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Effect of glatiramer acetate (Copaxone®) on the immunophenotypic and cytokine profile and BDNF production in multiple sclerosis: A longitudinal study

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Abstract

We assessed the effect of glatiramer acetate (GA) on the immunophenotypic and cytokine profile and the BDNF production by peripheral blood mononuclear cells, and their association with the clinical response in 19 *naïve*-treated MS patients prospectively followed-up after GA therapy. Two patients withdrew the therapy. After a median follow-up of 21 months, twelve were considered responders and five as non-responders. Non-responder patients had significant longer disease duration and a higher EDSS score at baseline. In the responder group, a significant decrease in the percentage of INF-γ producing total lymphocytes, $CD4^+$ and $CD8^+$ T cells, and reduced percentage of IL-2 producing $CD4^+$ and $CD8^+$ T cells were observed at 12, 18 and 24 months. These changes were associated with a significant increase in the percentage of $CD3^+$, $CD4^+$ and $CD4^+CD45RA^+$ T cells, and BDNF production from month 6 that remained significant throughout the study. We did not observe significant changes in the nonresponder group for any of the parameters studied. Our data suggest that GA treatment induces a downmodulation of proinflammatory cytokines associated with the regulation of the peripheral T cell compartment and with increased production of BDNF that might be related to the clinical response.

Keywords: Multiple sclerosis; Glatiramer acetate; BDNF; Cytokines; Lymphocyte immunophenotyping; Immunomodulatory treatment

Glatiramer acetate (GA, Copaxone®) is an approved agent for the immunomodulatory treatment of relapsing-remitting multiple sclerosis (RRMS). Although the mechanisms of action of GA are not fully understood, recent studies support both immunomodulatory and neuroprotective effects of the drug [17,24]. Besides the induction of anergy in autoreactive T cells, GA induces a shift of GA-reactive T cells from a Th1 to a Th2 phenotype [3,4,10,13,18] that are thought to release anti-inflammatory cytokines but also neurotrophic factors such as brain-derived neurotrophic factor (BDNF) within the CNS [1,3,25]. However, most of these immunological effects are based on the results of the analysis of GA-specific T-cell lines and less is known on the *ex vivo* systemic effects on peripheral blood mononuclear cells (PBMC) [9,11,15,16,23]. The aim of the present study was to assess the effect of GA on the immunophenotypic and cytokine profile and the BDNF production by PBMC, and to analyze the association of these parameters with the clinical response in a group of RRMS patients prospectively followed-up after GA treatment.

Nineteen *naïve*-treated patients with RRMS according to the Poser criteria [19] from the MS Units of the two participant hospitals were included in the study. All patients began treatment with GA because they had active disease with two or

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Table 1
Baseline clinical characteristics of responder and non-responder patients to GA therapy

	Overall	Responders	Non-responders[a]	p[b]
Number of patients	19	12	5	
Gender (W/M)	14/5	9/3	4/1	ns
Age (mean ± S.D.; years)	34.7 ± 9.4	34.8 ± 8.0	40.2 ± 11.3	ns
Disease duration (mean ± S.D.; years)	5.6 ± 3.3	3.8 ± 0.9	8.1 ± 2.6	<0.0001
Annual relapse rate (mean ± S.D.; in prior 2 years)	1.2 ± 0.6	1.2 ± 0.6	1.0 ± 0.3	ns
Baseline EDSS (mean ± S.D.)	1.5 ± 1.3	1.4 ± 0.8	3.1 ± 1.2	<0.0001

[a] Two patients withdrew the treatment in the first 3 months.
[b] Refers to comparison of clinical variables between responder and non-responder patients.

more relapses in the previous two years and with an Expanded Disability Status Scale (EDSS) score between 0 and 5.5. Baseline clinical characteristics are summarized in Table 1. Patients were regularly followed every 3 months and additional assessments were done in the event of a relapse. During the follow-up, patients who presented identical or higher annual relapse rate and/or increase of at least 1 EDSS point confirmed at six months, were defined as nonresponders to GA. PBMC were collected before treatment, at 6 months after GA therapy and then every 6 months. In case of coincidental relapse, the sample was taken 1 month after steroid administration. The study was approved by the Ethical Committee of the two participating hospitals.

PBMC phenotypical characterization was analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Sistems (BDIS), San José, CA, USA), as previously reported [2]. Monoclonal antibodies to the following surface markers were used: CD3, CD8, CD4, CD19, HLA-DR, CD62-L, CD45RA, CD45RO, (BD Biosciences, San Jose, CA, USA), and CD25 (BD, Pharmingen, San Diego, CA, USA).

Intracellular cytokine production was detected by flow cytometry as previously described [2,7] with slight modifications. Briefly, fresh peripheral blood was incubated for 4 h with phorbol 12-myristate (PMA) (25 ng/ml, Sigma–Aldrich, St. Louis) and ionomycin (1 μg/ml, Sigma–Aldrich) to stimulate cytokine production. Stimulated and unstimulated cells were treated with Brefeldin A (10 μg/ml, Sigma–Aldrich), an inhibitor of secretion, followed by superficial staining, cell fixation, permeabilization, and then intracytoplasmic staining for detection of the accumulated cytokines. T cells were first gated by their expression of CD8 or CD4 followed by the detection of IFN-γ, IL-2 (as markers of Th1 phenotype) and IL-4 (as marker of Th2 phenotype) (BD, Pharmingen, San Diego, CA, USA) positive cells.

BDNF release levels were measured in the supernatants from PBMC unstimulated and stimulated with anti-CD3 and soluble anti-CD28 antibodies by using an ELISA kit (Promega, Madison, USA) as previously described [2].

Non-parametric tests were used for all data comparisons: Friedman and Wilcoxon signed ranks tests for paired patients groups. Pearson's correlation coefficient was used for the correlation analysis. Significance levels were set at 5% ($p < 0.05$).

Two patients withdrew the treatment because of adverse events in the first 3 months. After a median follow-up of 21 months, twelve patients were considered as responders and 5 as nonresponders. Responder patients had a decrease of the annualized relapse rate (0.3 ± 0.4 during treatment versus 1.2 ± 0.6 before treatment) and did not present significant changes in the EDSS score (1.4 ± 0.8 at initiation of treatment versus 1.6 ± 0.8 at last follow-up). Non-responders had a mean of 1.3 ± 0.9 annualized relapse rate (1.0 ± 0.3 before treatment), and a mean increase of 0.6 ± 0.9 in the EDSS score (3.1 ± 1.2 at initiation of treatment versus 4.1 ± 1.4 at last follow-up). The only significant difference between both groups was that nonresponders patients had longer disease duration and a higher EDSS score at initiation of GA therapy ($p < 0.0001$) (Table 1).

Results of the immunophenotypic and cytokine profile along the evolution are presented as mean ± standard deviation in Tables 2 and 3 for both groups. There were no significant baseline differences between both groups of patients for any of the parameters studied. After GA therapy a significant increase in the percentage of $CD3^+$, $CD4^+$ and $CD4^+CD45RA^+$ (*naïve*) T cells was seen at 6, 12, 18 and 24 months in the responder group of patients compared with that found before treatment ($p = 0.003$, $p = 0.012$, $p = 0.05$; $p = 0.002$, $p = 0.01$, $p = 0.038$; $p = 0.002$, $p = 0.005$, $p = 0.036$; $p = 0.021$, $p = 0.05$, $p = 0.028$; respectively). The *naïve*/memory $CD4^+$ T cells ($CD4^+CD45RA^+/CD4^+CD45RO^+$) ratio also increased at 12 months and remained significant during the follow-up ($p = 0.028$, $p = 0.026$, $p = 0.011$). No significant changes were observed in the percentage of $CD4^+CD25^+$ T cells. No significant modification in T cells population frequencies were detected over the study period in the nonresponder group as compared with baseline values.

A significant decrease in the number of IFN-γ producing $CD3^+$, $CD4^+$ and $CD8^+$ T cells was observed in the responder group at 12, 18 and 24 months after GA therapy, compared with pretreatment values ($p = 0.028$, $p = 0.008$, $p = 0.008$; $p = 0.024$, $p = 0.008$, $p = 0.011$; $p = 0.012$, $p = 0.012$, $p = 0.012$; respectively) (see Fig. 1 and Table 1). In the same way, the number of IL-2 producing $CD4^+$ and $CD8^+$ T cells also decreased ($p = 0.013$, $p = 0.021$; $p = 0.037$, $p = 0.037$; $p = 0.036$, $p = 0.028$; respectively). No significant changes were observed in the number of IL-4 producing $CD4^+$ and $CD8^+$ T cells. In the nonresponder patients' group, no significant differences were found over the study period as compared with baseline values.

A significant increase of mean levels of BDNF in the supernatants of unstimulated PBMC was observed in the responder group from month 6 and throughout the follow-up after GA therapy as compared with baseline (Fig. 2). Similar results were found after stimulation with anti-CD3 and soluble anti-

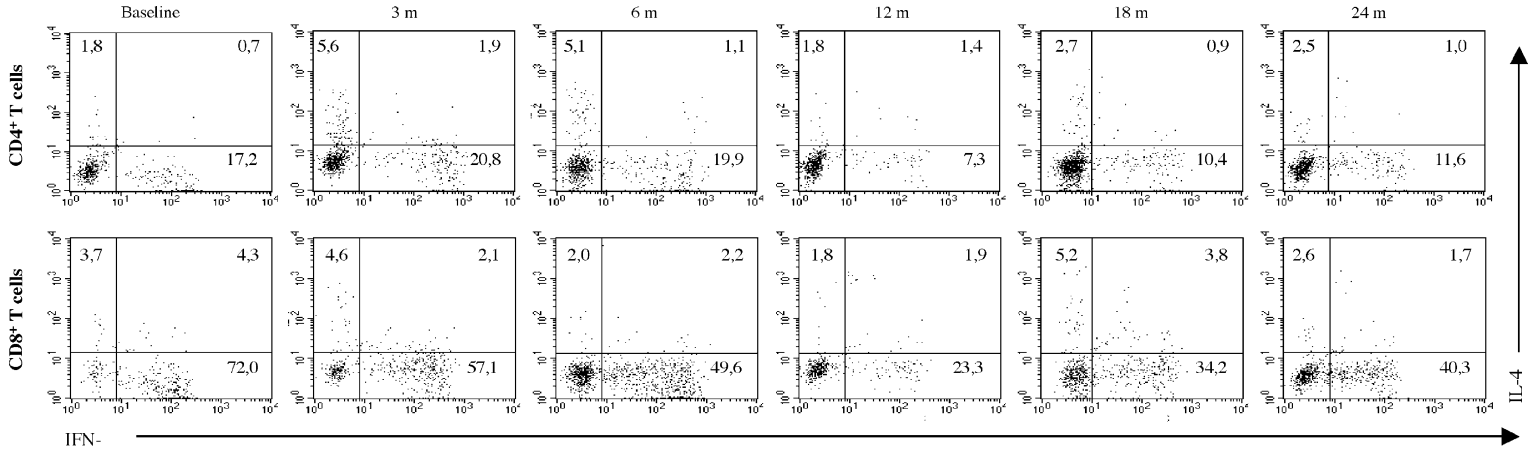

Fig. 1. An example of the flow cytometry analysis of cytoplasmic IFN-γ and IL-4 expression by $CD4^+$ and $CD8^+$ T cells of a responder patient before and after GA treatment, as described in methods. Quadrants were set based on the isotype control and on unstimulated samples. Values represent the percentage of positive cells.

Table 2
Peripheral T cell population before and after GA treatment

		Baseline	6 months	12 months	18 months	24 months
$CD3^+$	R	71.0 ± 5.1	76.1 ± 3.3[a]	75.7 ± 5.5[a]	75.2 ± 3.9[a]	76.5 ± 4.8[a]
	NR	76.1 ± 7.4	75.4 ± 3.3	79.8 ± 3.6	76.1 ± 1.1	77.2 ± 1.2
$CD4^+$	R	47.8 ± 9.1	53.3 ± 6.1[a]	53.9 ± 7.7[a]	53.7 ± 7.5[a]	54.4 ± 7.8[a]
	NR	51.5 ± 6.1	47.7 ± 5.2	51.2 ± 8.9	45.4 ± 11.9	54.1 ± 9.9
$CD4^+CD45RA^+$	R	35.3 ± 16.0	43.9 ± 10.1[a]	43.5 ± 10.2[a]	42.9 ± 9.4[a]	44.8 ± 12.8[a]
	NR	32.6 ± 15.0	34.1 ± 12.2	48.1 ± 17.5	30.6 ± 21.5	40.2 ± 18.4
$CD4^+CD45RO^+$	R	39.3 ± 8.0	38.8 ± 6.5	38.8 ± 8.1	40.6 ± 9.1	37.8 ± 9.0
	NR	49.7 ± 14.0	43.4 ± 7.4	41.5 ± 17.8	45.8 ± 11.0	46.0 ± 9.4
RA^+/RO^+ ratio[a]	R	0.9 ± 0.5	1.2 ± 0.4	1.2 ± 0.4[a]	1.1 ± 0.4[a]	1.2 ± 0.4[a]
	NR	0.8 ± 0.7	0.7 ± 0.3	1.1 ± 0.8	0.7 ± 0.6	0.9 ± 0.3
$CD8^+$	R	21.0 ± 7.0	22.5 ± 5.6	21.0 ± 5.9	21.2 ± 6.5	21.3 ± 5.0
	NR	26.7 ± 8.0	26.2 ± 11.6	27.4 ± 9.9	30.7 ± 13.1	24.1 ± 9.7
$CD8^+CD45RA^+$	R	58.4 ± 11.7	6.3.3 ± 11.0	60.2 ± 9.7	57.2 ± 12.4	57.8 ± 10.5
	NR	53.7 ± 17.0	58.1 ± 18.8	54.5 ± 23.7	49.0 ± 22.4	59.6 ± 17.3
$CD8^+CD45RO^+$	R	23.9 ± 8.3	24.2 ± 10.5	21.7 ± 6.6	24.8 ± 9.8	23.5 ± 5.7
	NR	23.2 ± 5.9	22.9 ± 4.9	17.8 ± 4.5	19.8 ± 3.0	23.0 ± 2.7
$CD4^+CD25^+$	R	31.7 ± 11.3	31.5 ± 8.9	28.7 ± 8.9	28.5 ± 6.3	31.3 ± 6.7
	NR	39.1 ± 6.3	31.5 ± 8.5	29.5 ± 4.8	24.3 ± 6.4	31.0 ± 5.1

Results are expressed as mean ± standard deviation of the percentage of positive cells for each surface marker; R = responder group; NR = non-responder group.
[a] $CD4^+CD45RA^+$ to $CD4^+CD45RO^+$ T cells ratio.

CD28 antibodies (data not shown). No significant changes were observed for the nonresponder group (Fig. 2).

No significant correlation was found between basal immunological data and clinical features (sex, age, disease duration, annualized relapse rate and EDSS score), nor there was any significant correlation between immunological measurements on treatment and clinical outcome (number of relapses, change of the annualized relapse rate, increase of the EDSS score and last EDSS score). None of the clinical and immunological data analyzed could significantly predict responsiveness to GA therapy.

Our study shows that *in vivo* therapy with GA induces a down-modulation of proinflammatory cytokine-producing T cells in the periphery, and increases the BDNF production by unstimulated PBMC but also after stimulation involving T-cell receptor-mediated activation, and this effect is sustained over time but only observed in patients with clinical response to the drug. These data are in line with the results previously shown by the more complex method of analysis of GA-specific T-cell lines [1,3,4,10,13,18,25], although few immunological longitudinal studies with clinical correlation such as we present have been reported so far [22]. In this sense, other authors observed

Table 3
Intracellular cytokine staining profile before and after GA treatment

		Baseline	6 m	12 m	18 m	24 m
IFN-γ $CD3^+$	R	19.7 ± 9.8	14.6 ± 5.5	0.028	0.024	0.012
	NR	24.0 ± 17.6	20.2 ± 17.1	15.1 ± 12.6	28.4 ± 11.4	20.9 ± 15.2
IFN-γ $CD4^+$	R	20.6 ± 9.4	14.1 ± 8.7	0.008	0.008	0.012
	NR	23.5 ± 12.4	14.2 ± 8.9	11.3 ± 2.5	18.0 ± 5.8	12.7 ± 9.7
IFN-γ $CD8^+$	R	47.3 ± 18.1	39.1 ± 14.8	0.008	0.011	0.012
	NR	51.1 ± 27.5	42.4 ± 30.2	45.2 ± 16.3	67.9 ± 14.8	49.9 ± 15.0
IL-2 $CD4^+$	R	40.9 ± 17.5	50.4 ± 15.4	0.013	0.037	0.036
	NR	44.3 ± 11.9	42.3 ± 5.6	43.0 ± 14.6	45.1 ± 12.4	47.9 ± 11.7
IL-2 $CD8^+$	R	17.2 ± 11.5	22.3 ± 9.6	0.021	0.037	0.026
	NR	14.6 ± 2.6	19.8 ± 3.5	15.4 ± 2.8	17.3 ± 3.2	13.9 ± 2.7
IL-4 $CD4^+$	R	3.0 ± 2.0	3.3 ± 0.8	4.7 ± 2.5	2.7 ± 1.1	1.1 ± 1.0
	NR	3.3 ± 1.3	3.7 ± 3.4	3.5 ± 1.1	4.2 ± 2.5	3.8 ± 1.3
IL-4 $CD8^+$	R	3.4 ± 1.2	2.4 ± 1.4	3.4 ± 2.7	2.5 ± 1.5	1.7 ± 1.0
	NR	2.0 ± 1.8	2.5 ± 0.6	1.8 ± 0.6	2.9 ± 0.4	1.0 ± 0.5

Results are expressed as mean ± standard deviation of the percentage of cells staining positively. R = responder group; NR = non-responder group.

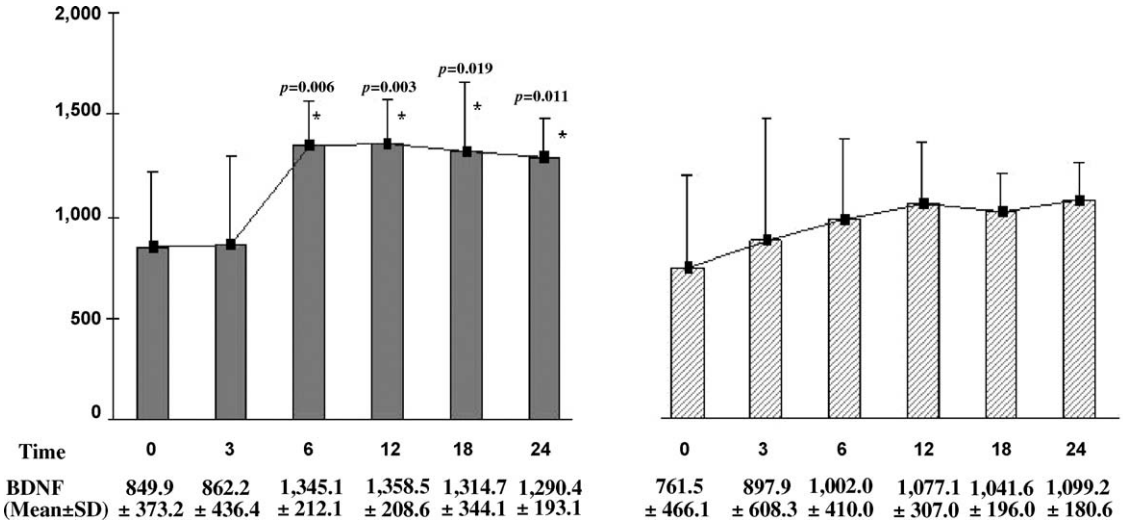

Fig. 2. BDNF levels in the supernatant of unstimulated PBMC of responder (left graph) and non-responder (right graph) group before and after GA treatment. Results are expressed in pg/ml. Time is expressed in months; time 0 refers to baseline. *p*-values are calculated in comparison with baseline and corrected for multiple comparisons. S.D.: standard deviation.

an increase in the IFN-γ secretion in responders to treatment; however the study was cross-sectional and retrospective [12]. Interesting, our significant immune changes started at month 6 for the immunophenotypic change and BDNF production, and at month 12 for the Th1 cytokine reduction, consistent with a delayed mechanism of action and a radiologic response, as was seen in the serial magnetic resonance imaging-based clinical trial [6].

However, the most interesting data we found was that the reduction of Th1-type cell number was accompanied by an *in vivo* change in the peripheral T cell compartment characterized by a significant increase of $CD4^+CD45RA^+$ (*naïve*) T cell frequency associated with an increased RA^+/RO^+ (*naïve*/memory) ratio. A finding that extends a previous *in vitro* study that showed that the response to GA was driven by the $CD4^+CD45RA^+$ T cell subpopulation [23].

The relevance of this increased frequency of peripheral $CD4^+CD45RA^+$ T cells after GA therapy to the clinical response we observed is intriguing. Nevertheless, in MS patients the *naïve* T cell frequency is lower than average [8], and have a reduced TREC (TCR excision circles) content in circulating T cells, especially for $CD4^+$ T cells, [14], suggesting that the release of *naïve* T cells from the thymus could be impaired in MS. Because $CD4^+CD45RA^+$ T cells number as well as TCR levels decline with age [14,21] it could be that the results we found were related to the different, although non significant, age of both groups of patients. However, it is unlikely because we did not observe an increase in the frequency of $CD4^+CD45RA^+$ T cells in 4 additional RRMS patients followed at least for 1 year after treatment with IFN-β who had a similar age (mean, 34.25 ± 7.93 years) to the GA responder group. Interesting, all four patients were considered as responders to IFN-β by using the same clinical definition.

Finally, if the differences we found on GA therapy merely reflect that the two patient groups were in different stages of the disease is unknown. However, it is likely because similar clinical results have been observed with other immunomodulatory therapies that showed higher therapeutic benefit in patients with shorter disease duration and lesser disability [20,5].

In conclusion, our study suggests that in addition to down-modulation of proinflammatory cytokines and increased secretion of BDNF, GA therapy may induce the regulation of the peripheral T cell compartment. Whether this regulation contributes to the clinical efficacy of GA needs further confirmatory studies in a larger patient sample.

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