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Glatiramer acetate (Copaxone®) regulates nitric oxide and related cytokine secretion in experimental autoimmune encephalomyelitis

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Abstract

Nitric oxide (NO) is an important mediator involved in the pathogenesis of experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS). We examined the effect of glatiramer acetate (GA), an agent with suppressing effect on EAE and of therapeutic value for the treatment of MS, on the secretion of NO, as well as of the NO regulating cytokines. We observed that induction of EAE leads to 4-fold elevation in NO secretion and that treatment of the EAE mice by GA indeed leads to a significant reduction in the NO secretion by the splenocytes in response to the encephalitogen. A parallel decrease was observed in the secretion of the NO inducing cytokine IL-1β. On the other hand, the secretion level of NO modulating cytokines IL-10 and IL-13 was significantly augmented. The correlation between these findings and the therapeutic effect of GA is discussed.

Keywords: Experimental autoimmune encephalomyelitis (EAE); Glatiramer acetate (GA); Copaxone®; Nitric oxide (NO); Th2 cytokines

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system (CNS) that shows many pathological and clinical similarities to multiple sclerosis (MS) [1]. The disease can be induced in rodents or primates by either immunization with neural antigens or by adoptive transfer of neural antigen-specific T cells. EAE is Th1-mediated autoimmune disease in which $CD4^+$ Th1 cells, infiltrating macrophages, proinflammatory cytokines (i.e. IFN γ, TNF α, IL-1β) and inflammatory mediators (i.e. nitric oxide (NO), superoxide) play a critical role in the pathogenesis of the disease [2–4].

NO is a mediator of a variety of biological functions, including the regulation of the immune response induction, permeability of the blood-brain barrier (BBB), trafficking of the cells to the CNS, tumoricidal and microbicidal activity and immunosuppression [5,6]. Its production is catalyzed in vivo by a series of three independent nitric oxide synthases (NOS) which have several fundamental roles in the biology of higher organisms [7]. Two constitutive NOS (cNOS) exist in various host cells and account for basal NO synthesis, whereas inducible NOS (iNOS) is primarily found in professional phagocytes stimulated by endotoxins and some cytokines. The cytokines: IFN γ, TNF α and IL-1β induce NO production while IL-13, TGF β, IL-4 and IL-10 are suppressive modulators of the NO production [8,9].

It is a matter of debate whether NO plays a pathogenic or protective role in EAE and MS. On one hand, it has been shown that NO is involved in the pathogenesis of EAE and MS [10,11]. The release of the proinflammatory cytokines IFN γ, TNF α and IL-1β in the parenchyma results in activation of astrocytes, macrophages and microglia cells. The consequent release of NO by those activated cells may impair the BBB function and may also contribute to primary demyelination via non-specific damage to the myelin sheath of axons as well as by promoting direct oligodendrocyte cell death [4,12]. On the other hand, NO can act as a suppressive molecule in CNS inflammation [13,14].

The synthetic random copolymer, Glatiramer acetate (GA, Copaxone®) composed of L-alanine (L-ala),

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L-glutamine (L-glu), L-lysine (L-lys) and L-tyrosine (L-tyr) exerts a marked suppressive and protective effect on EAE in various animal species including primates [15,16]. GA was also shown to slow the progression of disability and reduce the relapse rate in MS patients [17]. Although GA is completely non-encephalitogenic, it is immunogenic and induces both cellular and humoral immune responses in experimental animals as well as in humans [18,19]. The therapeutic effect of GA in MS and EAE has been attributed to the induction of specific Th2 suppressor cells that cross the blood brain barrier, accumulate in the CNS and become activated in situ by myelin basic protein (MBP) [20,21]. Once activated in the disease organ, they secrete Th2 anti-inflammatory cytokines that induce bystander suppression on the responses to other encephalitogenic components such as proteolipid peptides [22]. Treatment of patients with GA led to an elevation of TGF β, IL-4, IL-5, IL-10, and suppression of TNF α and IFN γ, indicating that its beneficial effect, manifested in a significant reduction of the relapse rate, may be due to shifting of the Th1 response to Th2 [23,24]. The anti-inflammatory cytokines secreted by Th2 cell are known to modulate NO secretion [4]. Hence, it is plausible that the induction of Th2 cells by GA may lead to the modulation of the NO secretion, and that this contributes to the effect of GA in EAE or MS. In view of the role of NO in the pathogenesis of diseases, it was interesting to study the effect of GA on NO secretion as well as on the pattern of cytokines involved in NO regulation, in EAE induced mice.

We wish to report herewith that induction of EAE causes elevation in NO secretion and that treatment of EAE mice by GA, indeed leads to significant reduction of NO secretion in response to MBP. This effect is accompanied by a decrease in the NO inducing cytokine IL-1β, with a concomitant increase in the NO modulating cytokines IL-10 and IL-13.

2. Materials and methods

2.1. Antigens and reagents

GA, Copaxone® containing four amino acids: L-ala, L-glu, L-lys and L-tyr [15] from batch 242990599 with an average molecular weight of 7300 kDa, obtained from Teva Pharmaceutical Industries (Petach Tikva, Israel), was used throughout the study. *E. coli* sero type O55:B5 lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). MBP was isolated from spinal cords of rat, as previously described [25,26]. Mouse spinal cord homogenate (MSCH) was prepared by homogenizing four parts of mouse spinal cord and one part of saline. The homogenate was strained through a sieve and lyophilized. Culture medium contains RPMI-1640 (Sigma) with 10% heat inactivated fetal calf serum (FCS, Biological Industries), 50 IU penicillin and 50 μg/ml streptomycin (Gibco).

2.2. Animals

(SJL/J × BALB/c) F1 mice, were purchased from Jackson Laboratories (Bar Harbor, ME). Female mice, 10–12-weeks-old, were used in all experiments.

2.3. Induction of EAE

In order to induce EAE, mice were injected in four footpads with 4 mg/mouse MSCH emulsified at 1:1 ratio with complete Freund's adjuvant (CFA, Difco), containing 1 mg/ml Mycobacteria H37Ra. Pertussis toxin (Sigma), 0.2 mg/mouse was injected intravenously (i.v) immediately after and 48 h later. Mice were examined daily from day 10 following the MSCH injection for disease signs. EAE was scored as follows: 0, no disease; 1, limp tail; 2, hind limb paralysis; 3, paralysis of all four limbs; 4, moribound condition, and 5, death.

2.4. Treatment of EAE

GA treatment was applied either by prevention treatment, in which GA (5 mg/mouse), emulsified in incomplete Freund's adjuvant (ICFA, Difco), was injected subcutaneously (s.c), 15 days prior to disease induction, or by blocking treatment, in which GA (0.25 mg/mouse) was injected concomitantly to the EAE induction within the encephalitogenic emulation [27,28].

In each experiment mice were divided into four groups: (1) EAE induced mice; (2) Mice rendered unresponsive to EAE by GA prevention treatment followed by induction of EAE; (3) Mice rendered unresponsive to EAE by GA blocking treatment administered at the time of induction of EAE; (4) Normal mice. Each group contained four mice. The presented results, in each case, are the mean values of three separate experiments performed.

2.5. Cell cultures

After removing the spleen under aseptic conditions, murine splenocytes were obtained by homogenizing the spleens, removing the connective tissues by filtering through a steel mesh and lysing the erythrocytes by treatment with 5 ml 0.2% NaCl for 30 s, followed by addition of 1.6% NaCl for 30 s. Cells were washed twice, resuspended in culture medium and plated at 2.5×10^6 cells per well in 24 well tissue culture plates (Costar). Splenocytes of each mouse were stimulated separately in duplicates with MBP in the presence or absence of GA for 48 h. Supernatants were harvested after 48 h to measure nitrite and cytokine levels. The results represent

the average of 12 mice obtained in three separate experiments.

Peritoneal exudate cells (PEC), were elicited with sterile thioglycolate medium (4%). After 4 days; PEC were harvested by lavage of the peritoneal cavities with ice-cold RPMI-1640 supplemented with 1% heat inactivated FCS, 50 IU/ml penicillin and 50 IU/ml streptomycin. Cells were washed twice and resuspended in culture medium. For in vitro stimulation 250 μl of the cell suspension was plated in 24 well tissue culture plates in final concentration of 0.5×10^5 cells per well. Cells were allowed to adhere for 4 h at 37 °C and the non adherent cells were removed by dispensing the culture medium. Remaining macrophages were ready to use after placing in culture medium.

2.6. Induction of cell lines

GA specific T cell lines were established from spleens of (SJL × BALB/c)F1 mice either injected or fed with GA, as described previously [21,28]. Briefly, cells were selected in vitro, every 14–21 days, by repeated exposures to GA presented on syngeneic irradiated splenocytes. This was followed by propagation in 10% supernatant of Con A -activated normal mouse spleen cells as T cell growth factor. These GA specific T-cells were characterized as Th2 regulatory cells by their ability to secrete IL-4, IL-5, IL-6, IL-10 and TGF-β, as well as by their capability to prevent EAE in vivo [22,28].

2.7. In vitro model

In order to investigate the effect of GA specific Th2 cells on NO secretion, LPS (1 μg/ml, vortexed for 30 min before using) stimulated RFC (0.5×10^5 cells/well) were co-cultured with GA specific Th2 cells (10^4 cells/well) in duplicate wells in the presence or absence of GA (50 μg/ml) at 37 °C for 48 h. Nitrite and cytokine levels were measured after 48 h. The presented results are the mean values of two separate experiments performed.

2.8. Measurement of nitrite levels

Since the secreted NO quickly reacts with oxygen to yield nitrite as a metabolic product, the presence of NO in splenocytes and macrophages was determined by measuring the amount of nitrite in culture supernatant, using a colorimetric assay based on Griess reaction [29]. Briefly, 100 μl of culture supernatant was mixed with 100 μl Griess reagent [1:1 (v/v) of 0.1% naphthylethylenediamine (NED) in H_2O with 1% sulfanilamide in 5% H_3PO_4] in flat bottomed 96 well microtiter immunoassay plates and incubate for 10 min at room temperature. Absorbance of the wells was measured at 550 nm in an ELISA reader (Titertek Multiskan MCC/340, Finland). The amount of nitrite was calculated according to a standard curve of sodium nitrite (Sigma) (3–200 μM). Results were expressed in μM as mean concentration of duplicate culture supernatants.

2.9. Cytokine assays

Cytokine measurements were determined by using commercially available cytokine ELISA kits according to the manufacturer's instructions. The levels of IL-1β (Biosource cytoscreen, Camarillo, CA), TNF α (Diaclone Research, Besenchon, Cedex), IL-10 (Pharmingen, San Diego, CA) and IL-13 (R&D systems, Minneapolis, MN) were measured; results were expressed in picogram as mean concentration of duplicate culture supernatants.

2.10. Statistical analysis

Statistical analysis was performed using Student's t-test with $P < 0.05$ considered as statistically significant.

3. Results

The effect of GA on NO secretion was investigated by testing the levels of NO secreted by splenocytes of EAE induced mice treated with GA. GA treatment was applied either before the disease induction by s.c injection of GA in ICFA (prevention treatment), or concomitantly with the disease induction, by mixing GA with the encephalitogenic emulsion (blocking treatment). Splenocytes were harvested 12–13 days after disease induction. At that time 75% of the untreated EAE induced mice manifested disease (average score-grade 4) while none of the GA treated mice showed any disease signs. Splenocytes were incubated in the presence of MBP with or without GA.

3.1. The effect of GA on the secretion of NO by splenocytes from EAE induced mice

Nitrite levels in splenocytes stimulated by MBP or MBP plus GA are demonstrated in Fig. 1. EAE induction resulted in elevation of NO secretion in comparison to the secretion by splenocytes from normal mice, after stimulation with either MBP alone or MBP plus GA ($P = 0.0042$ and $P = 0.0002$, respectively). In mice treated with GA, either by blocking or by prevention, and stimulated by MBP alone the levels of NO secretion decreased but not significantly. However, following an in vitro stimulation with MBP + GA, the decrease in NO secretion was significant in comparison to the cells from the same mice stimulated with MBP alone (20.9 and 27.2% reduction for the blocking and prevention treatments, respectively). Moreover, upon

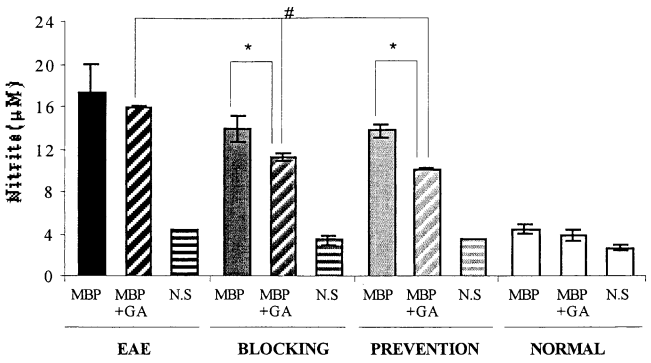

Fig. 1. The effect of GA on NO secretion by splenocytes from EAE induced mice. Splenocytes (2.5×10^6 cells/ml) were stimulated with 200 μg/ml MBP in the presence or absence of GA (50 μg/ml) for 48 h. Data are the mean ± S.D. of three independent experiments. *, Indicates statistical significance between MBP and MBP+GA stimulated splenocytes within a treatment groups. #, Indicates statistical significance of the MBP+GA stimulated splenocytes between the GA treated and untreated group. The actual P values for the various samples were; for the blocking treatment: *, $P = 0.009$ and #, $P = 0.005$; for the prevention treatment: *, $P = 0.0004$ and #, $P = 0.003$. N.S; non-stimulated.

stimulation with MBP+GA, splenocytes originated from GA treated mice secreted significantly lower amounts of NO than splenocytes from untreated EAE induced mice. This was manifested in 30.2% reduction for the blocking treatment and 36.3% reduction for the prevention treatment.

3.2. The effect of GA on NO regulating cytokines

The effect of GA on cytokines that are involved in NO regulation was also investigated. The levels of NO inducing cytokines i.e. IL-1β and TNF α, as well as NO modulating cytokines i.e. IL-10 and IL-13, were measured in the splenocytes supernatants. The results are presented in Fig. 2. As shown, EAE induction resulted in highly significant elevation of IL-1β as well as TNF α in comparison to the level in normal mice, both in the presence or absence of GA in the culture medium. GA did not affect the level of TNF α in any significant way. However, GA treatment by either blocking or prevention induced a significant reduction in the levels of IL-1β secretion in response to MBP (22.17 and 27.2%, respectively). This decrease was even more prominent when GA was present with the MBP in the culture medium, as there was 33.7% reduction in blocking and 34.6% reduction in the prevention treatments (Fig. 2A).

The opposite effect was observed concerning the NO modulating cytokines IL-10 and IL-1β. IL-10 levels were significantly elevated both by blocking and prevention treatments in comparison to the amounts secreted by EAE induced mice. This was observed when the stimulation was induced with MBP alone and even more so by stimulation with MBP+GA (Fig. 2B). The levels of the other Th2 cytokine, IL-13 was also increased after GA treatment compared with EAE group, both in the blocking and the prevention treatment groups. In this case stimulation with MBP alone was effective and no further increase was observed by adding GA to the stimulated cell cultures.

3.3. The effect of GA specific Th2 cell on PEC derived NO response

In order to understand the effect of GA specific Th2 lymphocytes on NO secretion we constructed an in vitro model in which syngeneic GA specific Th2 cells (established cell line) were cultured with LPS activated peritoneal macrophages. The PEC were exposed to the LPS alone or in the presence of GA, Th2 cells, or Th2 cells+GA. NO levels were measured 48 h after stimulating the PEC. As shown in Fig. 3, LPS induces a significant secretion of NO by the PEC. However, the nitrite levels were reduced by 50.1% when Th2 cells with GA were added to the LPS stimulated PEC ($P = 0.019$). Addition of either GA alone or, theTh2 cells without GA, led to a minor decrease in the NO secretion, which did not reach statistical significance.

3.4. The effect of GA specific Th2 cell response on NO regulating cytokines secretion

In order to test whether the down-regulation of NO secretion was accompanied by a parallel change in the levels of NO regulating cytokines, we measured the levels of IL-1β, TNF α, IL-10 and IL-13 in the supernatant of the LPS activated PEC after co-culturing with the GA specific T cells. Indeed, while LPS stimulated cells secreted excessive amounts of IL-1β and TNF α, the addition of GA together with Th2 cells significantly decreased the secretion of these cytokines (Fig. 4A).

The most prominent effect was observed on the levels of the NO modulating cytokines IL-10 and IL-13 (Fig. 4B). Here, LPS stimulation of the PEC did not lead to any cytokine secretion. However, when GA and the specific Th2 cells were added to the culture, a dramatic increase in both IL-10 and IL-13 was observed. This effect was not obtained by the addition of GA alone or by unstimulated Th2 cells. According to these results, only GA specific Th2 cells stimulated by their specific antigen were effective in the suppression of IL-1β and TNF α as well as in inducing the secretion of the modulating cytokines IL-10 and IL-13.

4. Discussion

NO is an important mediator implicated in pathophysiological processes. Various investigations have indicated the high expression of iNOS and excessive amount of NO production in the pathogenesis of murine

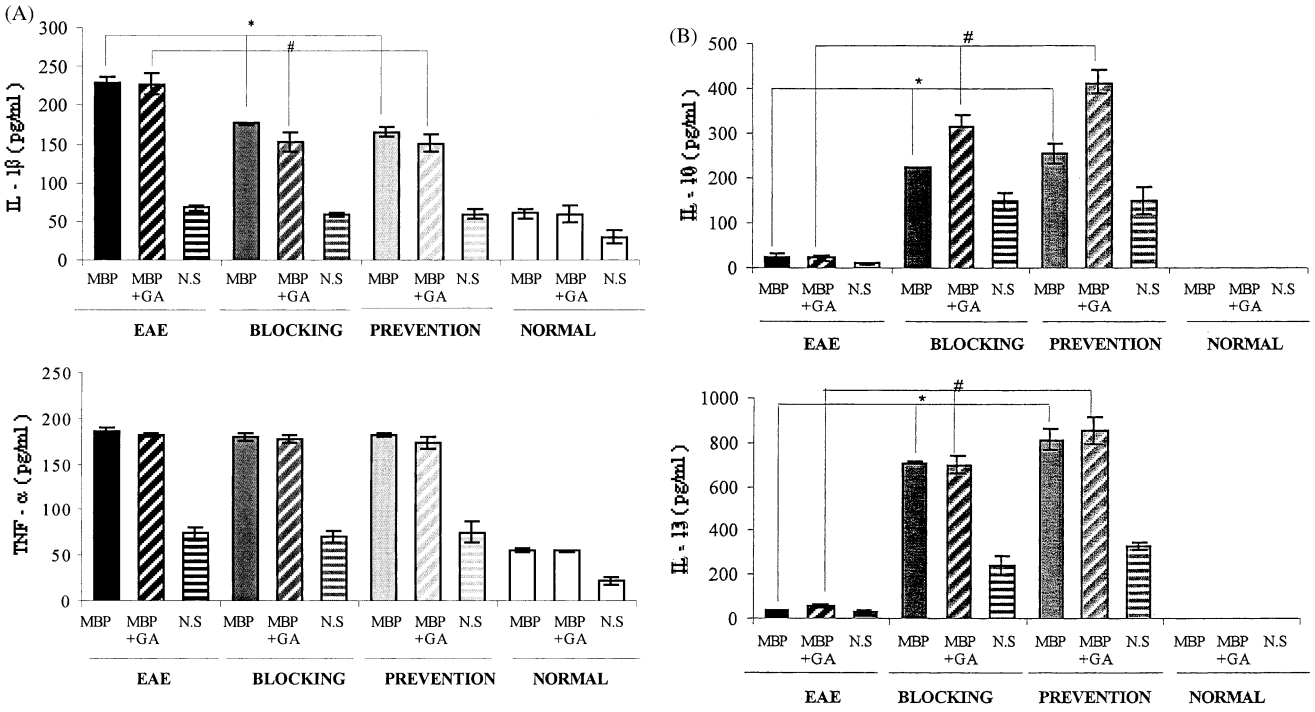

Fig. 2. (A) The effect of GA on IL-lβ and TNF α secretion by splenocyte from EAE induced mice. The levels of cytokine in the supernatant of cultures were determined by ELISA after 48 h incubation. Data are the mean ± S.D. of three independent experiments. *, Indicates statistical significance of MBP stimulated splenocytes between GA treated and untreated groups. #, indicates statistical significance of MBP+GA stimulated splenocytes between GA treated and untreated groups. The actual P values for the various samples were; for the blocking treatment: *, $P = 0.013$ and #, $P = 0.03$; for the prevention treatment: *, $P = 0.013$ and #, $P = 0.022$. N.S.; non-stimulated. (B) The effect of GA on IL-10 and IL-13 secretion by splenocytes for EAE induced mice. The levels of cytokine in the supernatant of cultures were determined by ELISA after 48 h incubation. Data are the mean ± S.D. of three independent experiments. *, indicates statistical significance of MBP stimulated splenocytes between GA treated and untreated groups. #, Indicates statistical significance of MBP+GA stimulated splenocytes between GA treated and untreated groups. Values for 'Normal' group were below detection level. The actual P values for the various samples were; for the blocking treatment: *, $P = 0.001$, $P = 0.0005$ and #, $P = 0.004$, $P = 0.003$; for the prevention treatment.*, $P = 0.005$, $P = 0.003$ and #, $P = 0.004$, $P = 0.005$; in IL-10 and IL-13, respectively. N.S., non-stimulated.

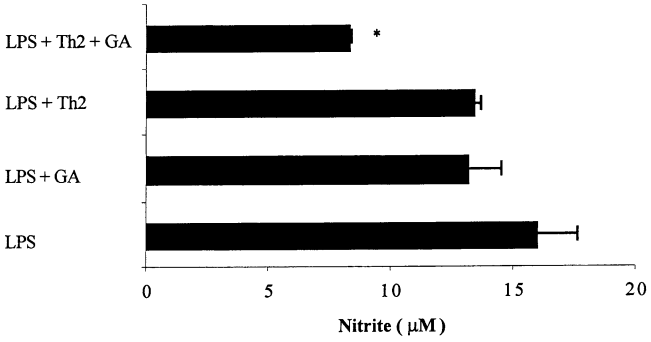

Fig. 3. The in vitro effect of GA and GA specific syngeneic Th2 cells on NO secretion from LPS stimulated peritoneal macrophages. GA (50 μg/ml), LPS (1 μg/ml) and GA specific cells (10^4 cells/well) were added simultaneously to the peritoneal macrophages cell cultures. Nitrite levels were measured after 48 h incubation. While GA+Th2 cells reduce nitrite levels (*, $P < 0.05$), neither GA alone nor non stimulated Th2 cells alter the levels. Data are the mean ± S.D. of two independent experiments.

inflammatory CNS demyelination [10,30]. Furthermore, an excessive increase of NO production was found in both the CSF and the peripheral blood of MS patients [31,32].

There is a controversy on the effect of NO in EAE and MS. While some of the studies suggest a protective role, some of them support a destructive role of NO. Regarding the protective effects, NO inhibits T cell proliferation [33], induces apoptosis or necrosis in T effector cells [34], and downregulates the expression of various adhesion molecules [35]. On the other hand, the detrimental effects of NO in EAE and MS include damage to different cell types such as oligodendrocytes, that leads to disruption of the myelin sheath and consequently to demyelination and cell death, increase in injury due to peroxynitrite formation and enhancement of TNF toxicity [4,12,36,32].

Several investigators have considered the possibility of interfering with the development and severity of EAE and MS by altering NO production. However, the therapeutic attempts in EAE mice, to directly inhibit NO production by analogue iNOS inhibitors, led to conflicting results which can be related to the type and dose of iNOS inhibitor, schedule of administration, and to the acute or chronic EAE model [37,13,38]. Therapeutic attempts to reduce the disease severity and in parallel NO secretion, by using Th2 cytokines and

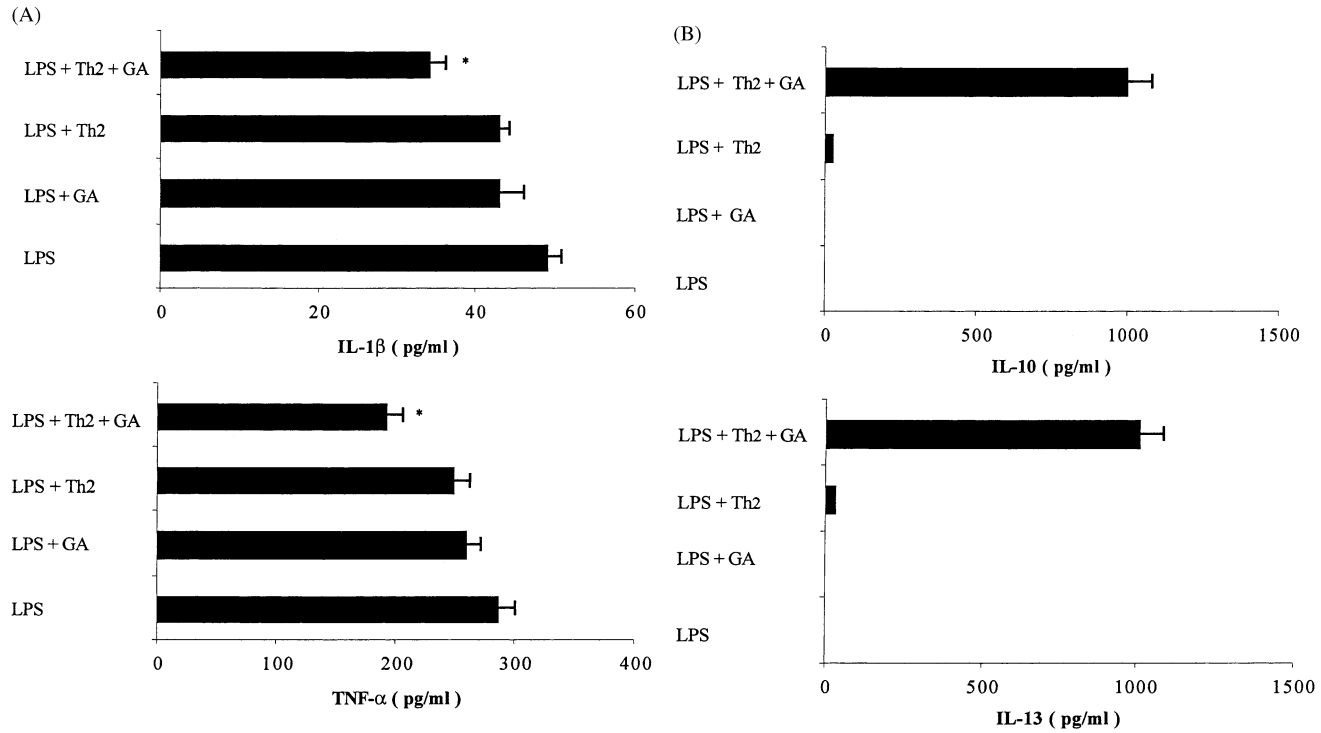

Fig. 4. (A) The in vitro effect of GA and GA specific Th2 cell line on the secretion of NO inducing cytokines IL-1β and TNF α. IL-1β and TNF a levels reduced significantly (*, $P < 0.05$) after stimulating peritoneal macrophages and Th2 cells with LP5 (1 μg/ml) and GA (50 μg/ml) simultaneously. Data are the mean ± S.D. of two independent experiments. (B) The effect of GA specific Th2 cell lines on the secretion of NO modulating cytokines, IL-10 and IL-13 levels increase significantly (*, $P < 0.05$) after stimulating peritoneal macrophages and Th2 cells with LPS (1 μg/ml) and GA (50 μg/ml) simultaneously. Data are the mean ± S.D. of two independent experiments. Vales for 'LPS' and 'LPS + GA' were below detection level. Actual P values for IL-10 and IL-13 were $P = 0.0001$ and $P = 0.0001$, respectively.

agents which induce Th2 cell response, were successful [39,40]. Hence, we tested here whether GA, an agent which demonstrates therapeutic efficacy in EAE and MS, reduces NO secretion. We also investigated whether such regulation of NO can be attributed to GA capability to induce Th2 response. GA was tested for its effect on the secretion of NO in splenocytes of EAE induced mice, in response to MBP, a major auto antigen in EAE and MS, that was previously demonstrated to induce NO production [41].

GA was administered to EAE induced mice either as prevention or blocking treatment [27,28]. Both treatments suppressed disease manifestation, and induced similar effect on NO secretion. Thus the amounts of NO, secreted by spleen cells of EAE induced mice, in response to the encephalitogenic antigen MBP was significantly reduced by treatment with GA (Fig. 1). This phenomenon was observed even when GA was not present during the stimulation with MBP, but the presence of GA in vitro significantly augmented this effect, which was probably caused by the activation of GA specific Th2 suppressor cells. Thus, maximal inhibition of NO secretion was obtained when splenocytes from GA treated mice were stimulated by MBP in the presence of GA.

To understand the effect of GA on NO secretion, we tested its effect on cytokines that are involved in the NO regulation (Fig. 2). GA treatment significantly reduced the secretion of IL-1β in response to MBP. This was manifested by IL-1β decrease obtained by GA treatment in vivo as well as by further decrease in the presence of GA in vitro (Fig. 2A). IL-1β is a major proinflammatory mediator and it plays a central role in the activation of iNOS expression. Thus, it has been demonstrated that IL-1β enhanced the expression of iNOS, induced by encephalitogenic cells in murine macrophages [10] and that it correlated with NO levels in peripheral blood monocytes of MS patients [42]. The decrease in IL-1β can, therefore, play a role in the observed reduction of NO induced by GA treatment. We did not find an effect of GA on TNF α in this study (Fig. 2B). It is noteworthy that, reduction of TNF α mRNA was previously demonstrated in peripheral blood of MS patients treated by GA for 3 months [23]. It is thus possible that longer treatment period is needed in order to induce changes in TNF α.

In contrast to the suppressive effect on IL-1β, GA induced a marked stimulation of Th2 cytokines. Hence, GA treatment significantly elevated the amounts of IL-10 and IL-13 in comparison with the amount secreted by non-treated EAE induced mice (Fig. 2B). Interestingly,

in vitro addition of GA to MBP stimulated cells from GA treated mice, further increased the secretion of IL-10, while the amounts of IL-13 secreted with or without GA were similar. These differences suggest that the induction of IL-13 occurred already during GA treatment in vivo, and did not require additional in vitro stimulation. IL-10 as well as IL-13 are essential modulators of macrophage functions, capable of reducing the production of NO [9,43,44]. The decrease in IL-1β as well as the increase in IL-10 and IL-13 levels, are in accord with other studies demonstrating the ability of GA to deviate the pathological Th1 to protective Th2 response [21–24,28]. It was found that GA induces specific Th2 suppressor cells that in addition to IL-10 and IL-13, secrete IL-4, IL-5 and TGF β. Thus, the inhibitory effect of GA on NO secretion could result from the reactivity of these regulatory Th2 cells.

To investigate whether GA induced Th2 cells can mediate the effect of GA on NO we constructed an in vitro model, in which GA induced Th2 cells from a line previously characterized as distinct Th2 regulatory cells [22], were cultured with LPS stimulated peritoneal macrophages (Fig. 3). Indeed, in this model the GA specific Th2 cells significantly blocked the secretion of NO by the PEC (Fig. 3A). Furthermore, this effect was obtained only when the suppressor cells were added together with their specific antigen GA, indicating that only activated T cells that actually secrete Th2 cytokines are effective. This requirement for T cells in an activated state can explain the perquisite of adding GA in vitro to splenocytes of GA treated mice, in order to obtain a significant decrease in NO (Fig. 1). The decrease in NO was paralleled by reduction in the NO inducing cytokines IL-1β and TNF α (Fig. 4A), as well as prominent increase in the NO modulating cytokines IL-10 and IL-13 (Fig. 4B).

A transcriptional regulation in which IL-4 and IL-10 abrogated the expression of nuclear factor κ (NF-κB), inducing inhibition in iNOS expression, was recently demonstrated [45]. Interestingly, it was found that GA itself blocks IL-1 dependent NF-κB activation in human astroglial cells [46]. Thus, GA can affect NO secretion either by blocking of NF-κB, or through the induction of specific suppressor cells which secrete anti-inflammatory cytokines that induce down regulation of NO level. Yet, it seems that the direct effect of GA on NF-κB is restricted to the periphery since it was obtained only in the presence of high concentration of GA that probably do not exist in the CNS. In contrast, GA induced Th2 cells cross the BBB and accumulate in the CNS, where they can be stimulated in situ by MBP, and thereby exert therapeutic effect in the diseased organ [20,21]. So far this therapeutic effect has been related to decrease in inflammatory cytokines and the secretion of anti-inflammatory cytokines. In the present study we demonstrated an additional novel aspect of the therapeutic effect induced by the GA specific Th2 cells, namely the down regulation of the detrimental metabolite NO, involved in axons demyelination as well as induction of cell death. The modulation of NO secretion by GA can, therefore, be relevant to its activity in the prevention of tissue damage in the treatment of MS [47].

It should be noted that the EAE induced mice that had been treated with GA did not show disease manifestation, in contrast to the control mice that were severely sick (average disease severity 4). It is, therefore, possible that in vivo, the reduction of NO reflects either the attenuation in disease activity, or a direct effect of GA on NO production. However, regardless of whether NO is an actual target for GA activity in vivo, or whether its decrease results from bystander effect reflecting GA therapeutic activity, the reduction in this detrimental metabolite is of considerable significance.

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