

Glatiramer Acetate-Specific Antibody Titres in Patients with Relapsing/Remitting Multiple Sclerosis and in Experimental Autoimmune Encephalomyelitis

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Abstract

Glatiramer acetate (GA) is an immunomodulatory drug approved for the treatment of clinically isolated syndrome (CIS) and relapsing/remitting multiple sclerosis (RRMS). As an antigen-based therapy, GA induces GA-specific antibodies in treated patients and animals. GA-specific antibodies do not neutralize therapeutic effects on relapses and disability. Rather, it has been suggested that GA-specific antibodies may be associated with improved clinical outcomes. We evaluated antibody responses in eight patients with RRMS treated with GA for 15 months and antibody responses in GA-treated C57BL/6 mice before and after induction of experimental autoimmune encephalomyelitis (EAE). There were no significant differences from pretreatment levels of total IgE or GA-specific IgE in patients with RRMS. Total IgG1, IgG3 and GA-specific IgG4 were significantly increased at 15 months of GA treatment. Antibody type and titre were not associated with clinical outcomes, i.e. expanded disability status scale (EDSS) score, disease burden on magnetic resonance images (MRI) or clinical relapses. In contrast, mice with EAE showed a marked increase in GA-specific IgE and GA-specific IgG1 antibody responses. GA-treated mice demonstrated improved clinical symptoms and lower mortality than untreated controls. Our results suggest that antibody responses to GA are heterogeneous among patients with RRMS, with no apparent association between antibody response and clinical outcomes. Clinical improvements in EAE-induced GA-treated mice suggest that GA-specific IgE and IgG1 may contribute to GA treatment effects in EAE.

Introduction

Multiple sclerosis (MS) is an inflammatory neurological disease of the central nervous system (CNS) that leads to a cascade of events ultimately resulting in myelin destruction and neuronal damage [1–3]. Glatiramer acetate (GA, Copaxone; Teva Pharmaceuticals, Petach Tikva, Israel) is an immunomodulatory drug approved for the treatment of relapsing/remitting MS (RRMS) and clinically isolated syndrome (CIS). GA is a random copolymer of synthetic polypeptides composed of the four amino acids, L-glutamic acid, L-lysine, L-alanine and L-tyrosine, in a defined molar ratio [4, 5]. GA was synthesized to simulate myelin basic protein (MBP), a major constituent of myelin in the CNS, which acts as an autoantigen in patients with MS. GA was originally created to induce

experimental autoimmune encephalomyelitis (EAE), the animal model of MS. However, GA unexpectedly inhibited EAE in rodents and monkeys [4, 5], and subsequently, clinical trials demonstrated that GA treatment was associated with reduced relapse rate and progression of disability in patients with RRMS [6–8].

Antibodies against GA can be detected in most treated MS patients and animals [9–11]. GA-reactive antibodies do not neutralize the effectiveness or compromise the safety of GA therapy in human patients [9, 10] and may enhance remyelination in an EAE model [11]. There has been little examination of whether the presence of GA antibodies correlates with GA clinical efficacy in patients with RRMS or with clinical outcomes in mice with EAE. An analysis by Brenner *et al.* [10] did indicate that GA-treated MS patients with high GA-reactive

antibody titres were more likely to be relapse free at 18 and 24 months than patients with lower GA-reactive antibody titres. However, whether GA-reactive antibodies are correlated with clinical outcomes has not been formally validated.

Glatiramer acetate is thought to primarily affect MS pathophysiology by inducing T helper type 2 (Th2) cells, which typically produce anti-inflammatory cytokines, such as IL-4 and IL-10 [12–17]. Anti-inflammatory cytokines regulate isotype switching to IgG4 and IgE in plasma cells. In this study, we investigated circulating levels of immunoglobulin isotypes, namely IgG1, IgG2, IgG3, IgG4 and IgE, in sera of patients and mice treated with GA, and whether a correlation exists between levels of these isotypes and clinical symptoms in patients with RRMS and in mice with EAE.

Materials and methods

Patients. This study included a subgroup ($n = 8$) of patients who had participated in a study comparing the effects of mitoxantrone induction followed by GA maintenance to effects of GA monotherapy that was conducted at the Barrow Neurological Institute (BNI) (<http://www.clinicaltrials.gov> identifier NCT00203086). Study design and conduct of the NC-100 trial are reported elsewhere [18]. Briefly, in the trial, patients were randomized to receive either 3 monthly infusions of mitoxantrone followed by daily GA (20 mg subcutaneous) therapy for 12 months or GA monotherapy for 15 months. The subgroup of patients in the current analysis included only patients who were randomized to receive GA for 15 months. Participants had RRMS as defined by the revised (2005) McDonald criteria [19]. Blood samples were collected pretreatment (Month 0) and every 3 months after initiation of GA treatment for the duration of the study. Patients underwent standard testing for expanded disability status scale (EDSS) score every 3 months, matching the times of blood draws. Magnetic resonance images (MRI) of the brain were obtained at entry into the study and were repeated at 1 year (MRI parameters are described in detail elsewhere) [20].

The protocol was approved by the BNI Human Research and Ethics Committee, and informed consent was obtained from all patients before participating in the study.

C57BL/6 mice. C57BL/6 mice were purchased from Taconic (Hudson, NY, USA). A total of 12 female mice (6–8 weeks old) were used, six each in two identical studies. In each study, wild-type C57BL/6 mice were injected with GA (150 μ g dissolved in PBS ($n = 6$)) or PBS alone (control, $n = 6$) for 7 consecutive days. On day 8, EAE was induced in all mice with MOG_{35–55} peptide (200 μ g/ml) and pertussis toxin (400 ng/ml). Serum samples were analysed for GA-specific IgE and IgG isotypes by ELISA.

All mice were housed in the BNI animal facility, and the study was approved by the BNI Animal Care and Use Committee and performed in compliance with guidelines of the BNI Committee on Animal Research and the Committee's approved protocol (A3519-01). Care was taken to minimize any discomfort of the mice. Food and water were accessible to the mice at all times.

Antibodies and reagents. Purified monoclonal mouse anti-human IgG and horseradish peroxidase (HRP)-conjugated IgG₁, IgG₂, IgG₃ and IgG₄ were purchased from Zymed Laboratories (San Francisco, CA, USA). Purified anti-human IgE, biotin anti-human IgE, streptavidin–HRP, and enzyme-linked immunosorbent assay (ELISA) plates were all purchased from BD Biosciences (San Jose, CA, USA). Ortho-phenylenediamine (OPD) was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). GA was obtained from Teva Pharmaceutical Industries, Petach Tikva, Israel.

Antibodies for mouse IgG ELISA analyses were purchased from Southern Biotech Associates (Birmingham, AL, USA). Mouse IgE ELISA was performed using an IgE ELISA kit (BD Biosciences).

ELISA for human IgG isotypes. ELISA for total IgG and GA-specific antibody isotypes was performed on serum samples from eight patients with RRMS. The micro wells were coated overnight at 4 °C with monoclonal mouse anti-human IgG or 20 μ g/ml GA. Patients' serum sample dilutions used for each isotype were as follows: total IgG₁ 1:640,000; total IgG₂ and GA-specific IgG₁ 1:320,000; total IgG₃ 1:160,000; total IgG₄ and GA-specific IgG₄ 1:2500; and GA-specific IgG₂ and IgG₃ 1:40,000. Plates were incubated for 1 h with HRP-conjugated monoclonal mouse anti-human IgG₁, IgG₂, IgG₃ and IgG₄ to detect the IgG isotype in the serum. Immune complexes were visualized colorimetrically after exposure to OPD and measured by reading the plate at 490 nm.

ELISA for human IgE. For the IgE antibody assay, mouse anti-human IgE was used to capture antibody. Patients' sera were used at 1:10 dilution and detected with biotin anti-human IgE followed by avidin–HRP. Immune complexes were visualized colorimetrically after exposure to OPD and measured at 490 nm.

Mouse IgG and IgE ELISA. The mouse IgG ELISA was performed according to the manufacturer's instructions (Southern Biotech) with mouse sera diluted at 1:400. The mouse IgE ELISA was performed using the kit according to the manufacturer's instructions (BD Biosciences) with mouse sera diluted at 1:10. IgG and IgE ELISA tests were performed in duplicate. For GA-specific antibody isotype detection, ELISA plates were coated with 20 μ g/ml GA overnight followed by addition of sera at a 1:400 dilution. The specific immunoglobulin isotype was then detected using IgE, IgG1, IgG2a and IgG2b conjugated to HRP. Immune complexes were

visualized colorimetrically after exposure to OPD and measured at 490 nm.

Statistical analysis. Data were compared at the different sampling time points by ANOVA using GraphPad Prism 5 (GraphPad, San Diego, CA, USA). *P* values of <0.05 were considered statistically significant.

Results

Patient demographic characteristics are shown in Table 1. All eight patients were compliant with GA treatment. One patient (ID112) was missing one blood sample at month 15, but the patient was evaluated by EDSS.

Patients with RRMS

No association was detected between RRMS disease activity and antibody titres. Mean EDSS score at baseline was 2.72, and no significant change in mean EDSS score from baseline was detected at the end of the study. Two of the eight patients (ID106 and ID120) had very active disease (3 relapses each) and in the case of patient ID120, the EDSS score increased from 3 (at baseline) to 5.5 (15 months post-GA treatment). In accordance with the clinical course, brain MRI in the same two patients (ID106 and ID120) showed new brain lesions and new areas of gadolinium enhancement at the 1-year scan (the other six patients did not). Changes in EDSS score and number of relapses for each patient are shown in Table 1. Two patients had 3 relapses over the course of the 15-month study; one patient (106) had a slightly improved EDSS score at 15 months from baseline score, the other (120) progressed from EDSS 3 to 5.5 (Table 1). The latter patient may have been evaluated too soon after an unresolved relapse.

As shown in Figs. 1–3, antibody responses among patients were heterogeneous, regardless of whether EDSS

scores were unchanged or reduced (ID 103, 106, 112, 119, 102, 108 and 116), or whether the EDSS score increased (ID 120).

There was no significant increase in the overall total IgE or in GA-specific IgE from pretreatment (Month 0) levels for these eight patients over the 15-month period. However, one patient showed an increase from baseline in total IgE at 3 months that persisted afterwards (ID108) (Fig. 1A). An increase in the induction of GA-specific IgE responses over the course of GA treatment was not significant: three of eight patients showed elevated levels of GA-specific IgE at 3 months compared with month 0 (Fig. 1B), but two of these three patients' GA-specific IgE levels returned to baseline levels by month 15, suggesting that GA-specific IgE production is variable. Patients with increased levels of GA-specific IgE did not exhibit notable changes in EDSS scores, MRI lesions or relapse rates (Table 1), indicating a lack of correlation between IgE levels and clinical outcomes in these patients with RRMS. No hypersensitivity reactions were reported in these patients.

Total IgG1, IgG3 and IgG4 increased over the course of GA treatment. Compared with pretreatment levels, patients' sera demonstrated a significant ($P < 0.01$) increase in total IgG1 antibody after 15 months of GA treatment (Fig. 2A), while there was no change in the level of total IgG2 isotype (Fig. 2B). In addition, total IgG3 isotype antibody levels were significantly ($P < 0.01$) increased at month 6 and remained high through the rest of the treatment (Fig. 2C). Total IgG4 levels showed no differences throughout the study period (Fig. 2D).

Mean levels of GA-specific IgG1, IgG2 and IgG3 did not increase over the course of GA treatment (Fig. 3A–C), whereas there was a significant ($P < 0.01$) increase at months 12 and 15 in levels of GA-specific IgG4 (Fig. 3D). The increase in GA-specific IgG4 (a Th2 type

Table 1 Patient characteristics, clinical outcomes, and changes in antibody levels from baseline to 15 months of GA treatment.

Patient ID	Age	Gender	Ethnicity	EDSS at entry	EDSS at month 15	Relapses during the study period	GA-reactive IgG level changes between Month 0 and Month 15 of GA treatment				
							IgG1 ^b	IgG2 ^b	IgG3 ^b	IgG4 ^a	IgE ^b
102	57	M	Hispanic	6	6	0	NC	NC	NC	NC	–
103	37	M	Caucasian	2	1.5	0	NC	NC	NC	NC	NC
106	34	F	Hispanic	2	1.5	3	NC	NC	NC	NC	–
108	47	F	Caucasian	4	4	0	NC	NC	NC	NC	+
112	59	F	Caucasian	3	2	0	(No 15-month sample available)				
116	49	M	Caucasian	1.5	1.5	0	NC	NC	NC	+	+
119	26	M	Caucasian	1.5	1	0	NC	NC	NC	+	–
120	39	F	Caucasian	3	5.5	3	NC	NC	NC	NC	–

EDSS, expanded disability status scale; GA, glatiramer acetate; NC, no change from month 0; +, increase from month 0; –, decrease from month 0.

^aMean change for all patients at 15 months was statistically significant ($P < 0.01$).

^bMean change for all patients at 15 months was not statistically significant.

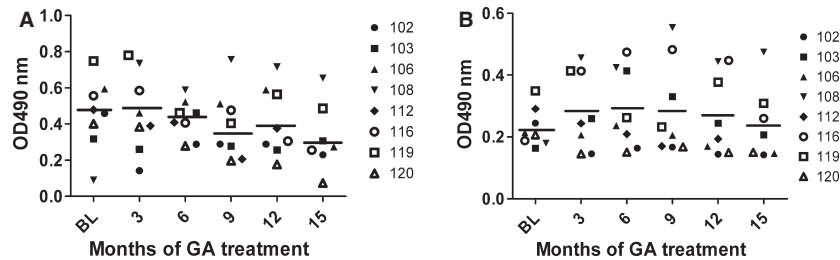


Figure 1 Sera of patients with relapsing/remitting multiple sclerosis (RRMS) treated with glatiramer acetate (GA) do not show significant increases in IgE levels. (A) Total IgE in sera over the course of GA treatment. (B) GA-specific IgE in sera over the course of GA treatment. [Horizontal lines represent mean values; BL = baseline (pretreatment)].

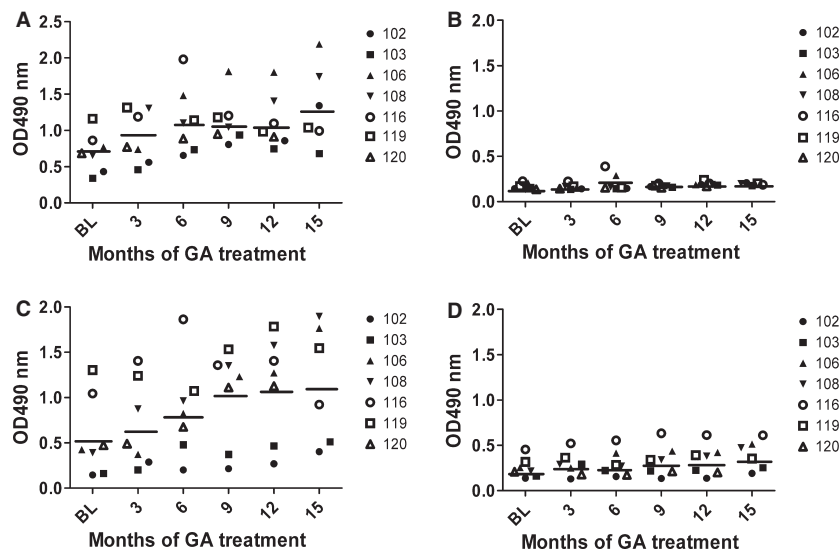


Figure 2 Sera of patients with relapsing/remitting multiple sclerosis (RRMS) treated with glatiramer acetate (GA) show significant increases in total IgG1 and IgG3 isotypes. (A) Mean total IgG₁ titres over the course of GA treatment were significantly increased at 6 months, and levels were maintained until the end of the treatment. (B) Mean total IgG₂ levels were not changed over the course of GA treatment. (C) Mean total IgG₃ antibody titres were significantly increased at 6 months with gradual increases to the end of GA treatment. (D) Total IgG₄ antibody levels were not significantly changed over the course of GA treatment. [Horizontal lines represent mean values; BL = baseline (pretreatment)] $P < 0.01$ for mean change from pretreatment to months 12 and 15 in total IgG₁ and total IgG₃ levels in GA-treated patients.

antibody that favours anti-inflammatory response) did not independently correlate with clinical outcomes in these patients (Table 1).

Mice with EAE

In the sera of C57BL/6 mice, there was a significant ($P < 0.005$) increase in both total IgE and GA-specific IgE levels (Fig. 4) in GA-treated mice ($N = 6$) compared with sera of untreated mice ($N = 6$). Results of the duplicate assay were consistent with these (data not shown).

GA-specific IgG₁ responses were significantly increased after GA treatment in three of six naïve and three of six mice induced with EAE (Fig. 5A, B). Levels of IgG_{2a} isotype antibodies were unchanged (Fig. 5C, D). The level of IgG_{2b} isotype, a Th1 type of antibody,

remained the same for the wild-type and the GA-treated mice, with and without EAE (Fig. 5E, F). Results were confirmed by duplicate testing (data not shown). These data suggest that GA induced a Th2 type of antibody response in this mouse model.

Increases in GA-specific IgE and IgG₁ antibodies were associated with low clinical scores and reduced mortality in the GA-treated mice with EAE (Table 2).

Discussion

Glatiramer acetate is partially effective in the treatment of MS, protective in EAE, and is known to induce GA-specific antibodies [4, 5, 7–11]. GA-specific antibodies do not neutralize the therapeutic effects of GA on MS or EAE [9, 10]; however, whether GA-specific antibodies correlate with clinical outcomes of MS or EAE continues

Figure 3 Sera of patients with relapsing/remitting multiple sclerosis (RRMS) treated with glatiramer acetate (GA) show significant increase in GA-specific IgG₄. Mean GA-specific IgG₁ (A), IgG₂ (B) and IgG₃ (C) antibody levels did not change significantly during GA treatment. Levels of GA-specific IgG₄ antibodies (D) were significantly increased at 12 and 15 months of GA treatment. [Horizontal lines represent mean values; BL = baseline (pretreatment)] $P < 0.01$ for mean change from pretreatment to 12 and 15 months in GA-specific IgG₄ levels in GA-treated patients.

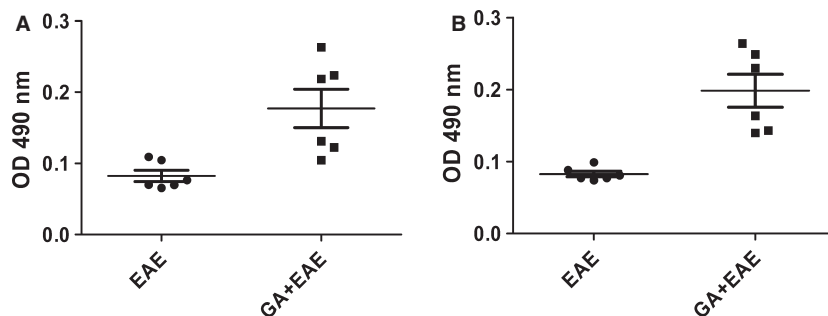
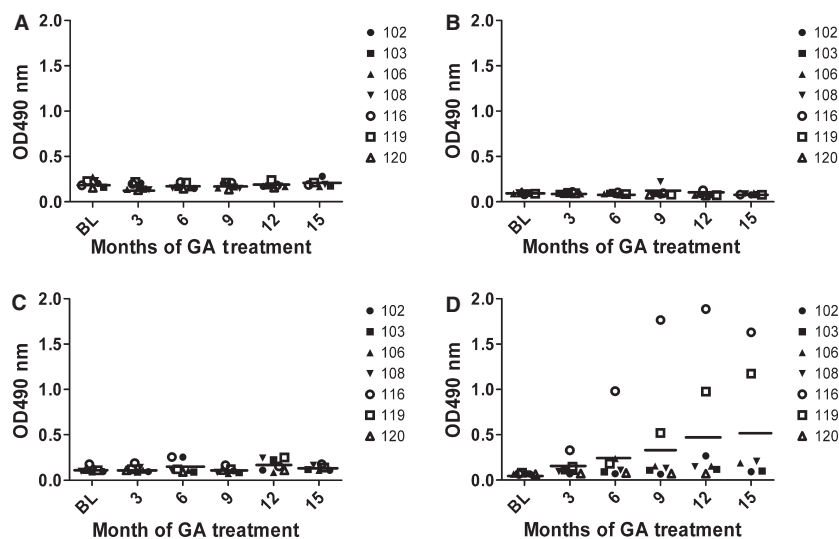


Figure 4 Sera of C57BL/6 mice treated with glatiramer acetate (GA) show increases in GA-specific IgE. There was a significant difference in mean levels of (A) total IgE and of (B) GA-specific IgE antibodies in GA-treated mice compared with untreated mice. These data represent results of one of two experiments that gave similar results; number of animals used for each experiment was 6. [Scatter plot shows mean and $\pm$ SEM (bold lines) values.] $P < 0.005$ for mean change in GA-specific IgE levels in GA-treated mice compared with untreated mice.

to be a subject of investigation. Our study did not show significant increases in mean levels of human GA-specific IgE, IgG₁, IgG₂ or IgG₃ antibodies. Results of larger studies by other groups indicate that GA-reactive IgG₁ antibody levels are predominant during early treatment [10, 21], a trend that was not apparent in the current study. Although speculative, this discrepancy may be due to differences in experimental conditions or the monoclonal antibodies employed, or to the small patient sample in this study and the heterogeneity of the human immune system.

In contrast, GA-specific human antibodies of the IgG₄ isotype were significantly increased at month 15 following continuous GA therapy, which is consistent with results of studies by Bar-Or *et al.* [22], Farina *et al.* [23] and Basile *et al.* [21]. Changes in antibody levels during GA therapy may be the result of treatment-related factors, for example changes in cytokine secretion or cell phenotypes. Of the four isotypes of human IgG, IgG₄ is the least abundant in the serum of healthy individuals,

comprising 1% to 4% of total IgG. IgG₄ antibodies do not activate the complement pathway and have lower affinity for phagocyte membrane receptors, making it less likely for these immunoglobulins to have pro-inflammatory effects [24]. It is conceivable that these antibodies have no significant impact on the therapeutic effects of GA in treated patients with MS. In this study, we found no significant correlation between antibody titres and clinical outcomes, although again, the small patient numbers and the heterogeneity of the MS disease course and of the human immune system may have obscured a relationship between them.

On the other hand, differences were noted when we compared our data in RRMS patients with our findings in mice with EAE, an animal model for MS. Mouse IgE and IgG₁ isotypes are among the Th2 type of antibodies and have a low capability of activating the complement pathway [25]. In mice with EAE, GA treatment produced elevated levels of both GA-specific IgE and IgG₁. The increase in GA-specific IgG₁ antibodies is consistent

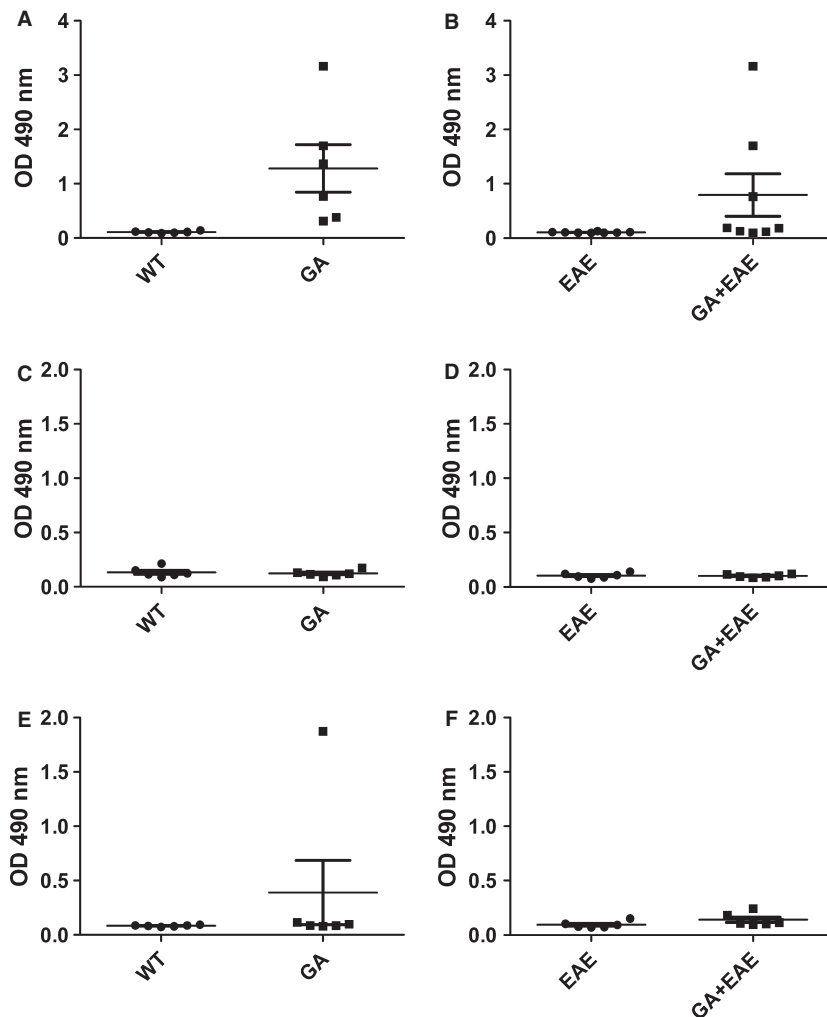


Figure 5 Sera of C57BL/6 mice treated with glatiramer acetate (GA) with or without experimental autoimmune encephalomyelitis (EAE) show significant increases in GA-specific IgG₁. (A) represents GA-specific IgG₁ levels in C57BL/6 mice with and without GA treatment. There was significant increase in GA-specific IgG₁ levels after GA treatment. (B) represents GA-specific IgG₁ levels in GA-treated and untreated mice after induction of EAE. There was significant increase in GA-specific IgG₁ levels in GA-treated mice with EAE. (C) represents GA-specific IgG_{2a} levels in C57BL/6 mice with and without GA treatment. The plot shows no difference in levels of GA-specific IgG_{2a} antibodies either with or without GA treatment. (D) represents GA-specific IgG_{2a} levels in GA-treated and untreated mice after EAE induction. The plot shows no change in levels of GA-specific IgG_{2a} antibodies when treated or not treated with GA. (E) represents GA-specific IgG_{2b} levels in C57BL/6 mice with and without GA treatment. The plot shows no change in levels of these antibodies. (F) represents GA-specific IgG_{2b} levels in GA-treated and untreated mice after EAE induction. There was no difference between GA-specific IgG_{2b} antibodies whether treated with GA or untreated. The plot shows mean and $\pm$ SEM (bold lines) values. $P < 0.01$ for mean change in mean GA-specific IgG₁ levels in mice, with or without EAE, treated with GA versus untreated mice.

Table 2 Clinical scores and antibody profiles of GA-treated and untreated mice with EAE.

	Incidence	Mortality	Days of onset	Mean clinical score
EAE ^a (mean)	100%	20%	9	3.24
GA + EAE	40%	0%	9	1.5

Clinical scores and antibody profiles in individual untreated and GA-treated mice with EAE

Untreated EAE mice						GA-treated EAE mice				
Mice	Clinical score	Antibody levels				Clinical score	Antibody levels			
		IgG1	IgG2a	IgG2b	IgE		IgG1	IgG2a	IgG2b	IgE
1	3.5	0.127	0.14	0.075	0.066	2	0.128	0.102	0.106	0.218
2	3	0.10	0.076	0.070	0.070	2	0.117	0.085	0.182	0.131
3	3	0.099	0.120	0.093	0.07	1.5	0.187	0.120	0.1	0.1045
4	3.5	0.111	0.107	0.15	0.109	1	3.163	0.090	0.242	0.122
5	3.5	0.106	0.096	0.072	0.077	1	1.700	0.12	0.114	0.263
6	3	0.096	0.088	0.08	0.104	2	0.102	0.096	0.087	0.224

EAE, experimental autoimmune encephalomyelitis; GA, glatiramer acetate.

^aWild type C57BL/6 mice; EAE induced with MOG₃₅₋₅₅ peptide (200 μ g/ml) and pertussis toxin (400 ng/ml).

with other data, indicating that they were elevated in GA-treated mice and seemed to promote myelin repair in the mouse model [11]. Although our data do not provide direct evidence of the consequences of GA-specific IgE and IgG₁ antibodies in the animal model, antibody-mediated myelin repair may be a mechanism for reduced demyelination in GA-treated mice. However, this hypothesis should be tested in a large animal model of EAE (e.g. non-human primates or swine), because their immune systems are more closely related to the human immune system [26].

Extensive data on GA mechanisms of action provide evidence of induction of GA-specific T cells with a Th2 phenotype [16, 27–29]. Whether GA-reactive Th2 cells promote the production of anti-myelin antibodies of certain IgG isotypes is not clear; nevertheless, our data suggest that GA treatment stimulates the production of GA-reactive IgG antibody subclasses that are primarily of the Th2 type (IgG₄ in humans and IgG₁ in mice). In earlier studies, our laboratory showed that GA-reactive T and B cells promote IL-10 secretion [27, 29]. IL-10 is known to be associated with the isotype switching of IgG to IgG₄ and IgE in plasma cells, as well as differentially regulating IgE versus IgG₄ production [30]. Although human data in the present study demonstrate the heterogeneity of immune responses to GA, the mice data support the view that GA induces B cells to secrete antibody isotypes with a type 2 immune profile [28].

In summary, our study provides evidence that GA-treated patients develop detectable IgG₄ antibodies to GA by 15 months after having started GA treatment. However, titres are variable among patients, and it is unclear whether antibodies have an impact on the clinical effects of GA. In contrast, the immunoglobulin responses to GA in mice with or without EAE were more robust compared with responses in untreated mice. Levels of both GA-specific IgE and GA-specific IgG₁ were significantly elevated in GA-treated mice compared with levels in control mice. Furthermore, clinical symptoms and mortality were improved in EAE-induced GA-treated mice compared with control animals. Results from the mouse model suggest that GA-specific IgE and IgG₁ could have important functional activities. Further research with larger sample sizes – patients with RRMS and animals with EAE – may elucidate the potential contribution of GA-reactive antibodies to the treatment effect of this medication.

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