. Duplicate values that differed from the mean by greater than 10% were not considered for further analysis.

Subjects

Twenty-one RR-MS consecutive patients visiting the Department of Neurology of the University of Chieti, Italy were selected. Inclusion criteria were: clinically definite MS [22,23], relapsing–remitting (RR) course [24], and a Kurtzke's Expanded Disability Status Scale (EDSS) score of 5.5 or less [25]. All participants were relapse free and had not used steroids for at least 30 days before screening or before randomization. The use of immunosuppressive drugs in the 12 months before study entry, or the use of interferons, intravenous immunoglobulins, more than 30 consecutive days of chronic steroid treatment, or participation in clinical studies of experimental drugs in the 6 months before study entry were not allowed. Twenty healthy donors (healthy controls (HC)) from the Transfusion Blood Bank Services of Chieti, Italy, matched for age, were enrolled. All subjects gave informed consent. None of the participants were smokers or vegetarians, none were known to be taking vitamin B$_{12}$ or folates (including multivitamins supplements) or prescribed drugs known to affect circulating homocysteine or NO concentrations, and none had been previously treated with immunosuppressive drugs. Signs of malnutrition were excluded on the basis of physical examination.

Fourteen patients were studied after 3 months of GA therapy (20 mg subcutaneously daily) and for 10 of them we performed additional experiments after at last 6 months of treatment. Patients characteristic and clinic disability measures are summarized in Table 1.

Table 1 Patients characteristic and clinic disability measures

Groups	n	Gender (male/female)	MEAN age (years ± SD)	MEAN pre-study disease duration (years)	MEAN EDSS baseline (T0)	MEAN EDSS (T3)	MEAN EDSS (≥ T6)
RR-MS	21	15F/6M	40.6 ± 6.4	7.9 ± 6.4	3.25 ± 1.04 (n = 21)	3.1 ± 0.88 (n = 14)	3.0 ± 0.98 (n = 10)
HC	20	12F/8M	42.3 ± 6	—	—	—	—

Statistics

To compare the level of iNOS between HC and RR-MS patients, unpaired Student's *t*-tests have been conducted in basal and LPS-stimulated conditions. In both cases the tests were statistically significant (*p*-values *p* < 0.01). The Wilcoxon's signed-rank tests were performed using statistiXL for testing pairs of data of RR-MS patients before and after short- (3 months) and long-term (≥ 6 months) GA treatment.

Results

NO, iNOS, 3-nitrotyrosine and O₂⁻ production

We compare the NO, iNOS, 3-nitrotyrosine, and O_2^- production in RR-MS patients and healthy controls.

Figure 1(A) shows the levels of NO in the supernatants of PBAMs from 20 HC and 21 RR-MS patients. Basal and LPS-induced production of NO is higher in RR-MS than in HC ($p < 0.001$). To verify whether the detected NO derived from iNOS, the WB analysis of iNOS expression was performed. Our data show that the level of iNOS was significantly higher in RR-MS than in HC, reflecting the higher production of NO. The densitometric analysis showed increased levels in basal (0.16 ± 0.002 versus 0.25 ± 0.03, $p < 0.001$) and in LPS-stimulated PBAMs from RR-MS (0.37 ± 0.03 versus 0.28 ± 0.031, $p < 0.001$; see Figure 1(B)). Increased iNOS expression in RR-MS may strongly support increased plasma NO levels detected in RR-MS patients with respect to the plasma levels detected in normal subjects (Figure 1(E)).

Basal superoxide released O_2^- levels from PBAMs obtained from 21 untreated RR-MS patients were
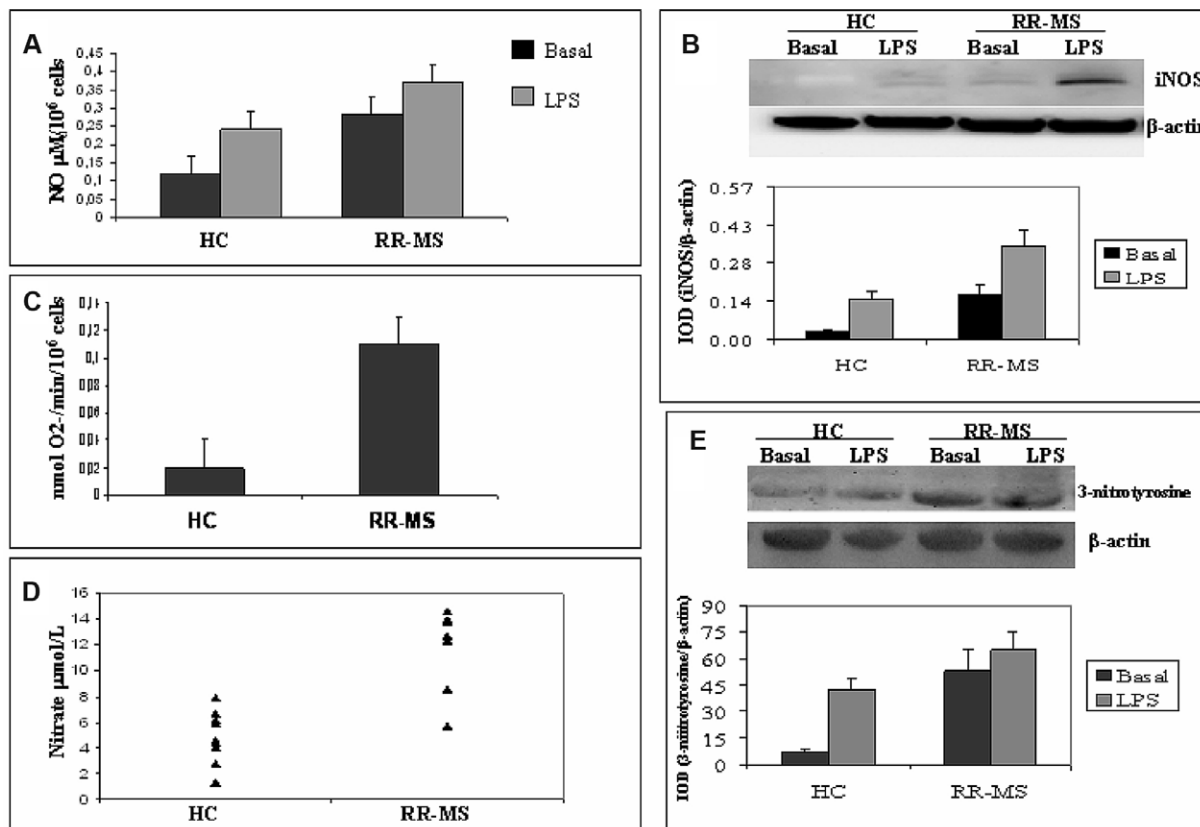

Figure 1   (A) NO release by basal and LPS-stimulated PBAMs from 20 HC and 21 RR-MS patients. All samples were analyzed in duplicate at the same time. (B) Shows analysis of iNOS protein in PBAMs of RR-MS patients and matched controls. An example of bands is shown as a representative experiment. Immunoreactive bands were analyzed by densitometry and normalized to β-actin levels. Each bar represents the mean of relative expression of iNOS by PBAMs from HC and RR-MS. Differences between groups were statistically significant. (C) Spectrophotometrically analysis of O_2^- production in HC and RR-MS. Cell counts were used to calculate results as nanomoles of O_2^- per 10^6 cells per minute. (D) Plasma levels of NO, express as μmol/l, in control and patient groups. NO was significantly increased in RR-MS patients as compared to controls. All assays were performed in duplicate. (E) WB and densitometric analysis were performed in order to derive levels of basal or LPS-induced 3-nitrotyrosine. An example of bands is shown as a representative experiment. Each bar represents the mean of percentage of relative expression of 3-nitrotyrosine by PBAMs from HC ($n = 20$) and from RR-MS ($n = 21$).

higher than PBAMs from 20 HC ($p < 0.001$; see Figure 1(C)), suggesting that NO production has a direct bearing on superoxide release. $ONOO^-$ formation evaluated as 3-nitrotyrosine, the stable end product of the $ONOO^-$ decomposition, was significantly higher in RR-MS patients than in HC subjects (Figure 1(D)), reflecting the higher production of both $ONOO^-$ and NO observed in RR-MS patients. The excessive diffuse immunological over activation may be responsible for less significant induction by LPS in PBAMs from RR-MS.

Effects of GA on NO, iNOS, 3-nitrotyrosine and O_2^- production

During preliminary experiments, cells were incubated at 37°C, 5% CO_2, overnight with LPS (range 1–25 µg/ml) and GA in the concentration range 5–100 µM/ml (Figure 2). No inhibitory effects of each GA concentration were observed in spontaneous production of nitrite, iNOS, 3-nitrotyrosine, and O_2^-, in all experiments performed. Our data are in agreement with reports showing that the good iNOS production was obtained in monocytes incubated with LPS concentration from 1 to 25 µg/ml for 8 to 48 h (see [29,30]). To assess the nitrite-blocking effect of GA, we monitored nitrite levels in culture media after stimulating cells with LPS (10 µg/ml) in the presence or absence of GA. LPS stimulation caused a significant accumulation of NO in culture media (2-fold) reduced by GA and the more significantly reduction was observed with GA at 50 µg/ml (3.3-fold); see Figure 3(A). We then examined whether the inhibition of NO production by GA was accompanied by a parallel change in the levels of iNOS protein expression. As expected, addition of GA (50 µg/ml) reduced iNOS protein expression (Figure 3(B)). Spontaneous O_2^- production by PBAMs was not significantly reduced by GA addition. To increase O_2^- production, the PBAMs were pre-treated with a sub-maximal concentration of H_2O_2 (20 µM) for 2 h and then maintained for an additional 16 h in the presence of GA (50 µg/ml). O_2^- generation by PBAMs significantly decreased after addition of GA ($p < 0.01$), as show in Figure 3(C). No influence by H_2O_2 on cell viability was observed. More than 98% cells were viable, as determined by trypan blue dye exclusion at the beginning of the culture, and more than 90% were viable at the end of the culture. 3-Nitrotyrosine, was significantly reduced after exposure of PBAMs to GA (50%, $p < 0.01$, mean of five donors; see Figure 3(D)).

In the next step we tested the down-regulating effect of GA in *ex vivo* PBAMs isolated from RR-MS patients. Of special significance is the restoration of iNOS and 3-nitrotyrosine to a basal level by GA treatment. iNOS production was significantly reduced in LPS-induced PBAMs (46%; Figure 4(A)) such as NO (42.8% reduction; Figure 4(C)), 3-nitrotyrosine (46,9% reduction; Figure 4(B)), and O_2^- production (36.4% reduction; Figure 4(D)).

NO, iNOS, 3-nitrotyrosine and O_2^- levels in MS patients treated with GA

After 3 months of GA therapy, there was a significant decrease of plasma NO levels in RR-MS patients, after 6 months or more of GA therapy the plasma NO levels remained low and the stability of the response to GA was confirmed in all patients (Figure 5). In order to test whether the down-regulation of circulating levels of NO was accompanied by changes in NO secretion by PBAMs, we measured the levels of NO in PBAMs from 14 GA-treated RR-MS patients after 3 months or more than 6 months of treatment. In MS patients we detected reduced NO production after 3 months or more than 6 months of treatment (Figure 6(D)). Reduction of iNOS protein expression by GA was confirmed in RR-MS patients treated for 3 months or more than 6 months with GA. The densitometric analysis shows basal and LPS-induced iNOS production reduction after 3 months of GA therapy (basal T0 = 0.25 ± 0.04 versus basal T3 = 0.15 ± 0.006, $p < 0.001$; LPS-induced T0 = 0.37 ± 0.03 versus LPS-induced T3 = 0.29 ± 0.007, $p < 0.001$). To assess whether the reduction of iNOS protein production was related to the duration of GA treatment, we examined iNOS protein levels in patients that proceeded with GA treatment for at least 6 months. The reduction iNOS production by GA observed at 3 months was confirmed after 6 months, suggesting that the main effect of proceeding with the treatment is to stabilize the reduction in both basal and LPS-induced iNOS expression (basal T3 = 0.148 ± 0.008 versus basal T≥6 = 0.146 ± 0.007; LPS-induced T3 = 0.286 ± 0.01 versus LPS-induced T≥6 = 0.282 ± 0.009; see Figure 6(A)). O_2^- formation was significantly reduced in PBAMs from RR-MS patients in the first 3 months of GA treatment (T0 = 0.11 ± 0.042 versus T3 = 0.04 ± 0.003). After another 6 months or more of treatment, the extracellular O_2^- value tends to stabilize (T≥6 = 0.05 ± 0.004; T≥6 versus T0, $p < 0.001$; T≥6 versus T3, $p < 0.05$; Figure 6(C)). Reduction of NO and O_2^- resulted in significant inhibition of $ONOO^-$ formation. In fact, in RR-MS patients after 3 months of GA treatment, basal and LPS-induced 3-nitrotyrosine levels are significantly decreased (basal condition = 27% of decrease, LPS stimulation = 15% of decrease), and similar results were observed in more long-term therapy (T≥6); see Figure 6(B).
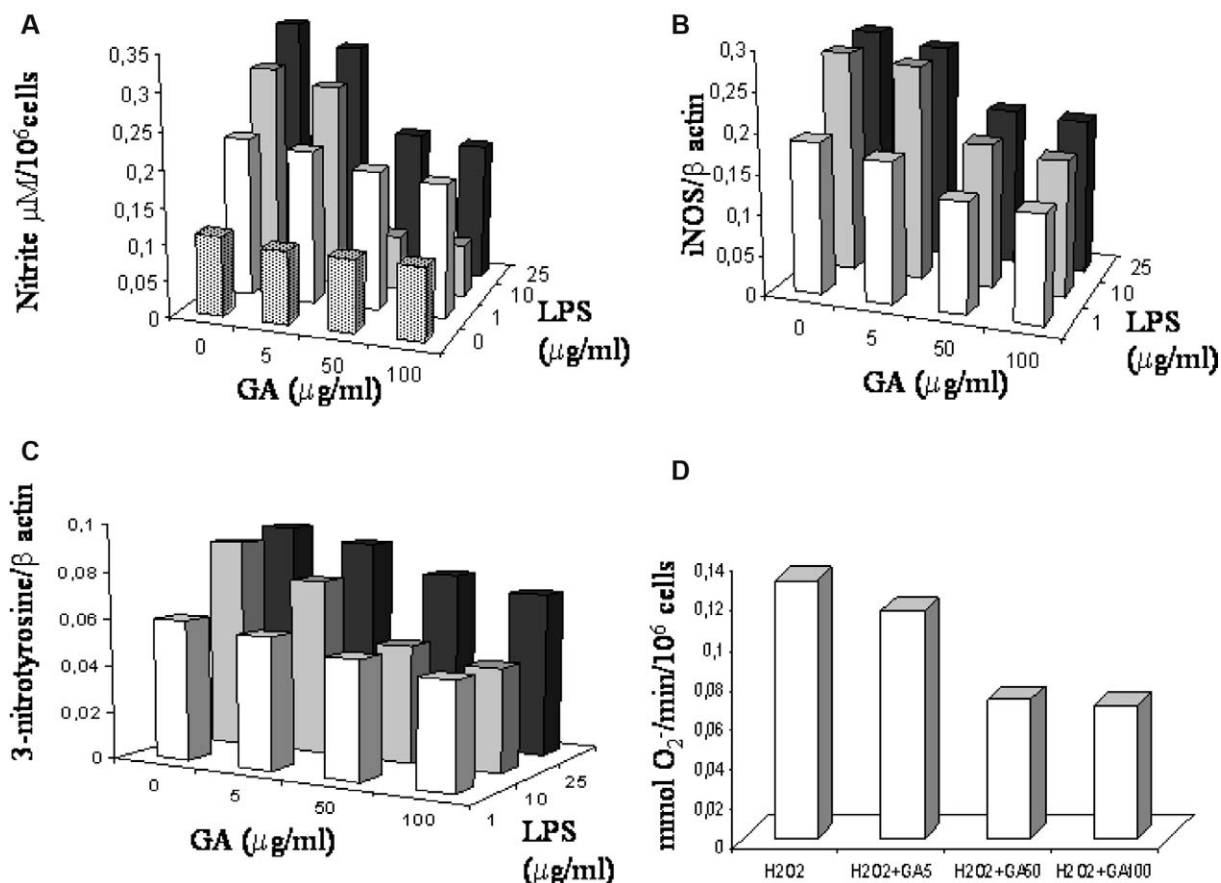
Figure 2 (A) Inhibition by GA (5–100 µg/ml) of the nitrite release by LPS-stimulated (1–25 µg/ml) PBAMs. Amount of nitrite in cell-free supernatants was quantified by a colorimetric assay based on the Griess reaction. Results are representative of four independent experiments, each using PBAMs isolated from different healthy donors. Inhibition by GA (5–100 µg/ml) of the (B) iNOS expression and (C) 3-nitrotyrosine by LPS (1–25 µg/ml)-stimulated PBAMs. Results are representative of four independent experiments each using PBAMs isolated from different healthy donors. Each cell proteins extract was blotted and stained at least twice. (D) Spectrophotometrically analysis of inhibition by GA (5–100 µg/ml) of O_2^- production in H_2O_2-stimulated PBAMs isolated from different healthy donors. Cell counts were used to calculate results as nanomoles of O_2^- per 10^6 cells per minute.

Discussion

Deregulation or overproduction of NO has been implicated in the pathogenesis of many disorders, including atherosclerosis, neurodegenerative diseases, and inflammatory autoimmune diseases. The role of oxidative stress in demyelinating condition such as those observed in MS is substantiated by pathological findings and animal models that have provided experimental paradigms to delineate the possible mechanisms. Recent observation showed that in experimentally produced autoimmune disease, EAE microglial cells destroy oligodendrocytes in an NO-dependent fashion. Localization of reactive nitrogen species in MS lesions and amelioration of EAE by administration of NOS inhibitors [26,27] prompted us to examine if the reported increase of iNOS in the CNS during MS occurred

also in PBAMs of MS patients. We performed a study aimed at evaluating the *ex-vivo* oxidative stress using PBAMs as a non-neural cell model able to produce proinflammatory cytokines and free radicals. We demonstrated that in RR-MS patients the expression of iNOS and 3-nitrotyrosine NO and O_2^- was elevated in comparison to HC, corroborating other studies that found increased levels of iNOS during the acute phase of EAE and MS [5,28]. Our data concur with those of Giovannoni, *et al.* [9], who found significantly higher serum levels of NOx and with those of Sarchielli, *et al.* [29], who detected the increased production of NO by PBMC of MS patients and considered NO a marker of mononuclear activation in the peripheral blood of MS patients and, indirectly, of disease activity.

This study provides new insights into the functional interaction between NO and O_2^- production
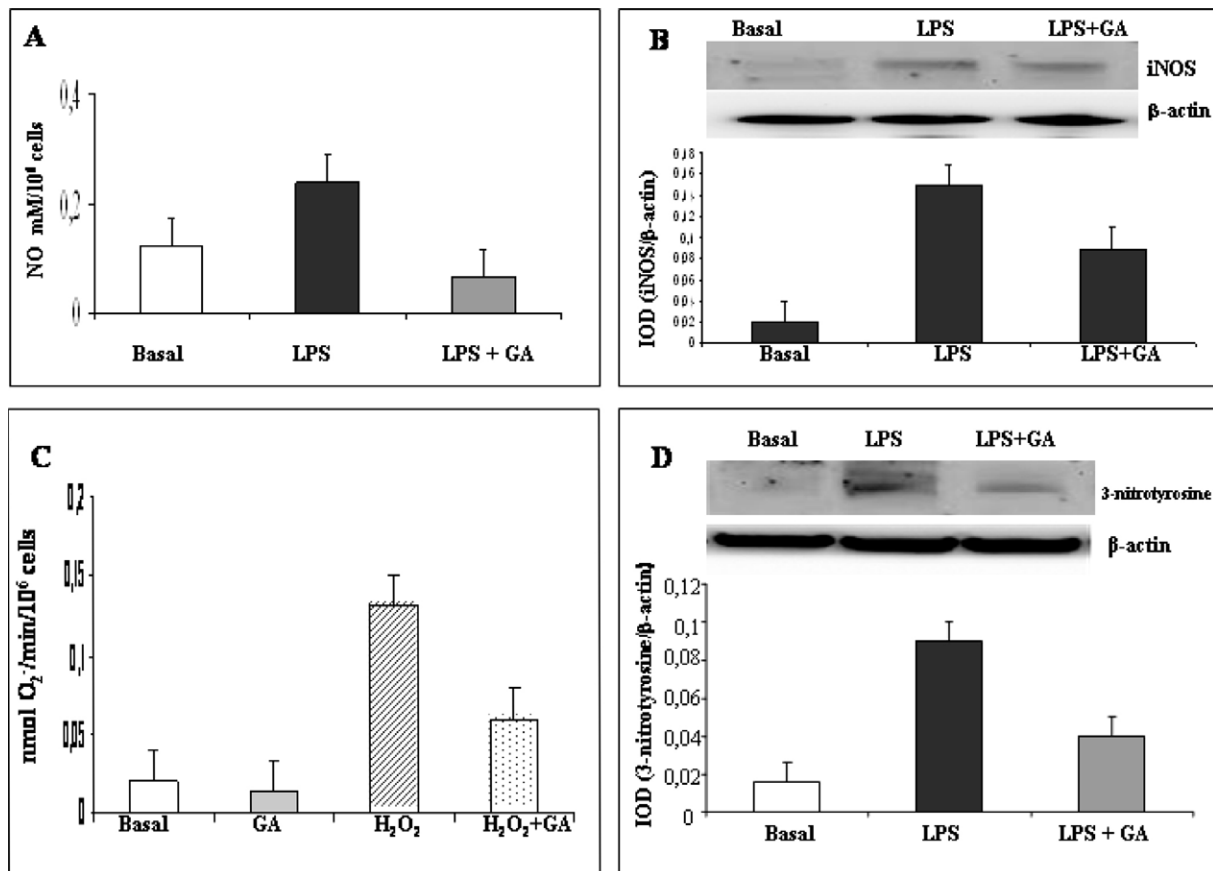
Figure 3 (A) *In vitro* effects of GA on NO production by PBAMs from HC subjects cultured overnight without (basal) or with LPS (10 μg/ml) and LPS + GA (50 μg/ml). Data are the mean ±SD of independent experiment performed in 20 HC subjects. (B) Representative blot of serial WB examination for iNOS expression. Integrated optical density (IOD) is reported as mean ± SD of 20 HC. (C) Baseline and H_2O_2 (20 μM) stimulated superoxide anion production in PBAMs was measured spectrophotometrically as superoxide dismutase inhibitable reduction of ferricytochrome c absorbance. GA (50 μg/ml) significantly reduced H_2O_2-induced superoxide anion production, and addition of GA led to a decrease in basal NO production which did not reach statistically significance. Data are the mean ± SD (nanomoles O_2^- per 10^6 cells per minute) of six separate experiments performed in triplicate. SD is lower than 10% of the mean. (D) Samples from HC were subjected to WB and densitometric analysis in order of the effect of GA on LPS-induced 3-nitrotyrosine. An example of bands is shown as a representative experiment. Each bar represents the mean of percentage of relative expression of 3-nitrotyrosine by PBAMs of HC.

and iNOS expression in peripheral mononuclear cells from RR-MS patients. We show that the increase of ONOO⁻ in PBAMs of RR-MS patients is related with increased NO and O_2^- production and iNOS activation. Greater O_2^- production is balanced by increased iNOS and NO production resulting in higher ONOO⁻ formation, suggesting that the increased production of free radicals and iNOS activation could be considered a marker of peripheral mononuclear cells activation in RR-MS patients. A decrease of iNOS production may constitute a mechanism to prevent excess of ONOO⁻ formation in a situation of high O_2^- and NO production.

Several investigators have considered the possibility of interfering with the development and severity of MS by altering NO production. Hence, we tested whether GA, an agent which demonstrates therapeutic efficacy in MS, may affect NO and iNOS levels. Our study shows that therapy with GA induces a down-modulation of iNOS, NO, 3-nitrotyrosine, and O_2^- in PBAMs from RR-MS patients. The increased NO plasma levels observed in RR-MS patients were reduced after 3 months (T3), and remain constant after 6 months (T6) of GA treatment. In GA treated MS patients, we observe the stability of the mean in EDSS. Previous studies have demonstrated that GA-reactive T-cell lines can be generated when PBMCs are isolated from non-GA-primed normal healthy individuals and incubated with GA in primary 7- to 14-day primary *in vitro* cultures. Immune modulation by daily injections of GA in MS treatment has been attributed to the generation of GA-reactive Th2 cells releasing anti-inflammatory cytokines that may induce
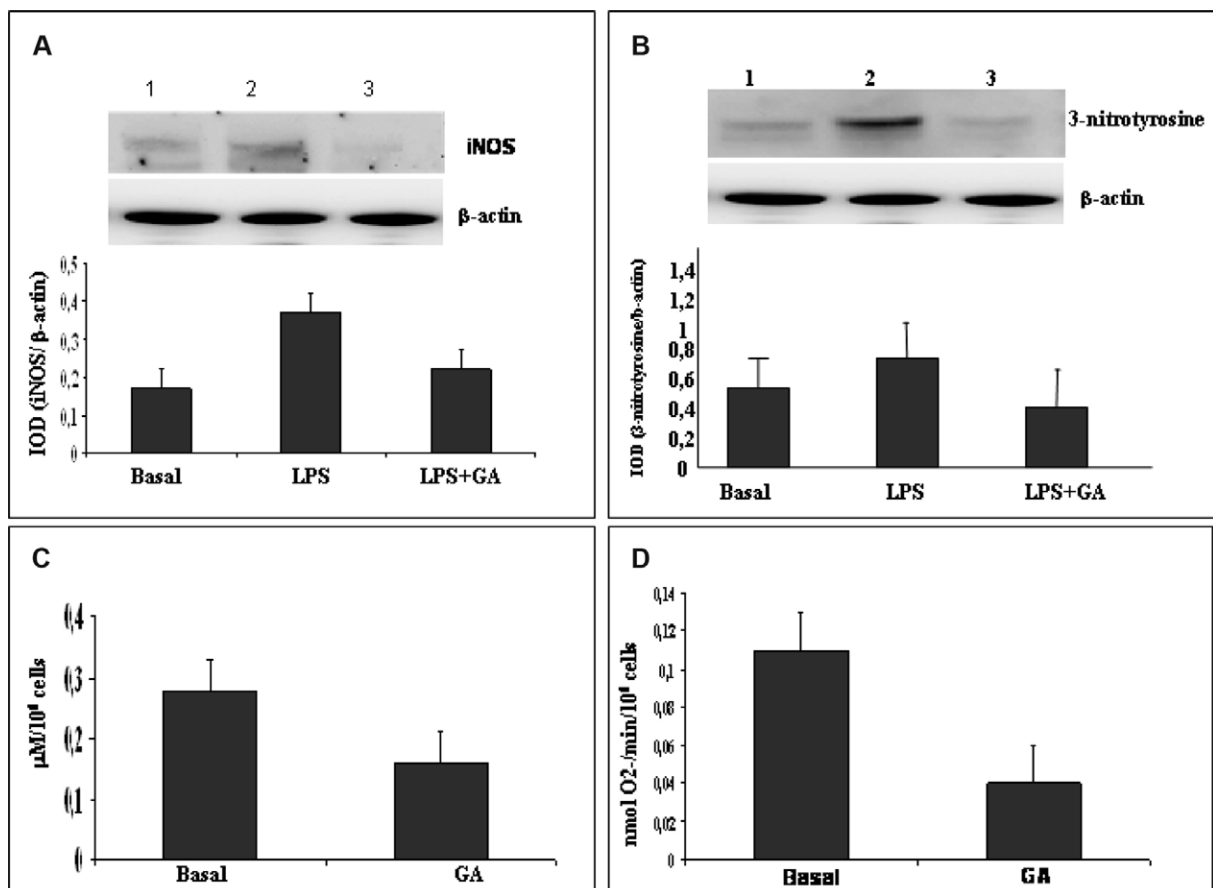
Figure 4   PBAMs from RR-MS patient were cultured without (basal) or with LPS (10 µg/ml). (A) The representative WB for iNOS is one of the independent experiments with similar results. Immunoreactive bands were analyzed by densitometry and normalized to β-actin levels. In densitometric analysis, each bar represents the mean ± SD of relative expression of iNOS by PBAMs. (B) WB and densitometric analysis, in order to derive levels of basal or LPS-induced 3-nitrotyrosine. An example of bands was shown as a representative experiment. Each bar represents the mean of relative expression of 3-nitrotyrosine (I.O.D.). (C) Effect of GA on basal release of NO by PBAMs. All samples were analyzed in duplicate at the same time. (D) O_2^- production was significantly reduced by GA addition. Values are mean ± SD.

down-regulation of free radicals [31]. Studies examining the effect of cytokines on NOS production have yielded conflicting results. This may be related
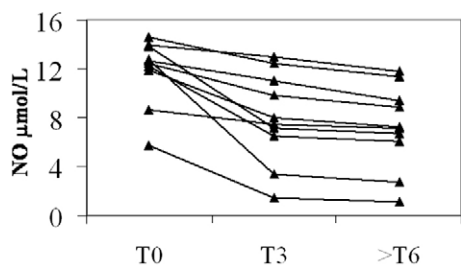

Figure 5   GA therapy: individual patient profiles of NO plasma levels. Each data point represents the NO level from each patient at the time of enrolment (T0) and after 3 months (T3) or 6 months and more of treatment (T≥6). All assays were performed in duplicate. Total NO was express as µmol/l.

to the production of IL-4 and IL-5 by Th2 cells, which can inhibit the iNOS induction by IFN-γ, while it has been demonstrated that both exogenous and endogenous IL-10 up-regulate the production of NO by LPS-activated macrophages. In contrast, Th1 cells characteristically produce IFN-γ, which can strongly activate macrophages to produce NO via iNOS. A regulatory role of NO in Th1/Th2 generation has long been recognized; NO selectively inhibits the expansion of Th1 cells by a negative feedback mechanism that lead to the increase of Th2 populations [32]. Therefore, regardless of whether free radical inhibition is a target for GA activity *in vivo*, or whether their decrease results from a bystander effect reflecting GA therapeutic activity, the reduction of free radicals *per se* could be beneficial in MS and may also represent an obvious therapeutic goal in other diseases caused by Th1/Th2 imbalance. By using *in vitro* cultured PBAMs obtained from HC
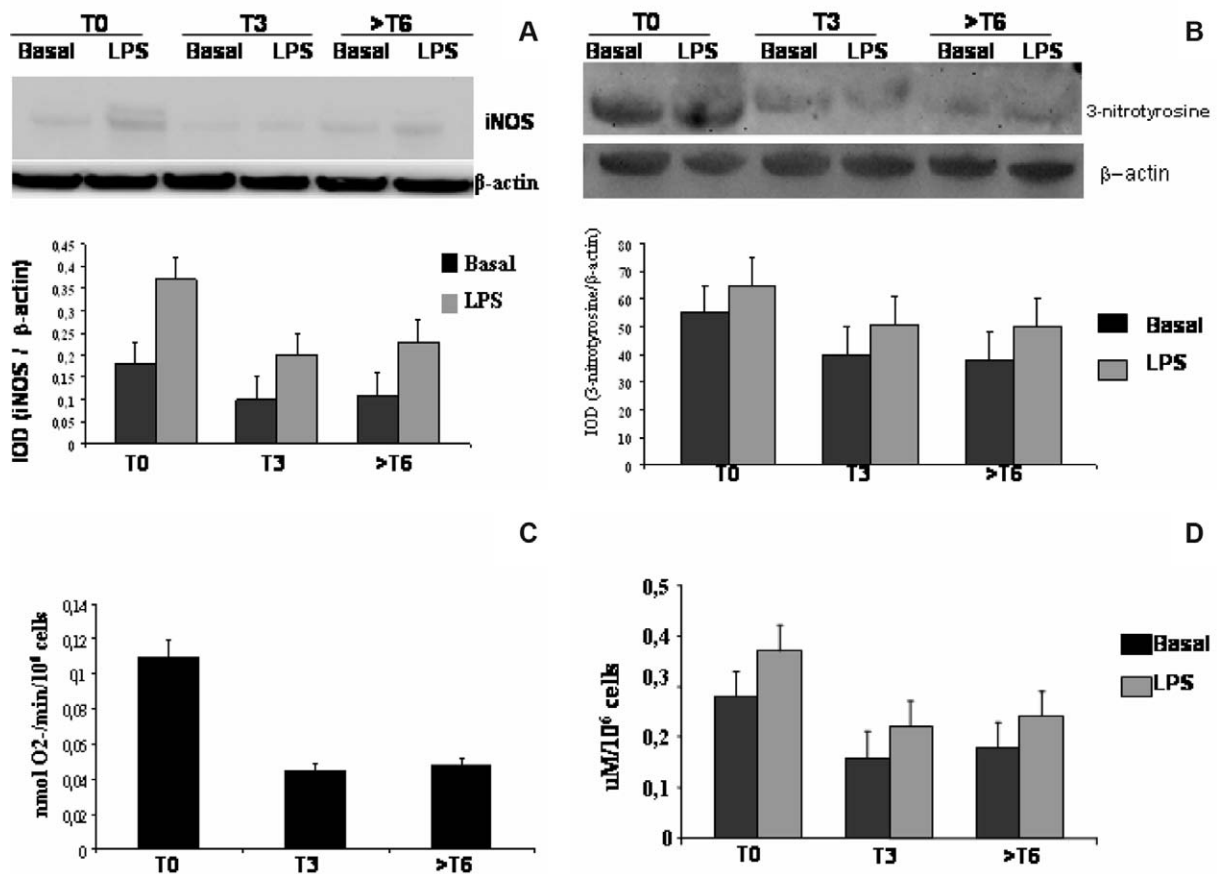
Figure 6   Effect of GA therapy on NO, iNOS, 3-nitrotyrosine, and O_2^- levels. PBAMs from pre-treated (T0) RR-MS patients and RR-MS patients treated with GA (20 mg subcutaneously daily) for 3 (T3) and 6 or more (T≥6) months were incubated with or without LPS for 24 h. A representative WB for iNOS of independent experiment involving identical comparisons is shown in (A) and that for 3-nitrotyrosine is shown in (B). (C) Baseline superoxide anion production was measured spectro-photometrically as superoxide dismutase inhibitable reduction of ferricytochrome c absorbance. Superoxide anion production in PBAMs was significantly reduced in RR-MS patients GA-treated for 3 months and 6 or more months. Data are the mean ± SD (nanomoles O_2^- per 10^6 cells per minute) of six separate experiments performed in triplicate. SD is lower than 10% of the mean. (D) Basal and LPS-induced release of NO by PBAMs from untreated (T0) RR-MS patients and RR-MS patients treated for 3 months and 6 or more months with GA. All samples were analyzed in duplicate at the same time.

subjects and stimulated with ligand for Toll-like receptor TLR-4 (LPS), we showed that GA (50 μg/ml) had a broad inhibitory effect on iNOS, NO, 3-nitrotyrosine, and O_2^- production. It is unlikely that this reflects a simple toxic effect, because cell viability was unaffected. To obtain further evidence on the down-regulation of free radicals production by GA, we examined PBAMs of RR-MS patients and the comparable down-regulating effect was observed. These findings establish the ability of GA to inhibit the free radicals in non-GA-primed PBAM populations restoring the impaired expression of free radicals induced by the disease process.

We do not know whether these observations made in peripheral mononuclear cells correlate with the situation in the CNS in our MS patients but we believe that this study may provide a rationale for estimating the peripheral iNOS expression, O_2^-, NO, and 3-nitrotyrosine production in RR-MS. Taken together, the results of this study support the conclusion that iNOS protein, NO, O_2^-, and 3-nitrotyrosine are abundantly produced in PBAMs by RR-MS patients and the assessment of their iNOS levels in PBAMs and NO levels in plasma needs to be considered as one part of the more complex pathophysiological process in MS. Furthermore, this has important implications for clinical monitoring and for therapeutic intervention in RR-MS.

Studies are in progress, in our laboratory, to strengthen these preliminary observations by studying a wider population of patients and with a longer treatment period.

Acknowledgments

References

1. Forstermann, U, Kleinert, H. Nitric oxide synthase: expression and expressional control of the three isoforms. *Naunyn Schmiedebergs Arch Pharmacol* 1995; **352**: 351–364.
2. Moncada, SR, Palmer, MJ, Higgs, EA. Nitric oxide: physiology, pathology and pharmacology. *Pharmacol Rev* 1991; **43**: 109–142.
3. Furlan, R, Rovaris, M, Martinelli Boneschi, F, Khademi, M, Bergami, A, *et al*. Immunological patterns identifying disease curse and evolution in multiple sclerosis patients. *J Neuroimmunol* 2005; **165**: 192–200.
4. Bagasra, O, Michaels, FH, Zheng, YM, Bobroski, LE, Spitsin, SV, Fu, ZF, *et al*. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. *Proc Natl Acad Sci U S A* 1995; **92**: 12041–12045.
5. Cross, AH, Manning, PT, Stern, MK, Misko, TP. Evidence for the production of peroxynitrite in inflammatory CNS demyelination. *J Neuroimmunol* 1997; **80**: 121–130.
6. Tran, EH, Hardin-Pouzet, H, Varge, G, Owens, T. Astrocytes and microglia express inducible nitric oxide synthase in mice with experimental allergic encephalomyelitis. *J Neuroimmunol* 1997; **74**: 1211–1229.
7. Smith, KJ, Lassmann, H. The role of nitric oxide in multiple sclerosis. *Lancet Neuro* 2002; **1**: 232–241.
8. Nazliel, B, Taskiran, D, Irkec, C, Kutay, FZ, Pogun, S. Serum nitric oxide metabolites in patients with multiple sclerosis. *J Clin Neurosci* 2002; **9**: 530–532.
9. Giovannoni, G, Miller, DH, Losseff, NA, Sailer, M, Lewellyn-Smith, N, Thompson, AJ, *et al*. Serum inflammatory markers and clinical/MRI markers of disease progression in multiple sclerosis. *J Neurol* 2001; **248**: 487–497.
10. Carlson, NG, Rose, JW. Antioxidants in multiple sclerosis: do they have a role in therapy? *CNS Drugs* 2006; **20**: 433–441.
11. De Groot, CJ, Ruuls, SR, Theeuwes, JW, Dijkstra, CD, Van der Valk, P. Immunocytochemical characterization of the expression of inducible and constitutive isoforms of nitric oxide synthase in demyelinating multiple sclerosis lesions. *J Neuropathol Exp Neurol* 1997; **56**: 10–20.
12. Hill, KE, Zollinger, LV, Watt, HE, Carlson, NG, Rose, JW. Inducible nitric oxide synthase in chronic active multiple sclerosis plaques: distribution, cellular expression and association with myelin damage. *J Neuroimmunol* 2004; **151**: 171–179.
13. Minagar, A, Alexander, JS. Blood-brain barrier disruption in multiple sclerosis. *Multi Scler* 2003; **9**: 540–549.
14. Comi, G, Filippi, M, Wolinski, JS. European/Canadian multicenter, double-blind, randomised, placebo-controlled study of the effects of glatiramer acetate on magnetic resonance imaging-measured disease activity and burden in patients with relapsing multiple sclerosis: European/Canadian Glatiramer Acetate Study Group. *Ann Neurol* 2001; **49**: 290–296.
15. Simpson, D, Noble, S, Perry, C. Glatiramer acetate: a review of its use in relapsing-remitting multiple sclerosis. *CNS Drugs* 2002; **16**: 825–850.
16. Teitelbaum, D, Fridkis-Hareli, M, Amon, R, Sela, M. Copolymer 1 inhibits chronic relapsing experimental allergic encephalomyelitis induced by proteolipid protein (PLP) peptides in mice and interferes with PLP-specific T cell responses. *J Neuroimmunol* 1996; **64**: 209–217.
17. Fridkis-Hareli, M, Strominger, JL. Promiscuous binding of synthetic copolymer 1 to purified HLA-DR molecules. *J Immunol* 1998; **160**: 4386–4397.
18. Weber, M, Starck, M, Wagenpfeil, S, Meinl, E, Hohlfeld, R, Farina, C. Multiple sclerosis: glatiramer acetate inhibits monocyte reactivity *in vitro* and *in vivo*. *Brain* 2004; **127**: 1370–1378.
19. Prat, A, Biernacki, K, Antel, J. Th1 and Th2 lymphocyte migration across the human BBB is specifically regulated by interferon β ☒and copolymer-1. *J Autoimmunol* 2005; **2**: 119–124.
20. Aharoni, R, Kayhan, B, Eilam, R, Sela, M, Arnon, R. Glatiramer acetate-specific T cells in the brain express T helper 2/3 cytokines and brain-derived neurotrophic factor *in situ*. *Proc Natl Acad Sci U S A* 2003; **100**: 14157–14162.
21. Pritchard, KA, Groszek, L, Smalley, DM, Sessa, WC, Wu, M, Villalon, P, *et al*. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Cir Res* 1995; **77**: 510–518.
22. McDonald, WI, Compston, A, Edan, G, Goodkin, D, Hartung, HP, Lublin, FD, *et al*. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the Diagnosis of Multiple Sclerosis. *Ann Neurol* 2001; **50**: 121–127.
23. Poser, CM, Paty, DW, Scheinberg, L, McDonald, WI, Davis, FA, Ebers, GC, *et al*. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983; **13**: 227–231.
24. Lublin, FD, Reingold, SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* 1996; **46**: 907–911.
25. Kurtzke, JF. Rating neurologic impairment in multiple sclerosis: an Expanded Disability Status Scale (EDSS). *Neurology* 1983; **33**: 1444–1452.
26. Al-Omaishi, J, Bashir, R, Gendelman, HE. The cellular immunology of multiple sclerosis. *J Leukoc Biol* 1999; **65**: 444–452.
27. Danilov, AI, Andersson, M, Bavand, N, Wiklund, NP, Olsson, T, Brundin, L. Nitric oxide metabolite determinations reveal continuous inflammation in multiple sclerosis. *J Neuroimmun* 2003; **136**: 112–118.
28. Liu, JS, Zhao, ML, Brosnan, CF, Lee, SC. Expression of inducible nitric oxide synthase and nitrotyrosine in multiple sclerosis lesions. *Am J Pathol* 2001; **158**: 2057–2066.
29. Sarchielli, P, Orlacchio, A, Vicinanza, F, Pelliccioli, G, Tognoloni, M, Saccardi, C, *et al*. Cytokine secretion and nitric oxide production by mononuclear cells of patients with multiple sclerosis. *J Neuroimmun* 1997; **80**: 76–86.
30. Lopez-Moratalla, N, Gonzales, A, Aymerich, MS, Lopez-Zabalda, MJ, Pio, R, de Castro, P, *et al*. Monocyte inducible nitric oxide synthase in Multiple Sclerosis. Regulatory role of nitric oxide. *Nitric Oxide: Biol & Chem* 1997; **1**: 95–104.
31. Duda, PW, Schmied, MC, Cook, SL, Krieger, JI, Hafler, DA. Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J Clin Invest* 2000; **105**: 967–976.
32. Niedbala, W, Cai, B, Liew, FY. Role of nitric oxide in the regulation of T cells functions. *Ann Rheum Dis* 2006; **65**: 37–40.

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