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# Blood levels of transforming growth factor-beta 1 (TGF-$\beta$1) are elevated in both relapsing remitting and chronic progressive multiple sclerosis (MS) patients and are further augmented by treatment with interferon-beta 1b (IFN-$\beta$1b)

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**SUMMARY**

The serum levels of TGF-$\beta$1, measured by solid-phase ELISA, were determined to be significantly augmented in patients with both relapsing remitting (RR) and secondary chronic progressive (CP) MS compared with sex- and age-matched healthy controls. Moreover, in RR MS patients, the blood levels of the cytokine were further augmented either during relapses or, in a rapid but reversible fashion, by s.c. injection with 8 million International Units (MIU) IFN-$\beta$1b. Because TGF-$\beta$1 possesses multiple anti-inflammatory activities, we hypothesize that the increase in its circulating levels in RR and CP MS patients might represent an endogenous anti-inflammatory mechanism aimed at counteracting ongoing immunoinflammatory events, and that IFN-$\beta$ may further potentiate this natural defensive apparatus.

**Keywords** autoimmune diseases immunotherapy interferon-beta multiple sclerosis transforming growth factor-beta

## INTRODUCTION

MS is a chronic disease characterized pathogenically by an immunoinflammatory reaction, probably autoimmune in nature, that is driven against the central nervous system myelin by T lymphocytes and macrophages (see [1] for a review). Recent studies indicate that the capacity of these cells to secrete proinflammatory cytokines such as IL-1$\beta$, IL-12, IFN-$\gamma$ and tumour necrosis factor-alpha (TNF-$\alpha$) might be central to their pathogenic potential [1]. It has also been hypothesized that the progression of the disease, and perhaps even its appearance, might depend on a critical balance between proinflammatory cytokines and their endogenous antagonists, either naturally occurring cytokine inhibitors (IL-1 receptor antagonist (IL-1Ra), soluble (s)TNFR) or anti-inflammatory cytokines (IL-4, IL-10, IL-13, TGF-$\beta$) [1].

TGF-$\beta$ is a cytokine produced by several lymphoid and non-lymphoid cells which exists in five different isoforms (TGF-$\beta$1 to TGF-$\beta$5) and which inhibits several aspects of the immune response [2]. For this reason much attention has been focused on its possible immuno-down-regulatory role in the pathophysiology of MS. Several studies conducted in rodent experimental allergic encephalomyelitis (EAE), the counterpart of human MS, have demonstrated that endogenous TGF-$\beta$ might play an important defensive role in the pathogenesis of the disease (see [3] for a review). In the Lewis rat EAE, the recovery phase of the disease is associated with the presence of splenic $CD4^+$ suppressor T cells that secrete TGF-$\beta$ (and IL-4) in response to the effector T cells that cause EAE. The production of IFN-$\gamma$ and TNF from these cells is also inhibited by TGF-$\beta$ [3]. Moreover, TGF-$\beta$ may also play a role in (auto)antigen tolerization, as suggested by the presence of either $CD4^+$ or $CD8^+$ suppressor T cells secreting TGF-$\beta$ in rodents rendered tolerant to myelin basic protein (MBP) [3]. Interestingly, Fukaura *et al.* [4] have demonstrated that oral administration of myelin in MS patients induces circulating MBP and proteolipid protein-specific TGF-$\beta$1-secreting Th3 cells. Finally, EAE development is prevented by exogenously administered TGF-$\beta$1 and is concordantly exacerbated by blockade of the endogenous cytokine with a neutralizing antibody [3]. That TGF-$\beta$ might also display a similar immuno-down-regulatory role in human MS is indicated by one study in which high levels of TGF-$\beta$ mRNA expression in MS patients' mononuclear cells were associated with no or slight disability [5].

In this study we measured the blood levels of TGF-$\beta$1 in MS patients with relapsing remitting (RR) and secondary chronic progressive (CP) forms of the disease. Moreover, the effects of short-term treatment with IFN-$\beta$1b, a compound which favourably modulates the course of RR MS [6], on circulating TGF-$\beta$1 levels were also examined.

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## PATIENTS AND METHODS

*Patients and controls*

We studied 55 patients (23 females) with definite RR MS and 15 patients (six females) with secondary CP MS as diagnosed according to Poser's criteria [7]. The mean age (± s.d.) of RR MS patients was 41 ± 14 years. The mean duration of the disease was 5·1 ± 2 years. RR MS patients were divided into two groups depending on whether their disease was in relapsing ($n = 24$) or in remitting phase ($n = 41$). Ten RR MS patients (four females) were sequentially sampled 4–6 months prior to, and within 48 h from the beginning of a relapse. The CP MS patients had a mean age of 48·6 ± 7·6 years. The mean duration of the disease was 15 ± 6 years. Controls consisted of 29 (14 females) age-matched healthy subjects and of 12 patients (six females) suffering from other neurological diseases (OND) including amyotrophic lateral sclerosis ($n = 2$), multisystemic atrophy ($n = 3$) and lumbar discopathy ($n = 7$). Neither MS patients nor control subjects had used immunomodulatory drugs during the 5 months before commencement of the study. The control subjects did not suffer from infectious, allergic or autoimmune diseases during the 6 months before blood sampling.

*Selection for IFN-β1b treatment*

Fourteen RR MS patients with stable disease who successively entered the MS centre of Catania for IFN-β treatment were selected for the study according to previously described criteria [8]. They were injected subcutaneously every other day for 10 consecutive days with 8 million International Units (MIU) recombinant human IFN-β1b (Farmades, Rome, Italy), so receiving five injections of the drug in total.

*Blood sampling*

Blood samples were obtained either within 48 h of exacerbation or during a remission. In the group of patients treated with IFN-β1b, blood samples were obtained prior to treatment (T0), 3 h (T1) and 12 h (T2) (h) thereafter, after 10 days (T3) of treatment and 48 h after the last injection. In addition, blood samples from 10 out of 29 untreated healthy controls were obtained at similar time points during consecutive 12 h to rule out possible diurnal variations in the circulating levels of TGF-β1. Venous blood samples from both MS patients and control subjects were collected between 09·00 and 10·00 a.m. to rule out possible circadian variation of circulating TGF-β1. Blood samples were allowed to clot at room temperature and serum was immediately separated by centrifugation at 1000 ***g*** and stored at −20°C until assay for the measurement of TGF-β1.

*TGF-β1 measurement*

TGF-β1 was measured by a solid-phase ELISA, purchased from Genzyme (Cambridge, MA) which has been previously used to measure the circulating levels of TGF-β1 in patients with spondyloarthropathies [9]. Serum samples, run in duplicate exactly as recommended by the manufacturer, were acidified with HCl for 1 h. This allows the biologically active form of TGF-β1 to be released from the biological inactive complex formed from non-covalent association of mature TGF-β dimer and a second dimer (latency associated protein). Intra- and interassay coefficients of variation were 4·1% and 7·2%, respectively. The limit of sensitivity of the assay is 0·05 ng/ml.

*Statistical analysis*

Results are shown as mean values ± s.e.m. Statistical analysis was performed by ANOVA. $P < 0·05$ was taken as significant.

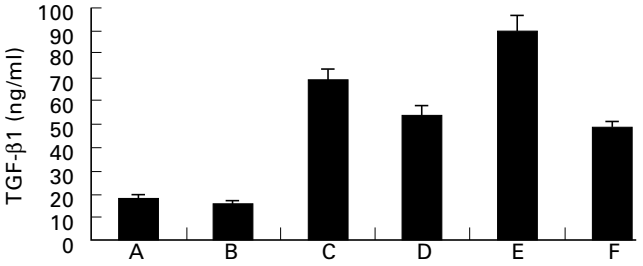


**Fig. 1.** Elevated serum levels of TGF-β1 in relapsing remitting (RR) MS patients. The serum levels of TGF-β1 were measured by solid-phase ELISA in RR MS patients (group C), either with stable disease (group D) or with relapses (group E), in chronic progressive (CP) MS patients (group F), in sex- and age-matched healthy controls (group A) and in patients suffering from other neurological diseases (group B). Results are shown as mean values ± s.e.m. Statistical analysis was performed by ANOVA. B *versus* A, not significant (NS); C *versus* B and A, $P < 0·0001$; C *versus* F, $P < 0·05$; D *versus* A and B, $P < 0·0001$; D *versus* F, NS; E *versus* A and B, $P < 0·0001$; E *versus* D and F, $P < 0·0001$.

## RESULTS

*Circulating levels of TGF-β1 in RR and CP MS patients*

The blood levels of TGF-β1 were significantly higher in both RR and CP MS patients compared with either healthy controls or patients with OND (Fig. 1). Moreover, when RR MS patients were considered on the basis of their clinical status, it was apparent that the patients suffering from MS attacks had significantly higher circulating levels of TGF-β1 than both the patients with either stable or secondary CP disease (Fig. 1). Because of the highly heterogeneous nature of MS, and to avoid the problem that patients who are naturally high or low producers of TGF-β1 may disproportionately influence the results, we performed longitudinal measurement of TGF-β1 in individual RR MS patients sampled during remission periods and relapses. Confirming the previous findings, these results again indicated that TGF-β1 blood levels substantially increased upon acute MS attacks (Table 1). TGF-β1 blood levels did not correlate with the sex of the patients, the duration of the disease or the Kurtybe's expanded disability status scale (EDSS) score.

**Table 1.** Longitudinal measurement of TGF-β1 blood levels (ng/ml) in individual relapsing remitting (RR) MS patients before and during acute MS attacks

| Patients (*n*) | Preattack (TGF-β1) | Attack (TGF-β1) |
|---|---|---|
| 1 | 45 | 88 |
| 2 | 54 | 102 |
| 3 | 34 | 52 |
| 4 | 41 | 77 |
| 5 | 66 | 90 |
| 6 | 41 | 64 |
| 7 | 71 | 109 |
| 8 | 55 | 89 |
| 9 | 34 | 41 |
| 10 | 63 | 110 |
| Mean value (s.e.m.) | 50·4 (4·2) | 82·2 (7·4)* |

* $P = 0·002$ *versus* mean preattack values by ANOVA.

**Table 2.** The effects of IFN-$\beta$1b on TGF-$\beta$1 blood levels (ng/ml) in relapsing remitting (RR) MS patients

| Patients (*n*) | TGF-$\beta$1 T0 | TGF-$\beta$1 T1 | TGF-$\beta$1 T2 | TGF-$\beta$1 T3 |
|---|---|---|---|---|
| 1 | 19 | 52 | 31 | 36 |
| 2 | 70 | 154 | 58 | 26 |
| 3 | 108 | 410 | 24 | 18 |
| 4 | 98 | 417 | 57 | 92 |
| 5 | 21 | 35 | 20 | 17 |
| 6 | 20 | 21 | 19 | 20 |
| 7 | 29 | 45 | 87 | 50 |
| 8 | 34 | 21 | 87 | 23 |
| 9 | 25 | 131 | 33 | 27 |
| 10 | 26 | 73 | 45 | 20 |
| 11 | 52 | 42 | 106 | 113 |
| 12 | 14 | 102 | 89 | 2·5 |
| 13 | 81 | 101 | 109 | 123 |
| 14 | 68 | 117 | 156 | 40 |
| Mean value (s.e.m.) | 47·5 (8·5) | 123 (34)* | 65·8 (11) | 43·4 (10) |

* $P = 0{\cdot}045$ *versus* pretreatment values by ANOVA.

TGF-$\beta$1 serum levels were measured prior to (T0), and 3 h (T1), 12 h (T2) and 10 days (T3) after treatment on alternate days for 10 days with 8 MIU of human IFN-$\beta$1b.

*The effects of IFN-$\beta$1b treatment on TGF-$\beta$1 blood levels*

Treating RR MS patients with IFN-$\beta$1b markedly augmented their blood levels of TGF-$\beta$1 and 12/14 of the patients experienced at least a 50% increase in circulating levels of the cytokine in response to the treatment at one of the time points considered (Table 2). Most often (7/14) the increase reached a maximal value 3 h after treatment, but in some patients (5/14) it was first apparent 12 h after IFN-$\beta$1b treatment (Table 2). These effects were unlikely to be due to diurnal variation of circulating TGF-$\beta$1, as its blood levels maintained comparable values in healthy individuals sampled within 12 consecutive hours at 9·00 a.m., 12·00 a.m. and 9·00 p.m. (data not shown). At the end of the study period (T3), i.e. after 10 days of treatment on alternate days but 48 h after the last injection, the blood levels of TGF-$\beta$1 were comparable in 6/14 of the patients and augmented or diminished in the other 4/14 and 4/14 relative to pretreatment values (Table 2). Thus, the IFN-$\beta$1b-induced increase in TGF-$\beta$1 blood levels seems to be strictly dependent upon continuous application of the drug. It also appears notable that the maximal 'pharmacological' increase in circulating levels of TGF-$\beta$1 observed after 3 h of treatment did not differ significantly from that 'naturally' occurring during MS attacks (see Fig. 1 and Table 1).

## DISCUSSION

We have demonstrated that both RR and secondary CP MS patients have more elevated blood levels of TGF-$\beta$1 compared with either healthy controls or patients with OND. Moreover, the blood levels of TGF-$\beta$1 were even more elevated in RR MS patients suffering from relapses than in those with stable disease. We also observed that IFN-$\beta$1b, when given to RR MS patients with stable disease, further augmented in a reversible fashion the circulating levels of TGF-$\beta$1. Pathophysiological and immunopharmacological points worthy of attention arise from these observations.

Although TGF-$\beta$1 blood levels were not found to correlate with clinical symptoms as assessed by EDSS score, it seems nonetheless possible that the observed increase might be biologically significant. This concurs with results from other clinical situations probably autoimmune in nature, such as spondylarthropathies and thrombotic thrombocytopenic purpura, where even two-to-three-fold lower circulating levels of TGF-$\beta$1, either dosed by our same ELISA or bioassay, respectively, appear to exert a biological effect [9,10]. It should be noted, however, that high variabilities have been reported to occur in the blood levels of TGF-$\beta$1 in both patients suffering from autoimmune diseases and healthy subjects. For example, in the above studies the circulating levels of TGF-$\beta$1 found in the healthy controls were also two-to-three-fold lower than those we have observed here. Moreover, using different ELISA kits other authors have previously found from about 100- up to 500-fold lower blood levels of circulating biologically active TGF-$\beta$1 in patients affected by Guillain–Barré syndrome, healthy controls and patients with other neurological diseases [11]. The reason for these differences remains unknown.

Along with the well known immuno-down-regulatory effects of TGF-$\beta$1 [3], its elevated blood levels in RR and CP MS patients could be envisaged as part of a complex endogenous anti-inflammatory apparatus that is activated during MS, and which might also comprise the sIL-1Ra [8], the sTNFR and the circulating form of intercellular adhesion molecule-1 (cICAM-1) [12]. It seems notable in this context that in a manner similar to sIL-1Ra and cICAM-1, the blood levels of TGF-$\beta$1 [8,12] were also augmented during relapses. As we have previously hypothesized for sIL-1Ra, this might be due to the activation of immuno-down-regulatory mechanisms elicited in a feedback fashion by the actively ongoing immunoinflammatory events which occur during a relapse. In fact, the immune system is tightly regulated '*in vivo*' and the exuberant production of proinflammatory cytokines is often counteracted by the release of either anti-inflammatory type 2 cytokines or naturally occurring cytokine inhibitors [13]. Interestingly, that endogenous TGF-$\beta$1 might have an important role in terminating demyelinating immunoinflammatory processes concords with data by Sindern *et al*., who reported that, in patients suffering from the acute inflammatory demyelinating polyradiculoneuropathy Guillain–Barré syndrome, maximal increases in the circulating levels of TGF-$\beta$1 occur just before onset of recovery [11].

That the blood levels of TGF-$\beta$1 are abnormally elevated in CP and RR MS patients, in particular in those with relapses, contrasts with other studies. Link *et al.* [14] reported comparable high levels of MBP- and proteolipid protein (PLP)-responsive TGF-$\beta$ mRNA-expressing cells in the blood of MS patients regardless of whether they were in exacerbation or in remission. In contrast, Beck *et al.* [15] found that TGF-$\beta$-like activity is produced during regression of exacerbation, and Mokhtarian *et al.* [16] observed that, relative to controls, TGF-$\beta$ production from MS patients' T lymphocytes is impaired, and that less TGF-$\beta$ is produced during a relapse than in stable disease. The different experimental conditions used ('*in vivo*' *versus* '*in vitro*') might probably account for these discrepancies.

The capacity of IFN-$\beta$1b to augment TGF-$\beta$1 blood levels in RR MS patients conforms to a preliminary study reporting that IFN-$\beta$ augmented production of TGF-$\beta$1 from cultured peripheral blood mononuclear cells [6]. Our finding is in contrast, however, with data of Rudick *et al.* [17], who recorded that adding IFN-$\beta$ to mononuclear cell cultures of healthy volunteers did not augment accumulation of TGF-$\beta$ mRNA. Here again, the different experimental conditions ('*in vivo*' *versus* '*in vitro*', transcripted *versus*

secreted protein) and the different population studied (healthy subjects *versus* RR MS patients) might explain the different results. Nonetheless, our present study, along with the similar stimulatory effects of IFN-$\beta$ on IL-1Ra and IL-10 secretion [6,8], further suggests that up-regulating the production of either naturally occurring cytokine inhibitors or anti-inflammatory cytokines might be one of the potential mechanisms by which this drug ameliorates the course of RR MS. Note, however, that TGF-$\beta$1 blood levels rapidly returned to pretreatment values upon interruption of the treatment; this might mean that under these experimental conditions the augmented blood level of TGF-$\beta$1 is a temporary phenomenon which has not determined long-lasting functional changes in TGF-$\beta$1-secreting cells. Hence, if induction of TGF-$\beta$1 is one of the mechanisms by which IFN-$\beta$ exerts its favourable effects in RR MS, and unless a more prolonged treatment does not permanently augment the production of this cytokine, a prompt reversal of the therapeutic action of IFN-$\beta$ might be expected when treatment is interrupted. Assessing whether or not the effects of IFN-$\beta$ in RR MS might be potentiated and eventually prolonged by combined treatment with TGF-$\beta$1 is an important point worthy of study.

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