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Sustained immunological effects of Glatiramer acetate in patients with multiple sclerosis treated for over 6 years

M. Chen [a], K. Conway [a], K.P. Johnson [a], R. Martin [b], S. Dhib-Jalbut [a,c,*]

[a] *University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[b] *Neuroimmunology Branch, NINDS, National Institutes of Health, Bethesda, MD 20892, USA*
[c] *Baltimore VA Medical Center, Baltimore, MD 21201, USA*

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Abstract

The availability of a group of multiple sclerosis (MS) patients at the University of Maryland, who had participated in the pivotal Copaxone® trial in the early 1990s, provided an opportunity to examine the long-term immunologic effects of Glatiramer acetate (GA) treatment in MS. Forty-eight GA-reactive T-cell lines (TCL) were generated from 10 MS patients who have been receiving GA treatment for 6–9 years. Proliferative responses, cytokine production, and cross-reactivity with myelin basic protein (MBP) and the MBP immunodominant peptide 83–99 were compared to responses obtained from 10 MS patients who were tested pretreatment and after a shorter period of treatment ranging from 1 to 10 months. The results indicate that while long-term treatment with GA results in a 2.9-fold decrease in the estimated precursor frequency of GA-reactive T-cells, the sustained response to GA remains Th2-biased and in part cross-reactive with MBP and MBP (83–99) as measured by proliferation and cytokine release assays. The results indicate that despite a drop in the precursor frequency of GA-reactive T-cells with long-term treatment, the sustained response remains predominantly Th2-biased and cross-reactive with MBP, which is consistent with the anti-inflammatory effects of the drug and bystander suppression.

Keywords: Multiple sclerosis; Glatiramer acetate; Copaxone®; Immune deviation; Immunotherapy

1. Introduction

Glatiramer acetate (GA) (Copaxone®), formerly known as Copolymer-1, is a synthetic amino acid copolymer that consists of four amino acids: L-alanine, L-lysine, L-glutamic acid, and L-tyrosine in fixed molar ratio and an average molecular weight of 4.7–11 kDa. The molecule was found to inhibit experimental allergic encephalomyelitis (EAE), and was subsequently developed for treating patients with relapsing–remitting multiple sclerosis (MS) [1–4]. When given subcutaneously in daily doses of 20 mg, the drug reduces the relapse rate, slows the accumulation of disability, and reduces MRI disease activity approximately 6 months after treatment is initiated [5–8].

Although the mechanism of action of GA is not fully understood, a number of studies both in EAE and in MS point to the induction of a GA-reactive T-cell repertoire with a protective Th2 anti-inflammatory phenotype as a likely mechanism of action [9–16]. The GA-reactive T-cells may exert their protective action by entering the CNS compartment and the production of anti-inflammatory cytokines in response to cross-recognition of myelin basic protein (MBP; bystander suppression) [17]. Additional mechanisms have been proposed, as a result of the ability of GA to bind promiscuously to HLA class II molecules associated with MS, including DR2 and DR4, on antigen presenting cells [18–20]. This includes inhibition of presentation of several myelin antigens to T-cells [4,11,15,21–24], induction of anergy or deletion of a population of GA-reactive T-cells [15,26].

Although peripheral blood mononuclear cells (PBMC) from the majority of MS patients proliferate in response to GA, this response declines approximately 6 months after treatment is initiated [13,25]. The surviving GA-reactive T-

* Corresponding author. Department of Neurology, University of Maryland Hospital, Rm. N4W46, 22 S. Greene Street, Baltimore, MD 21201, USA. Tel.: +1-410-706-4216; fax: +1-410-706-0186.
E-mail address: sjalbut@umaryland.edu (S. Dhib-Jalbut).

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cells demonstrate a high degree of degeneracy, with a Th2-polarized response characterized by IL-5 and IL-13 secretion [13]. In view of the decline in the proliferative response to GA with time, we investigated whether MS patients who had received the drug for 6–9 years, continue to generate the anti-inflammatory GA-reactive Th2 cells associated with the therapeutic effect of the drug, and whether these cells have the characteristics of bystander suppression.

2. Methods

2.1. Antigens

Glatiramer acetate (Copaxone lots 119135 and 119137) was from Teva Pharmaceutical Ind., Israel. Guinea pig MBP was purchased from Sigma (St. Louis, MO, USA). MBP peptide (83–99) (purity > 95%) was synthesized by Mixture Sciences (San Diego, CA, USA) using the simultaneous multiple synthesis methods [27]. Tetanus toxoid (TT) was obtained from Pasteur Merieux Connaught (North York, Ontario, Canada).

2.2. Subjects

Ten patients with clinically definite MS, who participated in the Copolymer-1 pivotal multicenter trial [7,8] and followed up at the Maryland Center for MS, were included in this study. Those patients received Glatiramer acetate treatment for 6–9 years and are termed the long-term treatment group. The clinical characteristics of the patients are presented in Table 1. Results were compared to those from another group of 10 MS patients who were studied 1–10 months after they have been on Glatiramer acetate treatment (short-term treatment group) and on whom we have reported earlier [16]. The study was approved by the University of Maryland Institutional Review Board, and the subjects' consent was obtained.

Table 1
Proliferative responses of GA–TCL from the long-term treatment group

Patient	Duration of GA treatment (year)	Relapses[a]	EDSS[b]		No. GA–TCL	Precursor frequency
			Entry	End		
MS 12(503)	9	1	1.5	1.0	5	$1.56/10^6$
MS 13(504)	6	1	1.5	1.5	7	$1.09/10^6$
MS 14(525)	8	1	2.5	2.5	4	$1.25/10^6$
MS 15(513)	6	7	1.5	4.5	1	$0.33/10^6$
MS 16(524)	8	3	5.0	4.0	17	$5.31/10^6$
MS 17(517)	8	7	1.5	4.0	6	$1.87/10^6$
MS 18(518)	8	0	1.5	1.0	4	$1.33/10^6$
MS 19(502)	9	1	2.0	2.0	2	$0.67/10^6$
MS 20(519)	6	2	2.5	2.5	2	$0.26/10^6$
MS 21(501)	6	1	1.5	1.0	2	$0.33/10^6$
Total					48	$1.40/10^6$

[a] Relapses during treatment period.
[b] EDSS: expanded disability status scale at entry and end of the reporting period.

2.3. Cells

Approximately 100 cc of heparinized blood was obtained by venipuncture from the 10 MS patients at each time point. Peripheral blood mononuclear cells (PBMC) were prepared using a Ficoll-Hypaque gradient as described in the supplier's protocol (ICN Biomedicals, OH, USA). For the generation of antigen specific T-cell lines (TCL), cells were cultured without prior freezing.

2.4. Generation of antigen-specific T-cell lines

Antigen-specific TCL were generated by the split-well assay as described previously [28]. Briefly, PBMC were seeded in complete medium RPMI 1640 (Biofluid, Rockville, MD, USA) containing 5% human AB serum (Sigma), 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin/streptomycin, at 1×10^5 cells/well into 96-well U-bottom microtiter plates (Nunc, Roskilde, Denmark) and stimulated with antigens (20 μg/ml of GA or 5 μg/ml of TT). Thirty microtiter wells were prepared for each antigen. Human recombinant IL-2 (Biosource International Camarillo, CA, USA) was added to the culture on day 8 at a final concentration of 20 U/ml. On day 15, 50 μl of the cell suspension were transferred into each of two adjacent wells of a separate 96-well U-bottom microtiter plate, and 150 μl of complete medium containing 1×10^5 autologous irradiated PBMC (3000 rad) was added. One well was stimulated with 20 μg/ml GA and the other with medium only. After 48 h, 1 μCi/well of ^{3}H-thymidine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added. Eighteen hours later, cells were harvested on an automated cell harvester (Tomtec, Hamden, CT, USA) and ^{3}H-thymidine incorporation was measured using a Betaplate counter (Wallac, Gaithersburg, MD, USA). Wells that showed stimulation index (SI, cpm of cells with antigen/cpm of cells without antigen) of >2, and background counts >200 cpm were further expanded and characterized.

2.5. Precursor frequency estimation

The precursor frequency of GA-reactive T-cells was estimated following the first stimulation cycle based on the number of proliferating microtiter wells and seeding 10^5 cells/well; precursor frequency = number of GA-reactive TCL/number of wells plated $\times 10^5$.

2.6. Cytokine production

IFN-γ and IL-5 were measured as markers of Th-1 and Th-2 phenotypes, respectively. Cells from GA-reactive wells identified after the first stimulation were restimulated with antigen, feeders, and IL-2 for 7–10 days. These cells were then harvested, washed, and cultured at 1×10^5 cells/well with 1×10^5/well of irradiated autologous PBMC with or without antigen for 48 h. Supernatants were harvested

and stored at −70 °C. IL-5 and IFN-γ were measured by ELISA according to the manufacturer's protocol (Biosource). The sensitivity of the ELISA was <4 pg/ml for both IL-5 and IFN-γ. The GA-specific TCL were classified as Th0-, Th1- or Th2-biased based on the ratio of IFN-γ/IL-5 secretion. A ratio >2 was arbitrarily defined as Th1 bias, <0.5 as Th2 bias, and a value between 0.5 and 2 as Th0.

2.7. Cross-reactivity studies

Cross-reactivity of GA–TCL with MBP and MBP peptide 83–99 were examined using 10 μg/ml of the antigens. These antigen concentrations had previously been found to give at least half-maximal stimulation in the vast majority of TCL [15]. Cross-reactivity of GA-reactive T-cells with MBP was determined by the split-well technique as described above. Proliferative responses ≥2 with a background of 200 cpm or higher were considered cross-reactive. Cross-reactivity by cytokine release was performed as follows: 1×10^5 cells/well of GA-reactive TCL were seeded in 96-well U-bottom microtiter plate together with 1×10^5 irradiated autologous PBMC in the absence of antigen or in the presence of GA, MBP, or MBP peptide 83–99 in triplicate wells. After cells were cultured for 48 h, 100-μl supernatants were removed from each well for cytokine level determinations. Cross-reactivity as measured by cytokine production was determined by pooling supernatants from triplicate wells and subsequent measurements of IL-5 and IFN-γ levels.

2.8. Statistical analysis

A software package (Graphpad, Prism™) was used in the statistical analysis. Differences in lymphoproliferative responses and cytokine production between groups were compared using the Student's *t*-test. A *p*-value <0.05 was considered significant.

3. Results

3.1. Frequency of GA-reactive T-cells

Forty-eight GA-reactive TLC were generated from 10 MS patients who have been receiving GA treatment for 6–9 years (long-term treatment group; MS 12-21). The estimated precursor frequency of the GA-reactive T-cells ranged from 0.26 to 5.31 in 1 million (Table 1). Precursor frequencies of GA-reactive T-cells generated from the long-term treatment group were compared to those generated from 10 MS patients who were studied pretreatment (81 TCL) and 1–10 months after initiation of treatment with GA (130 TCL) (short-term treatment group) and on whom we have reported previously [16] (Table 2). The mean estimated precursor frequency of the GA-reactive TCL was $1.4/10^6$ in the long-term treatment group, significantly lower than that of the short-term treatment group ($4.03/10^6$) ($p=0.027$). Regression lines for the estimated precursor frequency of GA-reactive T-cells generated for the short-term treatment group (1–6 months; $n=8$) and long-term treatment group (≥6 years; $n=10$) demonstrated a significant drop in the estimated precursor frequency of GA–TCL with increasing treatment duration ($P=0.010$). In contrast, the estimated precursor frequency for the TT–TCL did not change significantly with long-term treatment (Fig. 1).

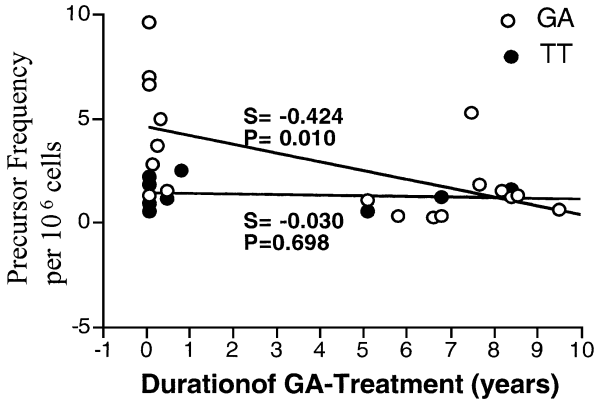

Fig. 1. Regression lines for changes in the precursor frequency of GA and TT reactive T-cells with increasing GA treatment duration in MS patients. GA responses were obtained from 18 MS patients and TT responses from nine. The numbers adjacent to the regression lines indicate slopes (*S*) and *p*-values (*P*).

3.2. GA-reactive TCL maintain a Th2 phenotype bias during long-term treatment with GA

We and others have previously demonstrated that GA treatment induced an immune deviation in MS patients, characterized by a cytokine profile shift from Th1 pretreatment to Th2 during GA treatment [16]. In this study, we examined the cytokine secretion profile of 34 GA-reactive TCL generated from the long-term treatment group. A comparison of the average levels of IL-5 and IFN-γ for GA and TT-reactive TCL from short- and long-term treatment groups is presented in Fig. 2. Mean IL-5 level was 47.14 pg/ml pretreatment, 90.78 pg/ml for the short-term treatment group, and 94.69 pg/ml for the long-term treatment group. Mean IFN-γ level was 171.2 pg/ml pretreat-

Table 2
Comparison of the estimated precursor frequency of GA-reactive T cells in the long-term and short-term treatment groups

GA–TCL	Precursor frequency/10^6		
	Range	Mean ± S.D.	*P*
Pre-Rx	0.31–9.0	2.66 ± 3.32	
S.T. Rx	0.94–9.67	4.03 ± 2.96	0.027
L.T. Rx	0.26–5.31	1.40 ± 1.48	

S.D.: standard deviation; Rx: treatment; S.T.: short-term; L.T: long-term; *P*: student *t*-test *p*-value.

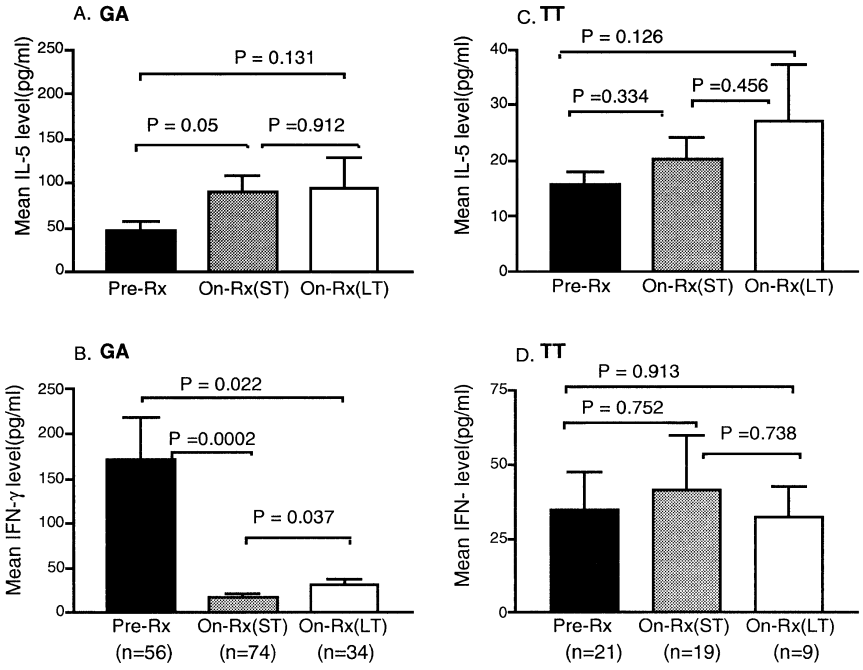

Fig. 2. Mean IL-5 (A and C) and IFN-γ (B and D) produced by the GA-reactive (A and B) and TT-reactive (C and D) TCL pretreatment with GA, during short-term and long-term treatments.

ment, 16.99 pg/ml for short-term treatment, and 31.19 pg/ml for long-term GA treatment. Both treatment groups had IFN-γ levels significantly lower than pretreatment levels. In contrast, cytokine levels produced by TT-reactive TCL did not change significantly in both treatment groups compared to pretreatment levels.

Next, the Th1 and Th2 phenotype bias of the GA-reactive TCL generated from the MS patients was analyzed. GA-reactive TCL producing IL-5 and IFN-γ above the detection limits of the ELISA were classified as Th0-, Th1- or Th2-biased based on the ratio of IFN-γ/IL-5 secretion as described in Methods (Fig. 3). The mean ratio

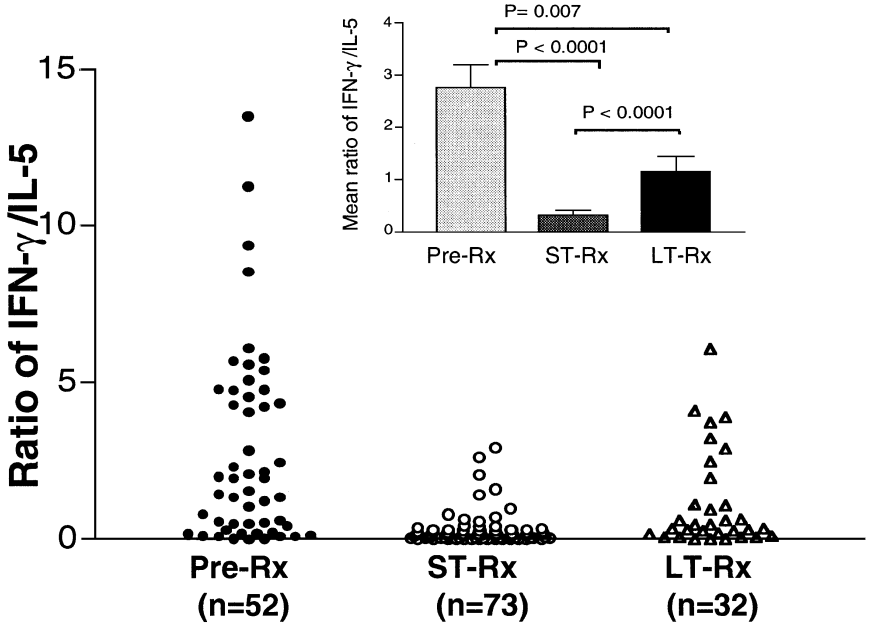

Fig. 3. Ratios of IFN-γ to IL-5 levels for each of the GA-reactive T-cell lines pretreatment and in both treatment groups. The insert figure shows the mean ratios from the three groups.

Table 3
Comparison of cross-reactivity of GA–TCL with MBP in short-term and long-term treated groups

MS patient	Cross-reactive by proliferation		Cross-reactive by cytokine secretion			
	No. TCL tested	No. TCL cross-reactive (%)	No. TCL tested	No. TCL cross-reactive (%) IL-5	IFN-γ	Total
Pre-Rx ($n=10$)	81	10 (12.34%)	51	5 (9.80)	4 (7.84)	8 (15.69)[a]
S.T. Rx ($n=10$)	130	21 (16.15%)	74	23 (31.08)	9 (12.16)	24 (32.4)[a]
L.T. Rx ($n=10$)	52	6 (11.54%)	32	10 (31.25)	2 (6.25)	12 (37.5)

[a] Some TCL were cross-reactive for both IL-5 and IFN-γ secretion, and therefore were counted as a single TCL in the "Total" column.

of IFN-γ/IL-5 of GA-reactive TCL was 1.15 and 0.32 for long-term and short-term GA treatments, respectively; both significantly lower than the corresponding pretreatment ratio (2.76). It is also noteworthy that this ratio was significantly different between the two treatment groups with less Th2 bias in the long-term treatment group. The percentages of GA-reactive TCL classified as Th1, Th0, or Th2 were 20.59%, 23.53%, and 55.88% for the long-term treatment compared to 8%, 9%, and 83% for the short-term treatment group. In contrast, the Th1/Th0/Th2 distribution of TT-reactive TCL did not shift post-treatment in either group (data not shown).

3.3. Cross-reactivity of GA-reactive TCL with MBP and MBP peptide 83–99

We have previously reported cross-reactivity of GA-reactive TCL with MBP and MBP peptide 83–99 in the short-term treatment group [16]. Cross-reactivity was observed by proliferation for some GA–TCL and by cytokine secretion for others. In this study, we determined whether GA–TCL generated from the long-term treatment group manifest cross-reactivity with MBP and MBP (83–99), since such cross-reactivity is a prerequisite for by-stander suppression. Cross-reactivity by proliferation (SI>2.0) was observed in 3 of the 10 patients, and in approximately 12% of the 52 GA–TCL examined (Table 3). Cross-reactivity of the GA–TCL as measured by cytokine secretion was examined in 32 and 24 GA–TCL for MBP and MBP (83–99), respectively. Cytokine secretion (>50% over background levels) in response to MBP or MBP (83–99) was considered a cross-reactive response. Approximately 25% of the GA–TCL cross-reacted with MBP, and 33% cross-reacted with MBP (83–99). The cytokines secreted by the cross-reactive GA–TCL in response to MBP or MBP (83–99) were biased in favor of the Th2 phenotype (Fig. 4). Comparison of the cross-reactive responses in the long-term treatment group with that for the short-term treatment group is presented in Table 3. While the percentage of cross-reactive T-cell lines as measured by proliferation was lower with long-term treatment, cross-reactivity as measured by cytokine secretion was not significantly different between the two groups. Interestingly, while 12% of the cross-reactive GA–TCL in the short-term

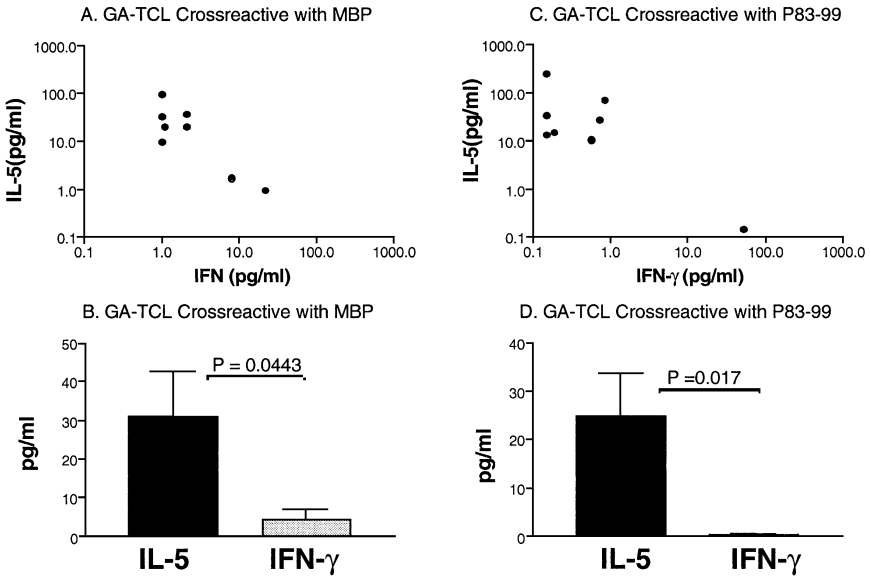

Fig. 4. IL-5 and IFN-γ levels produced by the MBP cross-reactive (A and B) and the MBP 83-99 cross-reactive (C and D) GA–TCL. A and C show the levels for each cross-reactive TCL. B and D show the mean levels for the cross-reactive TCL.

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