Multiple sclerosis: glatiramer acetate induces anti-inflammatory T cells in the cerebrospinal fluid

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Glatiramer acetate (GA) is believed to induce GA-reactive T cells that secrete anti-inflammatory cytokines at the site of inflammation in multiple sclerosis (MS). However, GA-reactive T cells have not been established from the intrathecal compartment of MS patients, and intrathecal T cells may differ from T cells in blood. Here, we compared the phenotype of GA-reactive T cells from the cerebrospinal fluid (CSF) and blood of five MS patients treated with GA for 3-36 months, and in three of these patients also before treatment. From the CSF of these patients, all 22 T cell lines generated before and all 38 T cell lines generated during treatment were GA-reactive. GA treatment induced a more pronounced anti-inflammatory profile of GA-reactive T cell lines from CSF than from blood. While GA-reactive T cell clones from CSF were restricted by either human leukocyte antigen (HLA) -DR or HLA-DP, only HLA-DR restricted GA-reactive T cell clones were detected in blood. No cross reactivity with myelin proteins was detected in GA-reactive T cell lines or clones from CSF. These results suggest that a selected subset of GA-reactive T cells are present in the intrathecal compartment, and support an anti-inflammatory mechanism of action for GA. *Multiple Sclerosis* 2008; **14:** 749–758. http://msj.sagepub.com

Key words: multiple sclerosis; immunology; glatiramer acetate; disease modifying therapies

Introduction

Glatiramer acetate (GA) has been shown to reduce both the relapse rate and the appearance of new central nervous system (CNS) lesions in multiple sclerosis (MS) [1,2]. GA is a polymer of the four amino acids most prevalent in myelin basic protein (MBP) and has been shown to bind several different human leukocyte antigen (HLA)-DR molecules [3]. Antigen presenting cells (APCs) within or outside the CNS may be the primary target of GA-mediated immune modulation [4–6]. The mechanism of GA is thought to involve induction of anti-inflammatory GA-reactive T cells in secondary lymphoid organs [7]. Anergy induction of activated and potentially pathogenic T cells is thought to play a role as long-term GA treatment leads to a drop in the precursor frequency of GA-reactive T cells [8,9]. Within the CNS, GA-reactive T cells may cross-react with myelin proteins [10–12], dampen the inflammatory response by secretion of anti-inflammatory Th2 cytokines such as interleukin (IL)-4, IL-5, IL-10, and IL-13, [10,13,14], induce regulatory T cells [5], and stimulate reparative processes through secretion of neurotrophic factors [15–18].

For these mechanisms to be relevant in MS, GA-reactive T cells must gain access to the CNS. Studies in mice have shown that GA-reactive T cells induced in the periphery penetrate the blood-brain barrier [13,14], and human GA-reactive T cells have been shown to migrate across an artificial blood-brain barrier *in vitro* [19]. However, because of difficulties in investigating migration of human T cells *in vivo*, current knowledge of human GA-reactive T cells stems from analyses conducted with lymphocytes derived from blood.

Studies in MS and other immune-mediated diseases have shown that T cells from the diseased organ may differ from T cells in blood [20–23]. To understand the effects of GA in MS, it is therefore important to investigate GA-reactive T cells from the intrathecal compartment. The aim of the present study was to compare GA-reactive T cells from

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blood and cerebrospinal fluid (CSF) in terms of cytokine profile, HLA restriction, and cross-recognition properties before and after initiation of GA treatment.

Materials and methods

Patients

Five patients (four women and one man) with relapsing-remitting MS (Expanded Disability Status Scale 1–2) gave informed consent to participate in this study. Study participants were treated by neurologists with no other involvement in the study and had received 20 mg GA daily for 0–36 months at inclusion. The study was approved by the local ethics committee.

Antigens

GA (copaxone©) was obtained from Teva Pharmaceuticals Ltd (London, UK). In all, 531 15-mer peptides, overlapping by 10 amino acids spanning the complete sequences of MBP (18.5 kDa and 21.5 kDa isoforms), protelipid protein (PLP), myelin-oligodendrocyte glycoprotein (MOG; including the β2 isoform), myelin-associated oligodendrocytic basic protein, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein, αβ-crystallin, S100β, and 2′, 3′-cyclic nucleotide 3′-phosphodiesterase (partly a gift from N. Karandikar, UT Southwestern Medical Center, Dallas, Texas, USA) were synthesized using standard techniques (BioSynthesis, Texas, USA) at 44.8–100% purity (0.5% were below 50% pure and 12% were above 90% pure) [24]. Peptides were dissolved in dimethyl sulfoxide (DMSC) at a concentration of 50 mg/mL and tested in pools of 8–10 peptides; each peptide was tested at a concentration of 2.5 μM. A chymotrypsin digest of gluten extracted from wheat flour (gift from O. Molberg, Institute of Immunology, Rikshospitalet Radiumhospitalet, Oslo, Norway), human MBP (Chemicon, California, USA), and a peptide from glutamic acid decarboxylase ($GAD_{57–72}$) served as control antigens.

Generation of T cell lines and T cell clones

In all, 40,000–160,000 cells were obtained from 23 mL CSF collected by lumbar puncture. No bleeding was observed during the procedures. To further avoid blood contamination, the first 2 mL of CSF were discharged. To minimize differences related to *in vitro* conditions, T cell lines from CSF and blood were cultured in parallel using identical procedures. Briefly, 1×10^5 peripheral blood mononuclear cells (PBMC) or 4000–6000 CSF cells were incubated with 10^5 irradiated (25 Gy) autologous PBMC, which had been pre-incubated overnight with 50 or 200 μg/mL GA. Cells were stimulated with 10 IU/mL IL-2 (RnDSystems, Minnesota, USA) on day 7 and restimulated on day 14 with 10^5 irradiated autologous PBMC and 50 or 200 μg/mL GA. The T cell lines were expanded with irradiated heterologous PBMC, 1 μg/mL phytohemagglutinin (Remel, Kansas, USA), and 10 IU/mL IL-2 on day 20 and tested for specificity and cytokine production on day 28 with autologous PBMC preincubated overnight with 50 or 200 μg/mL GA. T cell cloning and determination of the HLA-isotype presenting GA were performed using procedures and monoclonal antibodies described previously [25].

Proliferation assays

T cells were tested by incubating 10^5 T cells suspended in 200 μL medium with 10^5 irradiated autologous PBMC, which had been pre-incubated with antigen overnight in 100 μL medium. T cells were cultured for 72 h and proliferation tested on the basis of ^{3}H-thymidine incorporation as described previously [25]. A proliferative response was scored as positive if counts per minute (cpm) of stimulated wells minus cpm of unstimulated wells (Δcpm) exceeded 1000 and the stimulatory index (SI) exceeded 3 [23].

Cytokine measurement

Supernatants of T cells were frozen at –80°C for 48 h after antigen stimulation. Concentrations of IL-4, IL-5, IL-10, IL-13, IL-17, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were measured with a custom-made multiplex antibody bead kit (Biosource International Inc., Camarillo, California, USA) for Luminex™, according to the manufacturer's recommendations. The detection level for all cytokines was 3–5 pg/mL. Unstimulated T cells incubated with irradiated PBMC in the absence of antigen served as control. Irradiated PBMC incubated with antigen overnight produced no detectable levels of the cytokines. Cytokine production was calculated as cytokine concentrations in supernatants from antigen-stimulated cells (T + APC + GA) minus unstimulated cells (T + APC).

Statistics

Cytokine ratios were compared with a two-way ANOVA analysis with patients as random effect in

Statistical Package for the Social Sciences (SPS) version 15 (SPSS Inc., Chicago, Illinois, USA). To correct for deviation from the normal distribution, data were log transformed before analysis. The proportion of Th1-biased and Th2-biased GA-reactive T cell lines was analyzed using generalized estimating equation regression models in Statistical Analysis System (SAS) version 9.1 (SAS Inc., Cary, North Carolina, USA).

Results

GA-reactive T cells are present in the CSF and blood before and during GA treatment

To establish whether GA-reactive T cells are present in the CSF of patients before and after initiation of treatment, 22 T cell lines from CSF and 36 T cell lines from blood were generated from patients 1–3 before they started GA treatment, and 28 T cell lines from CSF and 31 T cell lines from blood of the same patients were generated after 3–6 months on treatment. All T cell lines generated both before and during treatment from both blood and CSF responded vigorously to GA both by proliferation and cytokine production (Figure 1). Although the number of CSF cells is too low to allow calculation of the frequency of GA-specific T cells, the observation that all T cell lines were GA-reactive indicates that the precursor frequency of GA-specific T cells in CSF both before and after initiation of GA treatment exceeds 1:5000.

Th2-bias is more pronounced in GA-reactive T cell lines from CSF than from blood

To establish cytokine profiles of GA-reactive T cells, concentrations of pro- and anti-inflammatory cytokines were measured in supernatants of proliferating GA-reactive T cell lines. Using an IL-5/IFN-γ ratio of 1 as the cut-off between a predominant Th1 versus Th2 phenotype, 13 of 22 GA-reactive T cell lines from CSF and 11 of 36 GA-reactive T cell lines from blood were Th2-biased before treatment. After treatment, the proportion of predominantly Th2-biased GA reactive T cell lines from the same patients was 25 of 28 (CSF) and 21 of 31 (blood). Thus, short-term GA-treatment induced an increase in the ratio of Th2-biased cell lines in both CSF and blood. The increase was only

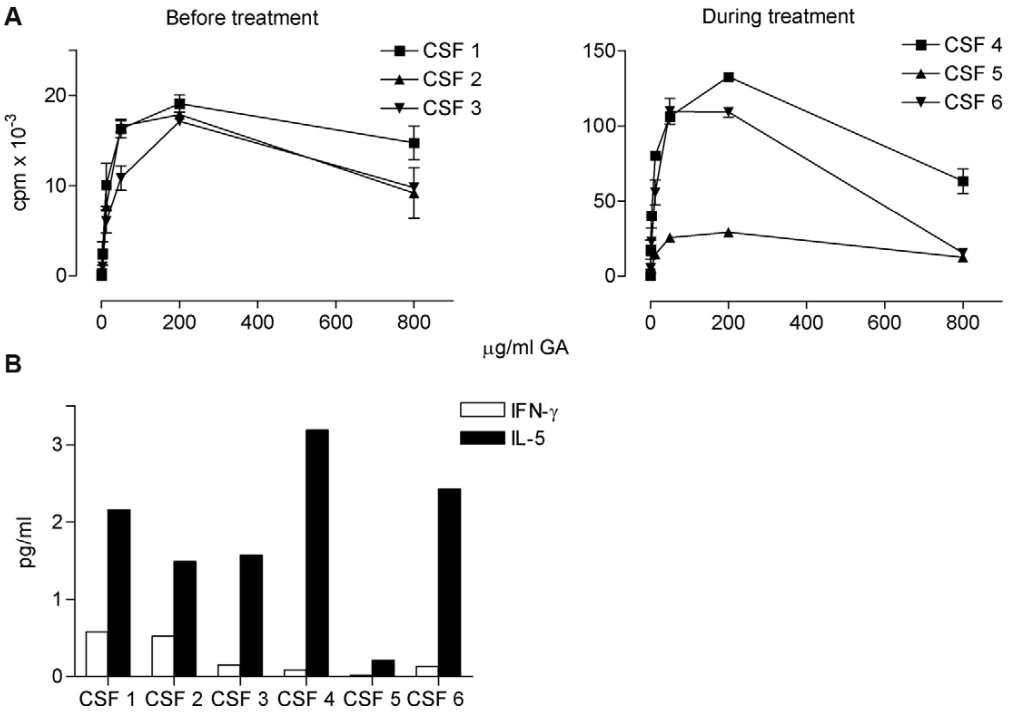

Figure 1 Glatiramer acetate (GA)-reactive T cell lines (TCL) can be generated from the CSF of GA-treated and treatment naive individuals. Proliferative responses (Δcpm) against four-fold serial dilutions of GA from 800–0.2 μg/mL GA in three representative CSF T cell lines from patient 1 generated before GA-treatment (CSF TCL 1–3) and after 3 months on GA treatment (CSF TCL 4–6) are shown (A). None of the T cell lines displayed any proliferation in response to 20–0.003 μg/mL myelin basic protein or gluten in the same experiment (data not shown). Production of IL-5 and IFN-γ is shown for all cell lines (B).

significant in the CSF ($P = 0.03$), where a shift towards almost total dominance of Th2-biased GA-reactive T cells was observed.

No consensus exists regarding the threshold ratios that define human T cells as Th1 or Th2. In addition to comparisons based on a potentially arbitrary classification into Th1-biased and Th2-biased T cell lines it is therefore also important to compare absolute ratios of pro- and anti-inflammatory cytokines produced by GA-reactive T cell lines from blood and CSF before and during GA treatment. Figure 2 depicts the secretion of IL-5, IL-10, and IL-13 compared with IFN-γ produced by GA-reactive T cell lines before and during treatment for patients 1–3. Results for the secretion of IL-5, IL-10, and IL-13 compared with IFN-γ and TNF-α in patients 1–3 combined are given in Figure 3. These results show that i) in T cells from CSF, GA treatment induced a highly significant increase in the secretion of IL-5, IL-10, and IL-13 compared with IFN-γ and TNF-α; ii) in T cells from blood, GA-treatment induced a highly significant increase in the secretion of IL-5, IL-10, and IL-13 compared with IFN-γ but not compared with TNF-α; iii) after 3–6 months on GA treatment, there is a prominent and significant Th2-polarization of GA-reactive T cells from both CSF and blood,

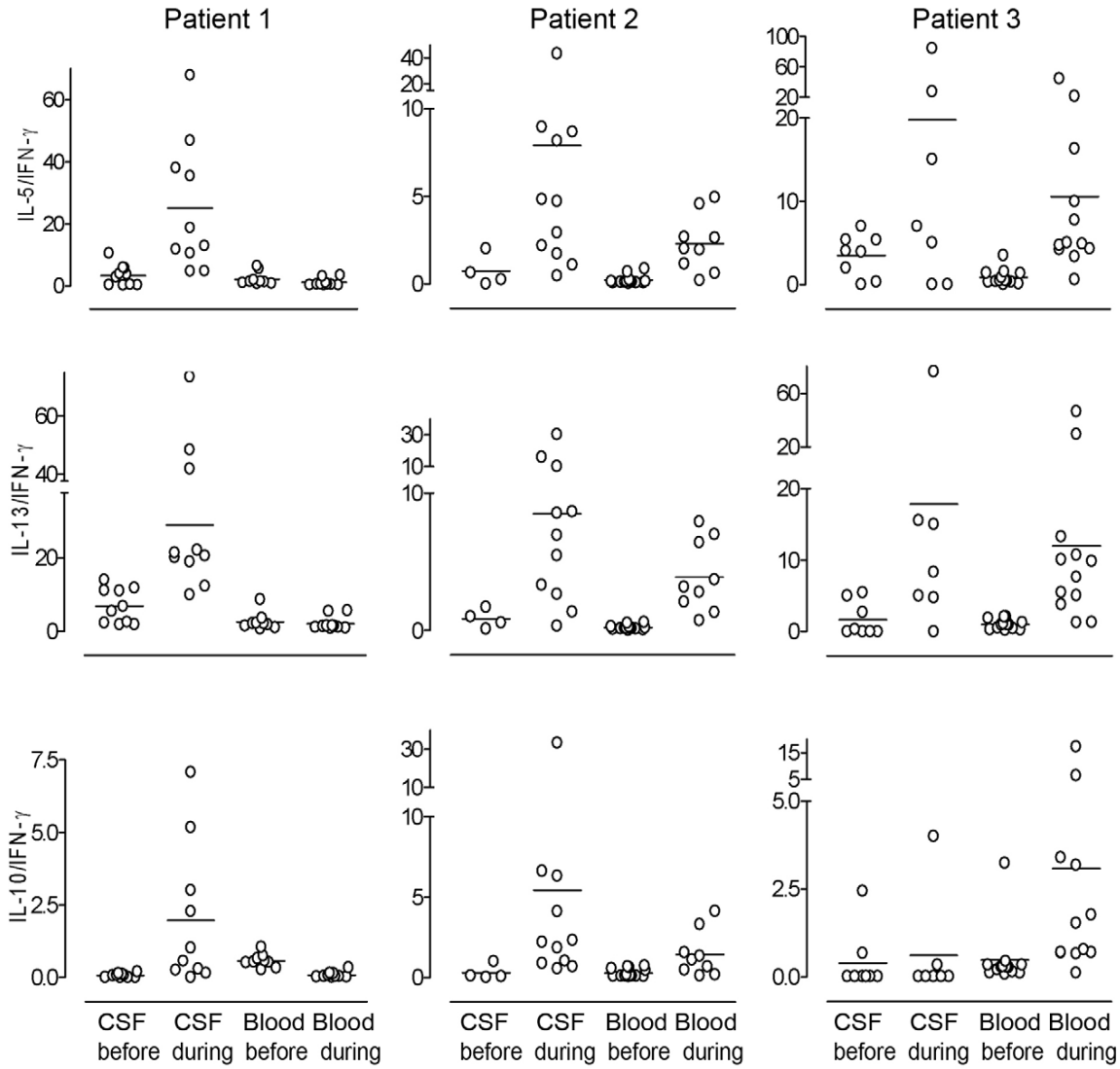

Figure 2 Short-term glatiramer acetate (GA) treatment induces a Th2-bias of GA-reactive T cells, which is most prominent in CSF. Each data point represents the indicated cytokine ratio for individual GA-reactive T cell lines before and after 3 months (patient 1) or 6 months (patient 2 and 3) of treatment.

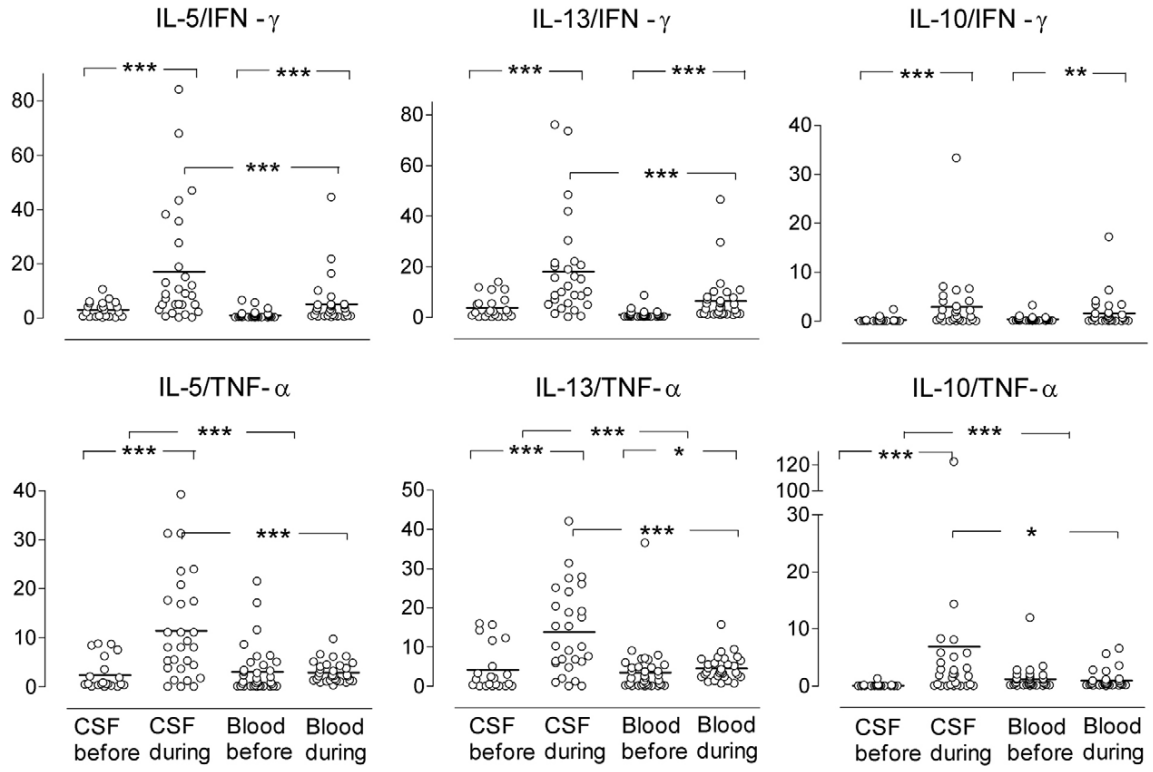

Figure 3 Glatiramer acetate (GA) treatment induces a pronounced and significant increase in the ratio between anti- and pro-inflammatory cytokines produced by GA-reactive T cells from CSF and blood. GA-reactive T cell lines were established from CSF and blood of patients 1–3 before and 3–6 months after initiation of GA treatment. Each data point represents the indicated cytokine ratio for one GA-reactive T cell line 48 h after GA stimulation. Lines represent mean ratios. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. The absolute cytokine concentrations (pg/mL, mean ± SD) in all cell lines (CSF and blood, before and after combined) were 2339, 3045 (interferon [IFN-γ]), 1981, 2703 (tumor necrosis factor [TNF-α]), 3024, 4209 (IL-5), 1188, 1385 (IL-10), and 3680, 3916 (IL-13). The mean absolute value for unstimulated cells, all cytokines combined was 15 pg/mL, SD = 31 pg/mL.

which is more pronounced in T cells from CSF. The Th2-polarization of GA-reactive T cells was evident in each of the three patients.

IL-17 has recently been identified as an important pro-inflammatory cytokine [26]. In CSF, IL-17 production was detected in 5 of 21 GA-reactive T cell lines during GA treatment compared with 10 of 28 before treatment. In blood, IL-17 production was detected in 11 of 31 GA-reactive T cell lines during GA treatment compared with 18 of 37 before treatment. These differences were not significant (results not shown).

Patients 1–3 had only received GA for 3–6 months. To assess the impact of prolonged treatment, we established 10 GA-reactive T cell lines from CSF and 23 GA-reactive T cell lines from blood obtained from two patients who had received GA for 24 and 36 months (patients 4 and 5). Interestingly, GA-reactive T cell lines from both CSF and blood of these patients displayed a significantly stronger Th2-bias than GA-reactive T cells from the short-term treated patients (Figure 4a). Although based on few patients, these results suggest that long-term treatment further enhances the Th2-polarization of GA-reactive T cells, which is most pronounced in CSF.

Combining results for all GA-reactive T cell lines generated after initiation of GA treatment from patients 1–5 further confirms that GA-reactive T cells from CSF display a more pronounced anti-inflammatory profile than GA-reactive T cells from the blood (Figure 4b). In sum, the mean IL-5/IFN-γ ratio of GA-reactive T cells from CSF was 35.8 (95% CI: 20.4–51.6), compared with 9.6 (95% CI: 6.3–12.8) for GA-reactive T cells from blood ($P < 0.005$).

GA-specific T cells from CSF are restricted by either HLA-DR or HLA-DP

To investigate the characteristics of individual GA-specific T cells, we cloned GA-reactive T cell lines

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