Clin Exp Immunol 2003; **133**:454–460

Correlation of serum IL-13 and IL-5 levels with clinical response to Glatiramer acetate in patients with multiple sclerosis

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(Accepted for publication 27 June 2003)

SUMMARY

Glatiramer acetate (GA) is effective in the treatment of Multiple Sclerosis (MS) presumably by the induction of an immunoregulatory T-cell response. We have previously shown that GA directly induces the Th2 cytokines IL-13 and IL-5 in T-cells *in vitro*. In the present study we compared the *in vitro* response to GA in healthy controls, untreated and GA-treated MS patients and tested whether the induction of IL-13 and IL-5 secretion is also detectable in the serum of 25 MS patients treated with GA. Patients were grouped into clinical responders and nonresponders in order to determine a possible correlation with the immunological response. As a result we found a significant increase of IL-13 in the serum of clinical GA-responders whereas IL-13 was not detectable in controls, untreated MS ($P < 0{\cdot}001$) and nonresponders ($P = 0{\cdot}015$). Similarly, GA-treatment increased serum levels of IL-5 ($P = 0{\cdot}001$). The correlation of serum IL-5 and clinical response was also significant ($P = 0{\cdot}039$), however, there was an overlap between the different groups. The selective induction of IL-13 and IL-5 but not IL-4 by GA treatment suggests that the specific biological functions of these cytokines might be important for the therapeutic mechanism of GA. Measurement of serum IL-13 and IL-5 levels is a simple and inexpensive tool for monitoring the response to GA in MS patients.

Keywords Multiple sclerosis Glatirameracetate IL-13 IL-5

INTRODUCTION

Glatiramer acetate (GA) has been shown in several clinical class-I evidence trials to reduce the biological activity of relapsing-remitting MS [1–3]. The main clinical effects are a reduction of relapse rate and new lesion formation in MRI. The mechanism of action is not completely understood but is thought to involve the induction of an immunoregulatory T-cell response [4,5]. Unfortunately, the clinical response in individual patients is difficult to predict and some patients do not respond to GA. Due to the variable clinical course of MS it would be helpful to have a paraclinical marker for therapeutic effectiveness early after beginning of immunomodulatory treatment. So far, no such marker could be established and usually patients will have to be treated for at least one year before the individual clinical response to GA can be judged.

In early studies of GA it has already been shown that *in vitro* GA induces a marked proliferative answer in healthy patients as well as in MS patients [6]. This *in vitro* response to GA is downregulated during GA-treatment [7,8]. We have previously shown that GA directly induces IL-5 and IL-13 cytokine secretion in T-cells isolated from peripheral blood of healthy control subjects and MS patients *in vitro* [9]. Other recent studies also described an induction of Th2 cytokines by GA in GA-reactive T-cell lines [8,10–13]. In PBMCs generated from GA treated patients that were stimulated with GA *in vitro* a strong induction of IFN-$\gamma$was observed at high concentrations of GA whereas IL-4 was induced at lower concentrations as detected by ELISPOT [8]. This immunological response to GA was not observed in untreated MS controls and in most clinical nonresponders to GA-treatment [14].

As a follow up on our *in vitro* studies with GA we here further examined the effects of GA on cytokine secretion of IL-13 and IL-5 *in vitro* in untreated and GA-treated MS patients. We also tested whether corresponding effects on cytokine levels were detectable in the serum of patients with MS undergoing therapy with GA. By correlating the changes in serum cytokine levels with the clinical response to GA in individual patients we identified serum levels of IL-13 and IL-5 as potentially useful paraclinical markers in MS patients.

MATERIALS AND METHODS

Patients and controls

For *in vitro* studies 43 untreated and 17 GA-treated patients with relapsing-remitting or relapsing-progressive MS were included in

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this study (biometric data are shown in Table 1). All patients had definite MS according to Poser criteria, none had received any immunomodulatory or immunosuppressive treatment within 6 months prior to the experiments. Controls were 25 healthy age- and sex-matched individuals and 10 patients with other neurological diseases (2 headache, 3 Parkinson's syndrome, 2 vertigo, 3 polyneuropathy). All subjects signed an informed consent that was approved by the Institutional Review Board prior to venipuncture. The clinical disease course was determined by recording clinical exacerbations and measurement of clinical symptoms on Kurtzke's Expanded Disability Status scale (EDSS) in the 2 years before treatment and then every 3 month after beginning of therapy.

Serum samples from 25 GA-treated patients were obtained from the serum bank of the Department of Neurology of the Medical School Hannover. These patients had participated in an open label study with GA. Since a complete longitudinal course with pretreatment and 3 month data was not available for these patients a crossover analysis was performed using 38 untreated MS patients from the MS outpatient clinic as controls. In the GA treated group the samples from month 6–15 after beginning of treatment were tested and the mean was used for statistical evaluation. For comparison the baseline data of untreated MS patients were used. To determine natural changes in serum levels in untreated MS patients serum was collected from 4 untreated MS patients every 3 month over a period of one year. These untreated patients were part of the CORAL study (TEVA GA7023) and had received placebo. All serum samples were stored at –80°C. The study was approved by the local Ethics Committee and all subjects signed a written informed consent form.

To analyse correlations between clinical efficacy of GA and changes in serum cytokine levels patients were grouped into responders and nonresponders based on the clinical course of disease. Patients with an increase of EDSS of at least one point sustained over 3 month or an unchanged or increased rate of exacerbations were classified as nonresponders.

Cell preparation and stimulation

For *in vitro* studies PBMCs were isolated from heparinized venous blood by density gradient centrifugation over Ficoll-Paque. PBMCs were cultured in triplicates under different conditions of stimulation in 24-well plates (Nunc, Wiesbaden, Germany) in RPMI1640 (Sigma, Taufkirchen, Germany) supplemented with 10% FCS (Biochrom, Berlin, Germany), 10 mM Hepes buffer, 100 U/100 μg per ml penicillin/streptomycin and 2 mM L-glutamine. 2×10^5 PBMCs/well were stimulated with GA (at 40 μg/ml, batch number 242992997, provided by Teva Pharmaceutical Industries, Israel) and anti-CD3 crosslinking (clone HIT3a, Pharmingen, at 1 μg/ml coated to plastic) as positive control. After 96 h of culture in humidified air containing 5% CO_2?? at 37°C cell supernatants were harvested and stored at –20°C until further use.

Proliferation of PBMCs was measured after 6 days by BrdU incorporation assay (Boehringer Mannheim, Mannheim, Germany) as described previously [9].

Cytokine ELISA

Cytokine secretion of IL-13 and IL-5 was measured in cell culture supernatants after 4 days in resting cells by ELISA. The following reagents were used: for IL-13 ELISA recombinant human IL-13 (R & D systems, Wiesbaden, Germany), IL-13 capture antibody (Ab) (2 μg/ml, clone JES10–5A2, Pharmingen, Heidelberg, Germany), IL-13 detection Ab (2 μg/ml, clone B69-2, Pharmingen, Heidelberg, Germany) and for IL-5 ELISA recombinant human IL-5 (R & D), IL-5 capture Ab (clone TRFK5, 1 μg/ml) and IL-5 detecting Ab (clone JES1–5A10, 0,5 μg/ml) (both from Pharmingen, Heidelberg, Germany). Sensitivity of both ELISAs was 10 pg/ml. ELISAs were performed as described in detail previously [9].

For measurement of IL-13 and IL-5 in serum the human IL-13 Module Set (Bender MedSystems, Heidelberg, Germany) and human IL-5 OptEIA Set (Pharmingen, Heidelberg, Germany) were used following the manufacturers instructions. Sensivity of serum ELISAs were: 1·5 pg/ml for IL-13 and 1·1 pg/ml IL-5. Serum cytokine levels were considered increased when greater than 2 pg/ml.

Statistical analysis

Median and mean values were calculated and the different groups of MS patients and controls were compared using the one-way ANOVA test. Relationship between serum cytokine levels and clinical progression of treated MS-patients was determined with Fisher's two-tailed exact test. For all tests SigmaPlot 2001 for Windows/version 7·0 and SigmaStat 1997 for Windows/version 2·03 software was used. P-values < 0·05 were considered as significant.

Table 1. Biometric data (healthy controls were the same for *in vitro* and serum studies)

						EDSS	
	n	Age (years)	Gender (m/f)	Duration of disease (years)	No. of relapses/year	pretreatment	posttreatment
Healthy controls (HC)	25	40 ± 14	6/19				
Other neurological diseases	10	43 ± 15	3/7				
Untreated MS patients							
In vitro study	43	40 ± 9	11/32	6 ± 2	2 ± 1	2·0 ± 1·5	
Serum study	38	37 ± 7	7/31	7 ± 1	1 ± 1	2·0 ± 1·0	
GA treated MS patients							
In vitro study 2·0 ± 1·0	17	45 ± 11	2/15	9 ± 4	0·5 ± 0·3	2·0 ± 1·0	2·0 ± 1·5
Serum study							
Responder	20	41 ± 9	2/18	8 ± 4	0·3 ± 0·1	2·0 ± 1·0	2·0 ± 1·0
Nonresponder	5	47 ± 8	2/3	9 ± 5	1·6 ± 0·8	3·5 ± 1·0	5·0 ± 1·0

RESULTS

GA induces IL-13 and IL-5 secretion. Reactivity to GA is reduced in untreated MS patients

For our *in vitro* studies we isolated PBMCs from 25 healthy controls (HC) and 43 untreated MS patients. They were stimulated with GA (40 μg/ml) and secretion of IL-5 and IL-13 was measured over a period of 4 days in culture supernatants. Proliferation was measured with BrdU ELISA as described in materials and methods. As positive control PBMCs were also stimulated with anti-CD3 Ab coated to plastic.

As we have demonstrated recently [9], stimulation with GA induced proliferation and secretion of IL-13 and IL-5. PBMCs from untreated MS patients showed a reduced reactivity to GA-stimulation *in vitro*. That result was reproduced in this study with a larger number of subjects. The net GA-induced IL-13 secretion in healthy subjects was 150 ± 27 pg/ml (mean ± SE, range 0–438) as compared to 77 ± 14 pg/ml (range 0–357) in untreated MS patients ($P = 0{\cdot}017$). The net IL-5 secretion showed a mean of 60 ± 12 pg/ml (range 0–204) in healthy controls and was reduced to 22 ± 7 pg/ml (range 0–191) ($P < 0{\cdot}001$) in MS patients. Mean proliferation was 0·3 ± 0·03 (range 0·07–0·52) in healthy controls and 0·18 ± 0·03 (range 0–0·58) in MS patients ($P = 0{\cdot}004$) (Fig. 1 and Table 2).

In vitro *response to GA in GA-treated patients*

To determine changes of the *in vitro* response to GA stimulation after treatment with GA, we repeated our experiments with PBMCs from 17 GA treated patients. As reported in previous studies [7,14], we also observed a loss of GA- induced T-cell proliferation in GA treated patients. Mean proliferation was reduced to 0·058 ± 0·03 (range 0–0·43, $P < 0{\cdot}001$ when compared to healthy controls, and p = 0·006 when compared to untreated MS patients). Cytokine production of IL-5 and IL-13 was also markedly reduced in GA treated patients (IL-5 26 ± 14; range 0–217 and IL-13 57 ± 16 pg/ml; range 0–257) as compared to healthy controls, however, there was no statistically significant difference in IL-13/IL-5 secretion between untreated and GA treated patients (Fig. 1 and Table 2).

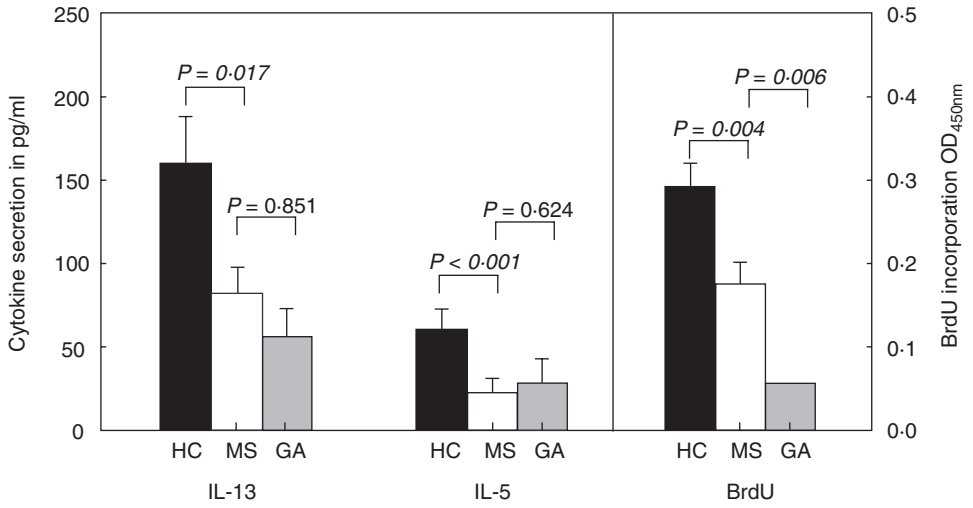

Fig. 1. Net GA induced cytokine secretion (in pg/ml) and proliferation (BrdU incorporation) *in vitro*. PBMCs from healthy controls (HC, ■ $n = 25$), untreated MS patients (MS, □ $n = 43$) and GA treated MS patients (GA, ▨ $n = 17$) were stimulated with GA (40 μg/ml). IL-13 and IL-5 secretion was measured after 4 days and proliferation after 6 days by ELISA as described under materials and methods. Mean values ± SEM are shown, for the BrdU data in GA treated patients no error bar is shown because the values were below the sensitivity level of 0·15.

Table 2. Secretion of IL-13 and IL-5 (in pg/ml for ELISA) and proliferation (BrdU incorporation OD_{450nm}) in unstimulated and GA stimulated (40 μg/ml) PBMC's from healthy controls ($n = 25$), untreated MS patients ($n = 43$) and GA treated MS patients ($n = 17$). Mean values ± SEM are presented. The GA-induced IL-13 and IL-5 secretion and proliferation was significantly reduced in MS patients without and with GA treatment as compared to HC

	Healthy controls		MS untreated		MS GA-treated	
	Unstimulated	GA	Unstimulated	GA	Unstimulated	GA
IL-13	37 ± 9	187 ± 35	45 ± 8	122 ± 20	41 ± 4	98 ± 8
IL-5	26 ± 9	86 ± 18	15 ± 4	37 ± 8	0 ± 0	26 ± 15
BrdU	0·39 ± 0·07	0·69 ± 0·08	0·35 ± 0·05	0·53 ± 0·05	0·37 ± 0·08	0·43 ± 0·07

Mean values ± SEM are presented. The GA-induced IL-13 and IL-5 secretion and proliferation was significantly reduced in MS patients without and with GA treatment as compared to HC

GA treatment increases serum levels of IL-13 and IL-5

To investigate the effects of GA therapy on serum cytokine levels of IL-13 and IL-5 serum samples of MS patients during treatment with GA and of untreated MS controls were examined by ELISA. We collected serum samples of 25 healthy volunteers, 38 patients with relapsing remitting MS without treatment, 25 patients treated with GA and 10 patients with other neurological diseases (Table 1). The disease durations were similar in the untreated and GA treated patients both in the *in vitro* study ($P = 0{\cdot}2$) and the serum study ($P = 0{\cdot}3$).

As shown in Fig. 2a IL-13 was not detectable in the serum of healthy controls, untreated MS patients and patients with other neurological diseases. After therapy with GA IL-13 serum levels increased to $4{\cdot}02 \pm 1{\cdot}18$ pg/ml (range 0–24·2 pg/ml; $P < 0{\cdot}001$).

IL-5 serum levels were $1{\cdot}1 \pm 0{\cdot}6$ pg/ml (range 0–14·1 pg/ml) in healthy controls, $1{\cdot}67 \pm 0{\cdot}81$ pg/ml (range 0–24 pg/ml) in untreated MS and $1{\cdot}87 \pm 0{\cdot}29$ pg/ml (range 0–6·86 pg/ml) in patients with other neurological diseases. There was no statistically significant difference between these groups. IL-5 serum levels increased to $2{\cdot}72 \pm 0{\cdot}61$ pg/ml (range 0–15·16 pg/ml) after GA treatment ($P = 0{\cdot}001$ when compared to untreated MS patients) (Fig. 2b).

Positive correlation between clinical efficacy and increased serum Th2 cytokine levels

To investigate whether changes in serum IL-13 and IL-5 cytokine levels correlate with the clinical response to therapy we grouped MS patients into responders and nonresponders as described in materials and methods. According to these criteria 20 out of 25 GA treated patients were classified as responders. Similarly, serum samples of patients were grouped into those with unchanged or increased levels of cytokine levels. As shown in Table 3 and Fig. 3a positive clinical response to GA-treatment correlated best with increased IL-13 serum levels: 13 out of 20 GA responders but none of the GA nonresponders had increased IL-13 serum levels after treatment ($P = 0{\cdot}015$). In addition GA treatment showed a correlation with increased IL-5 serum levels: 13 out of 20 GA responders and one nonresponder showed elevated IL-5 serum levels after treatment ($P = 0{\cdot}039$). These correlations were the same when the EDSS score alone was used to determine the clinical response, whereas the correlation with the relapse rates alone was not significant as 3 of the nonresponders had an EDSS progression of 1·5 without relapses.

Eleven of 20 GA responders had increased serum levels of IL-13 together with IL-5 and 5 had increased serum levels of either IL-13 or IL-5. 4 GA responders showed no changes in these two cytokines. In the GA nonresponder group none of the patients showed an increase in both IL-13 and IL-5. Therefore increased levels of IL-5/IL-13 had a sensitivity of 80% (for IL-13 and IL-5 alone sensitivity was 65%), specificity of 80% (for IL-13 alone 100%, for IL-5 alone 80%), a positive predictive value of 94·1% (for IL-13 alone 100%, for IL-5 alone 92·9%), but a negative predictive value of only 50% (for IL-13 alone 41·7%, for IL-5 alone 33·3%) for the detection of clinical responders (or nonresponders) during GA-treatment in our study.

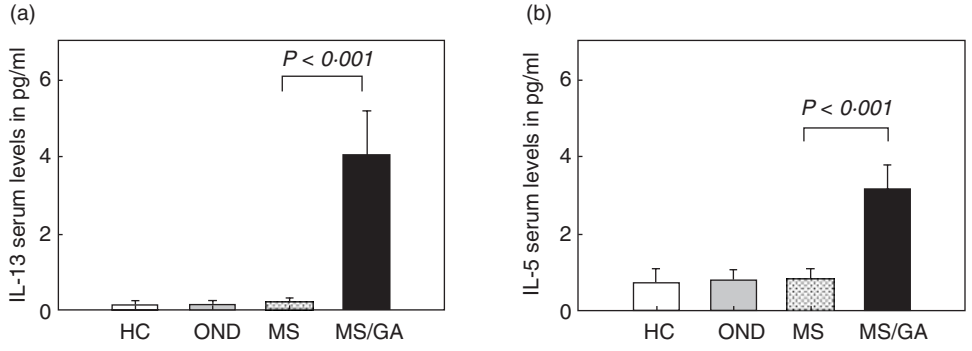

Fig. 2. Influence of GA treatment on serum cytokine levels in patients with MS. Serum samples were collected from 25 healthy donors (HC), 10 patients with other neurological diseases (OND), 38 untreated MS patients (MS) and 25 patients treated with GA (MS/GA). Serum levels of (a) IL-13and (b) IL-5 were measured by ELISA. Mean values ± SEM are shown.

Table 3 Correlation between increased and unchanged cytokine levels and therapeutical response to GA as described in materials and methods. Numbers of patients in each subgroup are shown.

	IL-13		IL-5		IL-4		IL-10		IFN-γ		IL-13 and/or IL-5	IL-13 & IL-5
	Incr.	Unch.	Incr.	Unch.	Incr.	Unch.	Incr.	Unch.	Incr.	Unch.	Incr.	Unch.
GA responders	13	7	13	7	14	6	8	12	3	17	16	4
GA nonresponders	0	5	1	4	2	3	0	5	0	5	1	4
***P*-value**	**0·015**		**0·039**		**0·312**		**0·140**		**1·000**		**0·023**	

Incr., cytokine level increased; Unch., cytokine level unchanged. *P*-values show correlations between increased cytokine production and responders, calculated with Fisher's two-tailed exact test

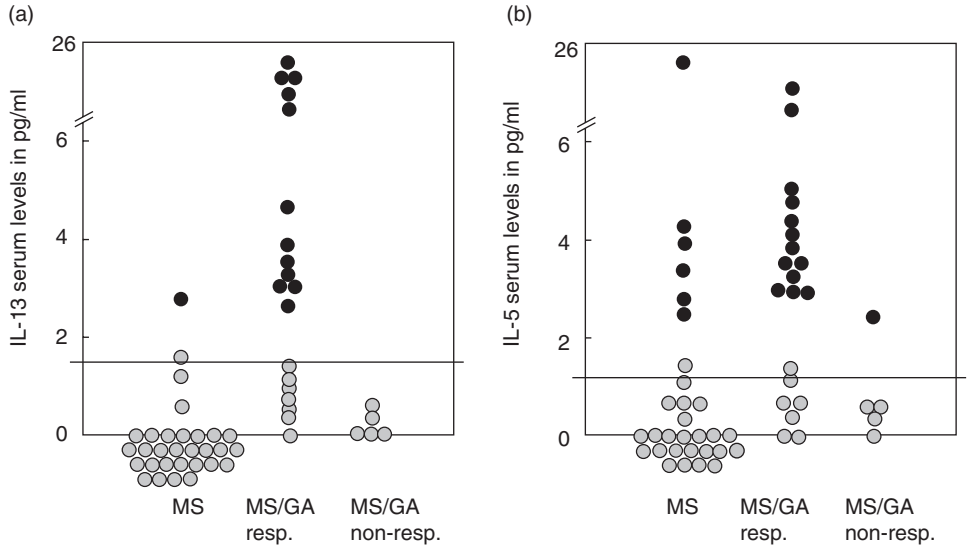

Fig. 3. Association of clinical response with increased serum cytokine levels. (a) IL-13 and (b) IL-5 serum levels of untreated MS patients (MS) and clinical responders (MS/GA resp.) or nonresponders (MS/GA non-resp.) are shown. Each circle represents one patient (● significant cytokine secretion, ○ no significant cytokine secretion, horizontal line: sensitivity of cytokine ELISA).

DISCUSSION

In this study we compared the *in vitro* and *in vivo* effects of GA on cytokine secretion of IL-13 and IL-5 and correlated changes in serum cytokine levels with the clinical response to GA-therapy in individual MS-patients. As reported previously [9] GA induces secretion of IL-13 and IL-5 by T-cells in healthy controls as well as in MS patients. However, this immunological *in vitro* response to GA was smaller in MS patients. To follow up on these findings we now tested a larger number of subjects (25 healthy controls, 43 untreated MS patients) and also examined the *in vitro* response to GA in PBMCs from GA-treated patients. In confirmation of our previous results we observed a significant difference in the *in vitro* response to GA in controls and untreated MS patients with reduced proliferation and IL-13/IL-5 secretion in MS patients. As $CD4^+/CD45RA^+$ T-cells are the major source of GA-induced IL-13/IL-5 and proliferation in our system [9] there are two obvious explanations for this finding: a reduced number of $CD4^+/CD45RA^+$ T-cells in MS and altered function of the $CD4^+/CD45RA^+$ T-cell subpopulation in MS. In support of our results, earlier findings reported a reduced number of $CD4^+/CD45RA^+$ T-cells, formerly termed 'suppressor-inducer' cells, in peripheral blood of MS patients particularly during relapses and a diminished function of these cells in MS patients [15–17]. In comparison to the findings in untreated MS patients the *in vitro* proliferation to GA was reduced in GA-treated patients whereas no changes were observed for cytokine secretion. A reduced proliferation to GA in treated patients has been described by several groups [7,8,11,18], although the mechanism is unclear. Since many patients treated with GA develop lymph node swelling [19] one possible explanation would be trapping of GA-reactive T-cells in the lymph nodes. Alternatively, chronically GA-stimulated T-cells might circulate in the peripheral blood and respond to repeated GA-stimulation with cytokine secretion without proliferation. Cytokine secretion without proliferation has been previously reported in MBP-specific T-cell lines in MS patients [20]. Moreover, as our *in vitro* results show loss of proliferation but unchanged levels of IL-13 and IL-5 in GA-treated patients it is unlikely that the GA-reactive T-cells in treated patients become anergic. Taken together our findings would support the hypothesis that the mechanism of action of GA in MS involves the induction of Th2-like regulatory T-cells in the periphery (e.g. lymph nodes) that recirculate and ultimately migrate into the CNS where cross-reaction with myelin antigens might occur resulting in a self-limited immunological response of Th2 cytokine secretion without T-cell proliferation.

The main objective of this study was to investigate whether the GA-induced cytokine secretion of IL-13 and IL-5 *in vitro* would be reflected by similar changes of these cytokines in the serum of patients treated with s.c. GA. Therefore we performed a small pilot crossover study with 25 GA-treated and 38 untreated MS patients. As a result we found low levels of IL-13 in the serum of GA-treated patients whereas this cytokine was not detectable in untreated MS patients and healthy controls. Similarly, the difference in IL-5 serum levels between untreated MS and GA-treated patients was statistically significant with a *P*-value of 0·005, although there was a relatively large overlap between the groups. As reported previously IL-4 and IFN-γ were not induced by GA *in vitro* and were neither detectable in the serum of GA-treated patients nor in any of the controls (data not shown).

In general, IL-5 and IL-13 have a high functional redundancy with IL-4 in Th2- and B-cell immune responses. They are clustered on the same chromosome together with IL-4 and the three cytokines are co-ordinately regulated [21]. On the other hand there are distinct mechanisms and biological functions mediated by either cytokine and they have different spatial and temporal patterns of expression in animal models of infection and inflammation [22,23]. In humans naive T-cells require IL-4 during primary stimulation to enable subsequent IL-4 expression and Th2-differentiation, while IL-13 plays a unique role in the induction of IgE production and has potent anti-inflammatory activities *in vivo* (reviewed in [24]). IL-5 seems to have a specific role in the regu-

lation of eosinophils [25]. Eosinophils do not seem to play a role in the pathogenesis of typical MS although eosinophils are detectable in the inflammatory brain lesions of Devic's disease [26]. In an EAE model distinct subpopulations of Th2 effector cells characterized by differential expression of IL-4 and IL-5 have been described [27]. The selective induction of IL-13 and IL-5 but not IL-4 by GA treatment suggests that the specific biological functions of these cytokines might be important for the therapeutic mechanism of GA. Further work is needed to determine whether a dysregulation of these cytokines might play a role in the pathogenesis of MS.

To determine whether the immunological response with increased serum levels of IL-13 and IL-5 in patients treated with GA correlates with the individuals clinical response we grouped the 25 patients in this study in clinical responders and nonresponders and analysed the cytokine response. The results show that an increase in serum IL-13 levels is strongly associated with a positive clinical response as 13 out of 20 clinical responders had increased IL-13 levels while none of the nonresponders did. For IL-5 there was also a positive although less clear correlation with the clinical response to GA. Our findings suggest that an increase in IL-13 and IL-5 is helpful to predict a positive but not a negative response to GA therapy (sensitivity 80%, specificity 80%, positive predictive value 94·1%, negative predictive value 50%). From the 17 GA-treated patients in our *in vitro* study all but 2 were clinical responders and therefore the numbers were too small for statistical comparison. The increases in serum cytokines in GA treated patients were significant after 6 month of therapy, however, since the 3 month data were not available in our patients it is still possible that a significant change might occur at even earlier time points. As patients serum can be obtained and stored very easily and the cytokine ELISAs are simple and inexpensive we propose IL-13 and IL-5 serum levels as valuable paraclinical markers to support the decision whether to continue GA therapy in patients where the clinical response is equivocal. Additional information might be gained from *in vitro* stimulation assays such as the ELISPOT assay for IL-4 and IFN-γ that was described recently [14], although these assays are more labourious and expensive. Although the number of patients in our serum study was small the results were highly significant and warrant larger prospective studies including earlier time points and MRI data to follow up on these findings.

ACKNOWLEDGEMENTS

We thank Franziska Bode for excellent technical assistance and Corinna Trebst and Martin Stangel for helpful discussions. This study was supported by an internal grant from the Medical School Hannover.

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