Clinical response to glatiramer acetate correlates with modulation of IFN-γ and IL-4 expression in multiple sclerosis

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Objective To determine whether glatiramer acetate (GA)-induced lymphoproliferation and IFN-γ and IL-4 modulation correlate with the clinical response in multiple sclerosis (MS).
Background GA therapy involves the induction of anti-inflammatory cytokine shifts. However, it is not known whether this response correlates with the clinical outcome.
Methods Thirty-six relapsing-remitting (RR) MS patients were treated with GA for at least two years, and classified clinically as GA-responders (GA-R = 22) or hypo/non-responders (GA-HR/NR = 14). Proliferation of peripheral blood mononuclear cells (PBMC) to GA and Tetanus toxoid (TT), as well as IL-4 and IFN-γ ELISPOT, were performed.
Findings There was no difference in PBMC proliferation to GA or TT between GA-R and GA-HR/NR before and during treatment ($P > 0.05$). The mean number of IFN-γ ELISPOTS in unstimulated, TT and anti-CD3/CD28-stimulated PBMC was lower among GA-R (unstimulated: GA-R = 10.1 ± 6.21 ($n = 22$) versus GA-HR/NR = 17.8 ± 12.7 ($n = 14$), $P = 0.04$; TT-GA-R = 12.2 ± 4.06 ($n = 12$) versus GA-HR/NR = 26.8 ± 21.0 ($n = 8$), $P = 0.028$; anti-CD-3/CD28 GA-R = 217.3 ± 140.4 ($n = 22$) versus GA-HR/NR = 368.5 ± 170.1 ($n = 14$), $P = 0.006$). In contrast, the number of IL-4 ELISPOTS remained unchanged in the GA-R group, but was progressively reduced in the GA-HR/NR group during GA therapy (GA-HR/NR IL-4: pre-Rx: 59 ± 34 versus 22 ± 11 at 12 months ($n = 6$), $P = 0.0429$). The IL-4/IFN-γ ratio in anti-CD3/CD28-stimulated PBMC was significantly higher among GA-R compared to GA-HR/NR ($P = 0.0474$).
Interpretation Lymphoproliferation to GA did not differentiate GA-R from GA-HR/NR. However, reduced IFN-γ expression and stable IL-4 expression in anti-CD3/CD28-stimulated PBMC, and an increased IL-4/IFN-γ ratio was associated with favorable clinical response. More data are needed to validate the prospective use of IL-4/IFN-γ expression in PBMC as a biomarker of clinical response to GA for individual patients. *Multiple Sclerosis* 2007; **13**: 754–762. http://msj.sagepub.com

Key words: multiple sclerosis; glatiramer acetate; interferon-gamma; Interleukin-4

Introduction

The mechanism of action of glatiramer acetate (GA) in the treatment of multiple sclerosis (MS) is not fully elucidated. The molecule binds to HLA class II molecules associated with MS, including DR2 and DR4, on antigen presenting cells [1–5]. Recent studies, both in experimental autoimmune encephalomyelitis (EAE) [6–8,12] and in humans [7,9–14], indicate that a likely *in vivo* mechanism of action of GA involves the induction of immunomodulatory regulatory T-helper type 2 (Th2) cells. The GA-specific T cells may exert their protective action systemically and by entering the CNS compartment, as evidenced by inhibition of new magnetic resonance imaging (MRI) gadolinium-enhancing lesions progressing into black holes [15], and the production of anti-inflammatory cytokines and brain-derived neurotrophic factor (BDNF) [16], in response to cross-recognition of

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myelin antigens (bystander suppression) [6,17]. We and others have demonstrated that GA treatment in MS results in the induction of GA-specific T cells with a predominant Th2 phenotype, both in response to GA and crossreactive myelin antigens [17,18]. These results strongly suggest that the mechanism of action of GA in MS involves the induction of crossreactive GA-specific T-cells with a predominant Th2 cytokine profile. Importantly, the induction of GA-specific Th2 cells was not universal in MS patients treated with the drug [19]. Since clinical response to GA varies among treated patients, this raises the possibility that the immunological effects of GA may correlate with the clinical response, and may distinguish responders from hypo or non-responders.

Recently, Farina *et al.* [20], reported a triad of immune responses to GA that predict treated from untreated patients. This triad consisted of: (1) a decline in GA-induced proliferation; (2) positive IL-4 $CD4^+$ cells; and (3) positive IFNγ $CD8^+$ cells in response to high dose of GA (100 μg/mL). Whether this triad correlates with the clinical response is not certain at present. In this study, we determined whether the lymphoproliferative response to GA and the GA-induced cytokine shifts in IL-4 and IFN-γ correlate with the clinical response to GA.

Materials and methods

Subjects

Thirty-six consecutive patients, diagnosed with relapsing-remitting (RR) MS and treated with GA for at least two years, were included in the study. Thirty-one patients were female with a mean age of 44 years, and five were male with a mean age of 49 years. Patients had at least one relapse during the year prior to initiation of GA therapy. All subjects were closely followed up at the University of Maryland Center for MS. The clinical characteristics of the patients are presented in Table 1. After at least two years on GA therapy, patients were classified as GA-responders (R) ($n = 22$) or hypo/non-responders (HR/NR) ($n = 14$) based on criteria arbitrarily set for this study. We adopted a more conservative criteria for patient classification into GA-R and GA-HR/NR compared to those recently reported in the literature [21,22]. A responder (R) is a patient with an annual relapse rate (ARR) < 0.5 and no evidence of disease progression, as measured by Expanded Disability Status Scale (EDSS). A HR/NR is a patient with an ARR > 0.5 and/or with progression in the EDSS of at least 1 point sustained for six months.

Cells

Approximately 60 cc of heparinised blood was obtained by venipuncture from each MS patient at different time points during treatment, as indicated in Table 1. In a subset of patients, blood samples were obtained before treatment, and at three, six, 12 and 18–24 months during treatment. The study was approved by the University of Maryland Institutional Review Board, and informed consent was obtained from each patient enrolled in the study. Peripheral blood mononuclear cells (PBMC) were purified using a Ficoll-Hypaque gradient, as described in the supplier's protocol (ICN Biomedicals Inc., OH, USA).

Lymphoproliferation assay

One $\times 10^5$ PBMC/well were seeded in a 96-well U-bottom microtiter plate together with 1×10^5 irradiated autologus PBMC, in the presence of antigen in triplicate wells (glatiramer acetate at 40 and 100 μg/mL, Teva Pharmaceutical Industries, Ltd., Israel). Tetanus toxoid (TT; 5 μg) obtained from Pasteur Merieux Connaught (North York, Ont., Canada) was used as a control antigen. After cells were cultured for 48 hours, 1 μCi ^{3}H-thymindine was added. Cells were harvested 18 hours later using TOMTEC cell harvester (TOMTEC, Hamden, CT). Incorporated radioactivity was measured using a liquid Scintillation counter (Wallac MicroBeta Trilux, Perkin Elmer, Boston, MA).

ELISPOT assay

Human IFN-γ (Th1 indicator) and IL-4 (Th2 indicator) were measured by the ELISPOT assay, according to the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). Briefly, ELISPOT plates (PVDF plate, Millipore Corp., MA, USA) were coated with 100 μL/well of 5 μg/mL capture antibody at 4°C overnight. The plates were then washed with PBS, and incubated with 200 μL/well of blocking solution (culture medium) for 2 hours. PBMC (2×10^5 cells/well for IFN-γ and 4×10^5 cells/well for IL-4 detection) and antigen (GA 40 and 100 μg/mL, TT 5 μg/mL, CD3Ab (2.5 μg/mL)/CD28 Ab (1 μg/mL)) was added and cultured overnight at 37°C in a 5% CO_2 incubator. Patients were tested in triplicate wells in response to each antigen. After overnight culture, the plates were washed twice with deionised water, and three times with PBS, then incubated with 100 μL of 2.0 μg/mL biotinylated antibody for 2 hours at room temperature. The plates were then washed three times with wash buffer (0.05% Tween-20 PBS), and incubated with

Table 1 Characteristics of MS patients and time points during treatment when blood samples were obtained

ID No.	Age/sex	Disease duration (years)	Rx duration (months)	Time sample analysed (months)					EDSS		ARR		Patient status
				0	3	6	12	18–24	Pre-Rx	On-Rx	Pre-Rx	On-Rx	
42	29/F	4	35			*			1	0	1.5	0.3	R
39	67/F	37	40		*				1.5	1.5	2	0	R
43	36/F	5	55		*				0	0	1.5	0.9	NR
44	41/F	Uncertain	27			*			1.5	1	1.5	0.4	R
45	39/F	18	60					*	6.5	6.5	3	0	R
46	40/F	19	24		*				2	2	2	0	R
47	37/M	13	56					*	1.5	1	1.5	0	R
48	64/F	25	27			*			2	1.5	1	0	R
49	46/F	12	24		*				2	1.5	1.5	0	R
9	49/F	6	24			*			2.5	4	2	1.5	NR
50	44/F	15	45					*	1.5	1.5	1.5	0	R
52	51/F	20	38					*	3	4	1.5	1.3	NR
7	52/F	5	38					*	1.5	2.5	1	0	NR
53	40/M	3	24				*		0	1.5	0.5	0	NR
54	46/F	5	30		*				2.5	2.5	1.5	0.4	R
55	41/F	5	25			*			2.5	2.0	0.5	0	R
56	22/F	2	24		*				2	0	2	0	R
57	53/F	32	57		*				6	7	1	0.4	NR
58	48/F	5	40		*				0	1	1	0.6	NR
59	43/F	Uncertain	41					*	1.5	1.5	0.9	1.5	NR
1	32/F	5	36	*	*	*	*	*	1.5	1.5	2.5	0	R
2	45/M	6	24	*	*	*	*	*	2	2.5	0.5	0.99	NR
3	45/F	23	24	*	*	*	*	*	1.5	1.5	2	0	R
4	32/F	37	26	*	*	*	*	*	3.5	1.5	1.5	0.46	R
6	60/F	5	48	*	*	*	*	*	7	1.5	3	0	R
7a	53/F	Uncertain	24	*	*	*	*	*	3.5	7	0.5	0.49	NR
8	53/F	18	29	*	*	*	*	*	2	2	1.5	0	R
10	43/F	13	36	*	*	*	*	*	1.5	2.5	1	0.99	NR
11	52/F	25	24	*	*	*	*	*	2	6	1.5	0	NR
12	40/F	12	24	*	*	*	*	*	1.5	1	0.5	0	R
14	51/F	21	24	*	*	*	*	*	1.5	2.5	1.5	1.33	NR
17	60/M	5	24	*	*	*	*	*	2.5	2	1	1.2	NR
18	29/F	5	24	*	*	*	*	*	1.5	1.5	1.5	0	R
21	49/F	5	24	*	*	*	*	*	2	2	1	0	R
22	63/M	6	27	*	*	*	*	*	2.5	2.5	2	0	R
24	36/F	5	24	*	*	*	*	*	1.5	1.5	2	0	R

Rx: treatment; pre-Rx, pre-treatment; post-Rx, post-treatment; ARR, annual relapse rate; R, responder; HR/NR, hypo/non-responder; LV, last visit.
*Blood sample obtained.

100 μL of 1:100 dilution of Avidin-HRP for 1 hour at room temperature, and washed three times with wash buffer. Finally, 100 μL of AEC (3-amino -9-ethyl-carbazole) substrate was added, and the reaction was stopped by washing plates with distilled water. Spots were counted and analysed by CTL Analysers LLC ELISPOT Plate Reader (Cleveland, OH, USA).

Statistical analysis

A software package (Graphpad, Prism™) was used in the statistical analysis. The differences in cytokine spots between responder and hypo/non-responder were compared using the Student's *t*-test and Bonferroni's multiple comparison test. A P value <0.05 was considered significant. Although only 2×10^5 cells/well were utilised for the IFN-γ ELISPOT, the values were adjusted to 4×10^5 cells/well for both the IL-4 and IFN-γ spots to account for the corrected dilution factor.

Results

Classification of clinical responders and hypo/non-responders

The patients were classified as GA-responders or hypo/non-responders after being treated with GA for at least 24 months, based on their annual relapse rate and progression in disability while on treatment (Table 1). The mean treatment duration was 34 months. The GA-responder group had a mean ARR of 1.68 and a mean EDSS of 2.31 before treatment compared to 0.07 ARR and an EDSS of 1.70 during treatment. The hypo/non-responder group had a mean ARR of 1.10 and a mean EDSS of 1.96 before treatment compared to 0.75 ARR and an EDSS of 3 during GA therapy. Overall, a 96% reduction in the ARR, and a 0.61 point decrease in the EDSS occurred in the GA-R, whereas a 31% reduction in the ARR, and a 1.14 point increase in the EDSS occurred in the GA-HR/NR during treatment.

Cytokine shifts

Initially, expression of IFN-γ and IL-4 was examined by ELISPOT in PBMC obtained *ex vivo* from GA-treated MS patients at random time points during treatment (Table 2). There was no significant difference in cytokine production between GA-R and GA-HR/NR in response to GA stimulation at 40 and 100 μg/mL. The mean number of IFN-γ spots in unstimulated TT (5 μg/mL) and anti-CD3/CD28-stimulated PBMC, was significantly lower among GA-R compared to GA-HR/NR (unstimulated: GA-R $= 10.1 \pm 8.21$ ($n = 22$) versus GA-HR/NR $= 17.8 \pm 12.7$ ($n = 14$), $P = 0.03$; TT-GA-R $= 12.2 \pm 4.06$ ($n = 12$) versus GA-HR/NR $= 26.8 \pm 21.0$ ($n = 8$), $P = 0.028$; anti-CD-3/CD28 GA-R $= 217.3 \pm 140.4$ ($n = 22$) versus GA-HR/NR $= 368.5 \pm 170.1$ ($n = 14$), $P = 0.006$). The mean number of IL-4 spots in anti-CD3/CD28-stimulated PBMC was not significantly different between GA-R and GA-HR/NR (GA-R $= 92.0 \pm 84.9$ ($n = 22$) versus GA-HR/NR $= 80.8 \pm 80.6$ ($n = 14$), $P > 0.05$). The IL-4/IFN-γ ratio in anti-CD3/CD28-stimulated PBMC was significantly higher among GA-R compared to GA-HR/NR (0.93 ± 1.02 versus 0.34 ± 0.40, respectively; $P = 0.0474$).

In 16 of the 36 patients (10 GA-R and six GA-HR/NR) pre-treatment and during treatment IL-4 and IFN-γ ELISPOTS were determined. Cytokine expression studies in anti-CD3/CD28-stimulated PBMC were performed at serial time points, ranging from three to 24 months for this sub-group (Figure 1). Mean IL-4 spots did not change significantly during treatment compared to pre-treatment levels in the GA-R group (Figure 1a). In contrast, mean IL-4 spots decreased from pre-treatment levels in the GA-HR/NR group beginning at three months of GA therapy, and the decrease became statistically significant at 12 months and beyond (Figure 1b). The mean IFN-γ spots steadily decreased during treatment in the GA-R group, beginning at three months, and this decrease became statistically significant at ≥ 12 months (Figure 1c). Although a decrease in IFN-γ was observed in the GA-HR/NR group, this decrease was delayed (≥ 12 months), and did not reach statistical significance (Figure 1d). The mean IL-4/IFN-γ ratio increased steadily during treatment in the GA-R group, and became statistically significant at six months and later (Figure 1e). In contrast, the mean IL-4/IFN-γ ratio remained unchanged at all time points in the GA-HR/NR group (Figure 1f).

Correlation of IL-4/IFNγ ratio with the clinical response

Linear regression analysis using Spearman correlation showed no significant correlation between the number of IL-4 spots and ARR (Figure 2a), while a significant positive correlation was observed between the number of IFN-γ spots and ARR ($P = 0.040$) (Figure 2b). A significant negative correlation between ARR and IL-4/IFN-γ ratio was observed ($P = 0.041$) (Figure 2c).

Table 2 IL-4 and IFN-γ expression by Elispot obtained from MS patients treated with GA

	R	HR/NR	R	HR/NR	R	HR/NR	R	HR/NR
No. Ag	5.40±3.90 ($n=22$)	4.00±2.56 ($n=14$)	10.1±6.21 ($n=22$)	17.8±12.7 ($n=14$)	0.5±0.60	0.25±0.40	0.25	0.20
GA (40 μg/mL)	9.27±5.54 ($n=12$)	8.06±2.84 ($n=8$)	10.83±7.2 ($n=12$)	13.0±9.53 ($n=8$)	1.77±2.15	0.658±0.29	0.73	0.60
GA (100 μg/mL)	11.31±6.45 ($n=12$)	11.89±5.14 ($n=8$)	14.0±8.04 ($n=12$)	16.5±9.41 ($n=8$)	0.967±0.57 ($n=12$)	0.82±0.39	0.85	0.74
TT (5 μg/mL)	10.96±5.79 ($n=12$)	8.33±3.23 ($n=8$)	12.2±4.06 ($n=12$)	26.8±21.0 ($n=8$)	1.35±0.94	0.76±0.27	1.12	0.41
CD3/CD28 (2.5 μg/1 μg/mL)	92.0±84.95 ($n=22$)	80.8±80.6 ($n=14$)	217.3±140 ($n=22$)	368.5±170 ($n=14$)	0.93±1.02	0.341±0.4	0.39	0.15

R, GA-responder; HR/NR, GA-hypo/non-responder; *n*, number of patients.
*Mean number of spots per 4×10^5 cells/well.
The mean IL-4 and IFN-γ spots shown in columns 2 and 3 were calculated as follows: first, the mean number of spots during the three to 24 month-treatment period was determined for each individual. Subsequently, the average of the mean number of spots for each group was calculated. The IL-4/IFN-γ ratio shown in column 4 represents the mean of the ratios for each group. Note that the ratio in column 4 is not the ratio of IL-4 and IFN-γ means shown in column 2 and 3.

Lymphoproliferative response

The *ex vivo* PBMC proliferative response to GA and TT are presented in Figure 3 as ΔCPM (mean cpm in antigen stimulated minus mean cpm in unstimulated wells) and as stimulation indexes (SI = mean CPM in stimulated wells/mean CPM in unstimulated wells). Overall, there was an initial increase in the proliferative response to GA followed by a decline after six months of treatment, which was statistically significant at 12 months ($P=0.04$) and 18–24 months ($P=0.01$). An initial decrease in the proliferative response to TT was observed at three months ($P=0.007$), but this response recovered after six months, and was not significantly different from pre-treatment values. There was no difference in PBMC proliferation to GA or TT between GA-R and GA-HR/NR before and during treatment at three, six, 12 months or two years (*P* values at all time points were >0.05).

Discussion

Previous studies suggested that a decrease in the lymphoproliferative response to GA and changes in IL-4 and IFN-γ expression are associated with GA treatment in MS [13,18,21]. The goal of this study was to determine whether the lymphoproliferative response to GA and GA-induced cytokine shifts correlate with the clinical response. While GA treatment initially induces a strong lymphoproliferative response during the first three months, this response declines over time [20,23]. It is believed that the residual T-cell response to GA after six months of treatment includes predominantly Th2-biased cells with significant degeneracy in their antigen recognition patterns [24]. These cells cross-react with myelin antigens [18], and are believed to mediate the GA-therapeutic effect through the release of anti-inflammatory Th2 cytokines, resulting in bystander suppression and by secretion of brain-derived neurotrophic factor (BDNF) [16]. However, it is not known at the present time whether GA-induced cytokine shifts correlate with the clinical response to treatment with GA.

Earlier reports have shown a reduction in PBMC proliferation to GA among GA-treated patients [20,24,31]. However, these results were not correlated with patients' clinical response to the drug. Subsequently, Farina *et al.* [20], and Weder *et al.* [31], examined the proliferative response to GA in GA-treated MS patients versus healthy controls and untreated MS patients, and found that T cells from GA-treated MS patients had a significant reduction in stimulation indexes. Although the PBMC proliferation data presented by Farina *et al.* showed a trend towards a higher GA-induced stimulation index among the GA non-responders, the difference between GA-responders and GA-non-responders was not statistically significant, and failed to differentiate the two groups. In addition, they found that a strong *in vitro* response for both IL-4 and IFN-γ correlates with a successful clinical outcome [29]. Our data showed an initial increase in both the ΔCPM and stimulation indexes within three months of treatment. However, a significant reduction was observed in the ΔCPM and stimulation indexes after six months of GA therapy, which is consistent with the findings reported by Duda *et al.* [24]. Although our study and those of others suggest that the reduced proliferation is specific to GA, the proliferation of GA and TT-stimulated PBMCs did not differentiate GA-responders from the hypo/non-responders. This raises the possibility of a mechanism unrelated to proliferation involved in the differential

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