J Neurol (2002) 249 : 1587–1592
DOI 10.1007/s00415-002-0904-0

ORIGINAL COMMUNICATION

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Immunological assay for assessing the efficacy of glatiramer acetate (Copaxone) in multiple sclerosis

A pilot study

Abstract Recently we described an enzyme-linked immunoadsorbent spot (ELISPOT) assay allowing us to define an immunological response profile observed in multiple sclerosis patients treated with Copaxone (glatiramer acetate; GA) but not untreated subjects [4]. The profile encompasses three criteria, a) reduced proliferative response to GA (as observed with a standard primary proliferation assay); b) strong in vitro activation of interferon-γ-producing T cells at high concentrations of GA (as detected by interferon-γ ELISPOT); and c) activation of interleukin–4-producing T cells over a wider range of concentrations of GA (as detected by interleukin–4 ELISPOT). It is at present unknown whether the *immunological* response to GA correlates with the *clinical* response. To address this question we performed the pilot study reported here. We asked the major German multiple sclerosis centres to send us blood samples from all GA-treated patients who were going to discontinue treatment because of treatment failure. The clinical nonresponders either had an unchanged or increased exacerbation rate, or developed a secondary progressive course during GA treatment. Over more than one year, we prospectively collected 9 samples from clinical non-responders. We compared the immune response to GA of peripheral blood mononuclear cells from the 9 clinical nonresponders with 15 clinical responders, using a standard proliferation assay combined with ELISPOT assays for detection of interferon-γ and interleukin–4 secreting cells. Thirteen (86 %) of the 15 clinical responders met at least 2 of the immunological response criteria mentioned above. In contrast, only 2 (22 %) of the 9 clinical nonresponders met two of the immunological criteria ($p = 0.0006$). We conclude that the ELISPOT assay may provide a promising additional tool for monitoring the treatment response in multiple sclerosis patients treated with GA.

Key words multiple sclerosis · glatiramer acetate · elispot assay

Received: 31 January 2002
Received in revised form: 27 June 2002
Accepted: 8 July 2002

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Introduction

Glatiramer acetate (GA, Copolymer–1, Cop–1, Copaxone) inhibits experimental allergic encephalomyelitis (EAE) and has therapeutic effects in multiple sclerosis (MS) (reviewed in refs. [1, 2, 7, 8, 10, 13–16]). In MS patients, GA is given by daily s. c. injection of 20 mg. Observations in EAE and MS suggest that GA induces a population of T-helper (TH)-2 cells which can enter the central nervous system (CNS) [3, 6, 11, 12]. It is thought that the GA-reactive regulatory T cells are (re-)activated in the CNS by cross-reacting myelin antigens presented by local antigen-presenting cells [10, 11]. Consistent with this hypothesis, analysis of the cytokine production profile of short-term and long-term T-cell lines has recently shown that GA-reactive T cell lines from GA-treated patients are preferentially TH2-type, whereas

GA-specific T cell lines from untreated patients and healthy controls are predominantly TH1 [11]. Furthermore, GA-treated patients develop anti-GA antibodies of the IgG4 subclass [5], which are regulated by TH2 cells.

Recently we described an enzyme-linked immunoadsorbant spot (ELISPOT) assay for the ex-vivo detection of cytokine-producing T-lymphocytes in blood of multiple sclerosis patients treated with GA [4]. This allowed us to define a characteristic immunological response to GA observed in GA-treated patients but not in untreated control subjects [4]. However, this initial study [4] did not address the question whether the *immunological* response profile, as defined in our paper, correlates with the *clinical* response to GA. Obviously, formal assessment of this possibility would require a prospective trial in a large number of patients. Presently, no such data are available. However, as a first step in this direction, we performed a small pilot study. Over the course of more than one year, we prospectively collected blood samples from clinical non-responders and compared their immunological response profile to the response of clinical responders.

Patients and methods

Patients

We contacted the major German MS centres and asked them to send us fresh blood samples from all patients who were identified as clinical non-responders and therefore advised to discontinue treatment. In each patient, the decision to stop treatment was made purely *on clinical grounds*, independently of the results of laboratory assays, and before the ELISPOT results were available. The criteria for determining that a patient was a clinical non-responder were a) unchanged or increased exacerbation rate, or b) development of a secondary progressive course. Blood samples were obtained with informed consent before GA treatment was stopped. Blood samples from a control group of clinical responders were obtained in the same way during routine outpatient visits.

Over the course of more than one year, we received nine consecutive samples from clinical non-responders. All samples were analysed, and none was excluded. The group of non-responders included 2 men and 7 women (mean age = 37.5 ± 9.1 years; mean EDSS = 3.7 ± 2, mean GA treatment duration = 22.5 ± 12.6 months) (Table 1). These patients had an unchanged or increased exacerbation rate, or developed a secondary progressive course (in Germany patients with secondary progressive MS with or without additional exacerbations are not treated with GA). The control group consisted of 15 patients (14 from our initial study [4]) (4 men and 11 women; mean age = 34.9 ± 7.9 years, mean EDSS = 2.9 ± 1.7), who had been treated with GA for at least one year (mean duration = 32.4 months ± 13.7) and were classified as clinical responders because they had a reduced relapse rate compared with the pre-treatment period. None of the patients was treated with immunosopressive agents during at least the last three weeks prior to the assay.

Table 1 Overview of the patients investigated in the study.

	Name	Gender	Age	EDSS	GA treatment (Months)	
Clinical responders	R1	F	41	3.5	44	
	R2	M	47	6.5	42	
	R3	F	33	1.5	39	
	R4	F	25	2	45	
	R5	F	27	2	38	
	R6	F	43	6	43	
	R7	M	33	2	42	
	R8	F	39	2	40	
	R9	F	31	2	43	
	R10	F	47	5.5	39	
	R11	M	22	1	25	
	R12	F	42	1	13	
	R13	F	29	2.5	12	
	R14	M	29	4	12	
	R15	F	36	2.5	10	
						Discontinuation due to
Clinical non-responders	NR1	F	31	0.5	12	Increased relapse rate
	NR2	F	40	4	16	Progression
	NR2	F	44	6	18	Progression
	NR4	M	40	4	16	Progression
	NR5	F	31	1	24	Increased relapse rate, Flush symdrome
	NR6	M	53	4.5	24	Progression
	NR7	F	21	2.5	30	Progression
	NR8	F	36	6	11	Progression
	NR9	F	42	5	52	Increased relapse rate and progression

Preparation of PBMC

Blood samples were shipped by express courier and processed within 24 hours. Peripheral blood mononuclear cells (PBMC) were isolated on a discontinuous density gradient (Lymphoprep, Nycomed, Oslo, Norway). Viable cells were counted with Trypan Blue (Sigma-Aldrich, Deisenhofen, Germany) and resuspended in culture medium (RPMI 1640 supplemented with 5 % FCS, 1 % glutamine and 1 % penicillin/streptomycin; Gibco, Karlsruhe, Germany). The same batch of FCS was used throughout the study.

Proliferation assay

PBMC (1×10^5 cells/well) were cultured in 96-well microtitre plates for 5 days in the presence of one of the following antigens: glatiramer acetate (GA, 6.25, 12.5, 25, 50 and 100 µg/ml; batch 242992899, Teva Pharmaceutical Industries, Petah Tiqva, Israel), tuberculin purified protein (PPD, 20 µg/ml; Statens Serum Institut, Copenhagen, Denmark), and staphylococcal enterotoxin B (SEB, 1 µg/ml; Toxin technology, Sarasota, FL) as a positive control. [3H]Thymidine (0.2 µCi/well) was added during the last 18 h of culture. Cells were harvested and [3H]thymidine incorporation was measured using a direct β-counter (Matrix 9600, Packard, Frankfurt, Germany). All experiments were performed in triplicate. Standard deviations (SD) were below 20 % of the mean.

ELISPOT assays

The enzyme-linked immunoadsorbent spot (ELISPOT) assays were performed in parallel with the proliferation tests and analysed with an automated imaging system and appropriate computer software (KS ELISPOT automated image analysis system, Zeiss, Jena, Germany) [4]. As shown in Fig. 1, 96-well polyvinylidene difluoride plates (Millipore, Eschborn, Germany) were coated at 4°C overnight with 10 µg/ml capture antibody (anti-interferon-γ Ab clone 1-D1K; Mabtech, Nacka, Sweden; or with anti-interleukin-4 Ab clone MP4-25D2, Pharmingen, Hamburg, Germany). The plates were then washed and blocked with culture medium for 1h at 37°C. PBMC (2×10^5 cells/well for the interferon-γ and 4×10^5 for the interleukin-4 ELISPOT assay) were cultured for 18h at 37°C and 5 % CO_2. For each subject, quadruplicate wells were exposed to the same antigens used in the proliferation assay. After culture, the plates were washed and incubated first with 1 µg/ml biotinylated detector Ab (anti-interferon-γ Ab clone 7-B6-1 or anti-interleukin-4 Ab clone 12-1; Mabtech), then with 1:1000 Streptavidin-alkaline phosphatase (Mabtech), and finally with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, Sigma-Aldrich). The frequency of cytokine-producing, antigen-reactive cells was expressed as the difference between the mean number of spots after antigen stimulation and the mean background for each experiment. A value equal to zero was assigned to spot frequencies smaller than the mean background of the individual assay plus two standard deviations. All SDs were below 20 % of the mean.

Statistical analysis

The t-test for independent samples was used to compare the two patient groups. The Welch test was used if variances were significantly different. All p-values were two-sided and subjected to a significance level of 0.05.

Results

We compared the immune response of 9 clinical nonresponders with that of 15 clinical responders to GA treatment. Overall, 13/15 (86 %) clinical responders, but only 2/9 (22 %) clinical non-responders met at least two of the three immunological response criteria ($p = 0.0006$;

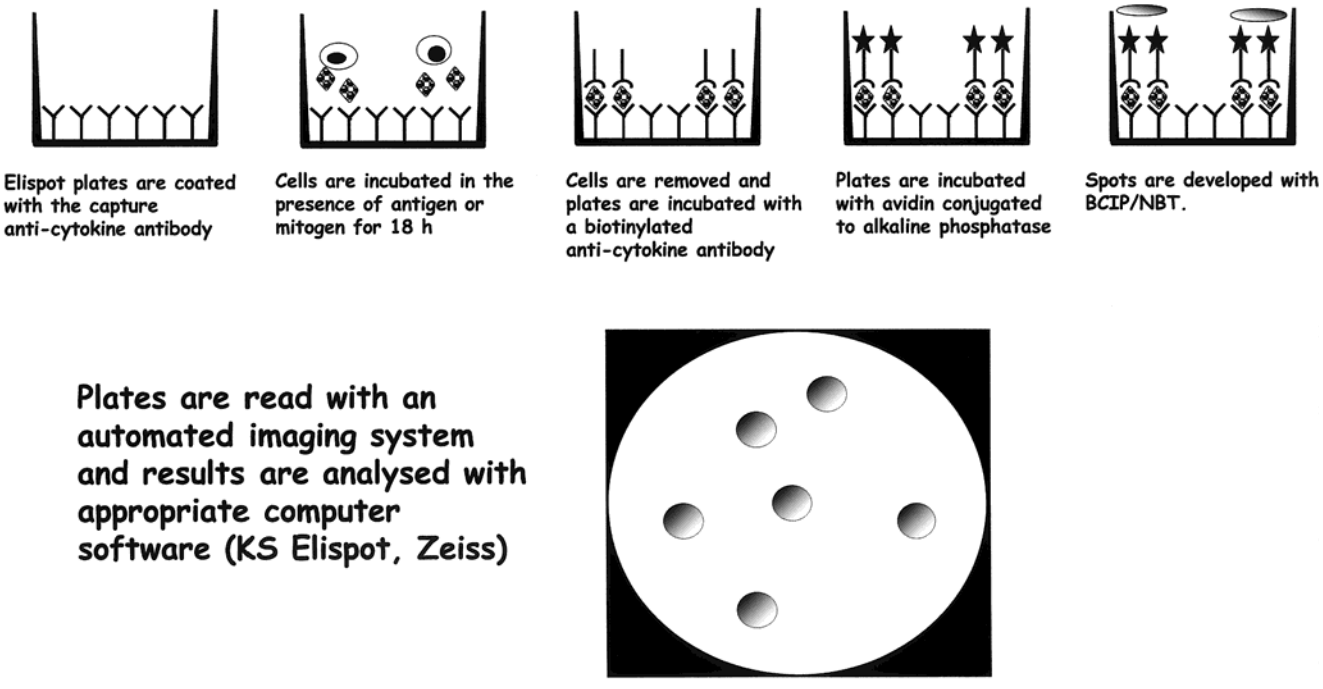

Fig. 1 Schematic overview of the ELISPOT assay used in the study.
Abbreviations: BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium

Table 2). The three criteria for a positive immunological response to GA were defined in our previous paper [4]. They comprise a) a reduction of GA-induced proliferation of PBMC (as compared to untreated normal subjects or MS patients [4]); b) activation of a strong GA-specific interferon-γ ELISPOT response at high in vitro concentrations of GA, and c) stimulation of specific interleukin–4 ELISPOT response over a wider range of GA concentrations [4].

With regard to proliferation, there was a trend towards higher GA-induced stimulation indices (SI) in the clinical non-responders, but the difference between responders and non-responders was not statistically significant (Fig. 2).

With regard to the ELISPOT results, only 2/9 clinical non-responders, but 13/15 clinical responders showed the typical, previously described, [4] dose response curve of the GA-stimulated interferon-γ ELISPOT response at increasing antigen concentrations (Fig. 3, upper panels) ($p = 0.019$ and 0.001 at 50 and 100 µg/ml GA, respectively). Furthermore, whereas 13/15 clinical responders showed a typical GA-specific interleukin–4 response, this was seen in only 2/9 clinical non-responders (Fig. 3, lower panels) ($p = 0.003, 0.001, 0.003, 0.011, 0.002$ at 6.25, 12.5, 25, 50 and 100 µg/ml GA).

PBMC from clinical responders and non-responders did not show any significant difference in the proliferative or ELISPOT responses when stimulated with the recall antigen tuberculin (data not shown).

Table 2 Comparison of the immunological response to GA in clinical responders and nonresponders.

		Test Results			Overall Classification
		SI < 2.5*	High IFN-g**	Positive IL-4***	Immunological Responder****
Clinical responders	R1	+	+	+	+
	R2	+	+	+	+
	R3	–	+	+	+
	R4	–	+	+	+
	R5	–	–	+	–
	R6	+	+	+	+
	R7	+	–	–	–
	R8	+	+	–	+
	R9	+	+	+	+
	R10	+	+	+	+
	R11	+	+	+	+
	R12	+	+	+	+
	R13	+	+	+	+
	R14	+	+	+	+
	R15	+	+	+	+
	NR1	+	–	+	+
	NR2	+	–	–	–
	NR3	+	–	–	–
	NR4	+	–	–	–
	NR5	–	+	+	+
	NR6	–	–	–	–
	NR7	–	–	–	–
	NR8	–	+	–	–
	NR9	–	–	–	–

R and NR indicate responder and non-responder, respectively
* at > 3 GA concentrations
** > 50 antigen-specific cytokine-producing cells x $200{,}000^{-1}$ at 100 µg/ml GA
*** at > 2 GA concentrations
**** classified positive if at least two tests are positive

Discussion

Like interferon-ß, glatiramer acetate (GA) is approved as a prophylactic long-term immunomodulatory treatment to ameliorate the course of multiple sclerosis [1, 2, 7–9, 12–15]. Usually, one year or more of treatment is necessary before it is possible to judge the clinical efficacy in individual patients. In patients who continue to have further exacerbations, the treatment is often continued for various lengths of time despite uncertainty about its clinical effectiveness. In patients who seem to respond to treatment, the apparent clinical response might simply reflect the natural disease course. For these and other reasons, any laboratory test that would help to distinguish between clinical responders and nonresponders would be most welcome.

In a previous paper [4] we described a simple ELISPOT test that defines an immunological response profile present in the vast majority of GA-treated patients but not in untreated patients or normal control subjects [4]. In the previous study [4] we compared the immunological response to GA in treated and untreated subjects. We did not, however, address the question

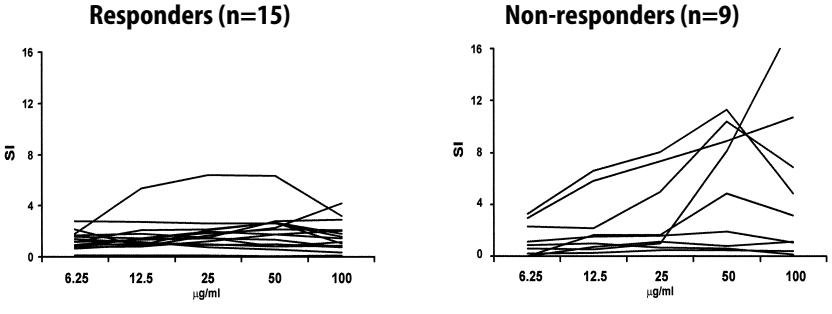

Fig. 2 Proliferative response (shown on the ordinate as stimulation index, SI) of peripheral blood mononuclear cells from 15 clinical responders and 9 non-responders at increasing concentrations of GA (abscissa). The cells from clinical responders showed a trend for decreased proliferation, but this was not statistically significant.

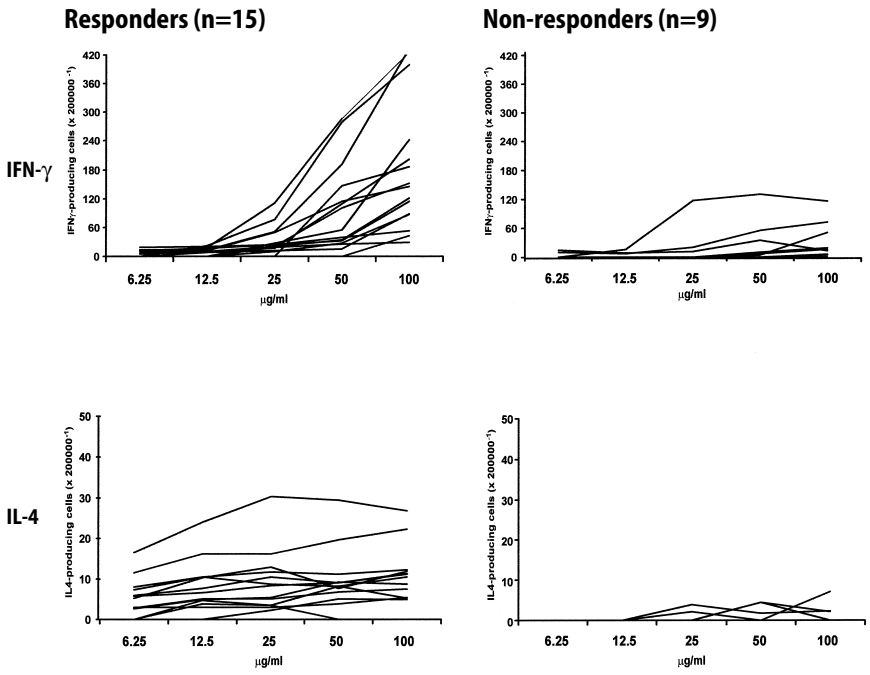

Fig. 3 Interferon-γ (IFN-γ) and interleukin-4 (IL-4) ELISPOT response of peripheral blood mononuclear cells from 15 clinical responders (left panels) and 9 non-responders (right panels) at increasing GA concentrations (shown on the abscissa). The clinical responders show much more pronounced ELISPOT responses than the clinical nonresponders.

whether the immunological response to GA correlates with the clinical response. In the present pilot study we sought to test this hypothesis. Our results show that indeed, the immunological response profile correlates with the clinical response to GA treatment.

We are fully aware of the limitations of our study protocol. Complete evaluation of the clinical response would require a rigorous prospective study design, including regular standardised clinical and MRI assessments. This can only be done in the context of a controlled trial, which is not currently in sight. We therefore performed a small pilot study to see whether our immunological assays are promising and worth further evaluation. Although the observed correlation between the immunological and the clinical response was not absolute, it was statistically highly significant. Because of the obvious limitations of our study, especially the small number of patients and lack of objective MRI evidence, the results need to be interpreted with caution. However, it should be noted that all nine non-responders were identified on clinical grounds, because they either had an unchanged or increased exacerbation rate or developed a secondary progressive course. In this regard, the study conditions and treatment decisions realistically reflect the current clinical routine.

Perhaps it is helpful to discuss briefly the relationship of the ELISPOT results to the proposed mechanism of action of Copaxone. It is thought that Copaxone induces a population of TH2-like T cells which secrete TH2 cytokines like interleukin–4, 5, 6 and 10 and cross-react with myelin-basic protein and perhaps, other myelin antigens [9]. The activated GA-reactive T cells enter the central nervous system, where they are restimulated by locally processed myelin autoantigens, secrete TH2-like cytokines and thereby suppress neighbouring pathogenic T cells ("bystander suppression"). At first sight, the strong interferon-γ ELISPOT response seen in GA-treated patients might seem to contradict this proposed mechanism of action, because interferon-γ is a typical TH1 cytokine. It should be noted however, that the interferon-γ ELISPOT response is seen only after stimulation with very high concentrations of GA in vitro. In the in vivo situation, it is unlikely that very high concentrations of GA are reached because of rapid and efficient enzymatic degradation. However, it is of note that both the IL-4 and the strong IFN-γ in vitro response correlate with a successful clinical outcome. In [4], CD4 and CD8 cells were found responsible for IL-4 and IFN-γ production, respectively. A recently published paper [4] confirms the effects of GA treatment on the stimulation of specific IFN-γ producing CD8 cells, although this effect was not correlated with the clinical efficacy.

In conclusion, the results of our pilot study suggest that there is a strong correlation between the immunological and clinical responses to GA treatment. In patients who fail to show a convincing clinical response, a negative ELISPOT test might help in the decision to stop treatment. This should help to save time, effort and resources, which would otherwise be spent on an ineffective therapy. Conversely, a positive immunological test might also provide useful information in patients who have an equivocal or doubtful clinical response. Ultimately, the decision to continue or discontinue the therapy must be made on the basis of the clinical response,

but a positive immunological response will be reassuring to both the patient and treating physician, especially during the first year of treatment when it is too early to judge the clinical response. We are now planning further studies in a larger number of patients to see whether the immunological assays reported here will keep their promis.

Acknowledgements We thank Drs. N. Heydari (Klinikum Grosshadern, Munich), A. Segert, M. Seiferheld and J. Haas (Jewish Hospital, Berlin), G. Ahle (Klinikum der Ruhr-Universität, Bochum), C. Stueckmann (Kamillus-Klinik, Asbach) for providing clinical samples; Professor A. Neiss (Institute of Medical Statistics and Epidemiology, Technical University Munich) for expert statistical advice; Drs. E. Meinl, M. Bradl and K. Dornmair (Max-Planck-Institute of Neurobiology, Martinsried) for helpful discussions and comments; and Ms B. Semlinger for excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft, SFB 571, and TEVA Pharma/Hoechst Marion Roussel. The Institute for Clinical Neuroimmunology is supported by the Hermann and Lilly Schilling Foundation.

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