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Effects of rIFN-β-1b on serum circulating ICAM-1 in relapsing remitting multiple sclerosis and on the membrane-bound ICAM-1 expression on brain microvascular endothelial cells

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rIFN-β reduces the frequency of the gadolinium-enhancing (Gd+) magnetic resonance imaging (MRI) lesions in relapsing remitting (RR) MS. Its mechanism of action on improving the integrity of the blood-brain barrier (BBB) remains unclear. We investigated the effect of rIFN-β-1b on the soluble intercellular adhesion molecule-1 (sICAM-1) serum levels (ELISA) in 36 RR MS patients receiving treatment with rIFN-β for 1 year, and also the TNF-α–induced membrane-bound ICAM-1 (mICAM-1) expression on cultured rat brain microvascular endothelial cells (BMECs). *In vivo* data showed that sICAM-1 serum levels at baseline significantly increased ($P < 0.01$) in 12 months of rIFN-β-1b treatment. The increase paralled a clinical and MRI improvement. In the second semester of the treatment the integrated area under the curve of Expanded Disability Status Score normalised to entry baseline (ΔEDSS AUC) was significantly ($P < 0.05$) smaller than in the first semester. The percentage of patients with Gd+MRI decreased significantly ($P < 0.05$) in the first (33%) and second (29%) semesters of treatment compared to baseline (62%). *In vitro* experiments showed that the incubation of BMEC monolayer with 100 u/ml of TNF-α for 24 h significantly ($P < 0.05$) increased mICAM-1 expression, whereas 2000 u/ml of rIFN-β-1b for 72 h did not modify the baseline levels. The incubation of BMEC with 2000 u/ml of rIFN-β-1b for 48 h followed by combined IFN-β-1b and TNF-α for 24 h significantly ($P < 0.05$) downregulated TNF-α-induced mICAM-1 expression. These results suggest that the effect of rIFN-β-1b on the BBB may be mediated by changes in both sICAM-1 serum levels and mICAM-1 BMEC expression. *Journal of NeuroVirology* (2000) **6,** S47–S51.

Keywords: multiple sclerosis; rIFN-β-1b; soluble ICAM-1; membrane-bound-ICAM-1; brain microvascular endothelial cells culture

Introduction

Disruption of the blood-brain barrier (BBB) is a consistent initiating event in the development of demyelinating lesions in relapsing remitting (RR) multiple sclerosis (MS) (Kermode *et al*, 1990). Soluble mediators such as interferon γ, interleukin-1-β, and TNF-α released by activated T-cells probably contribute to the BBB breakdown by upregulating the expression of adhesion molecules on BBB endothelium (Wong and Dorovini-Zis, 1992). The opening of the BBB, corresponding to infiltration of inflammatory cells and extravasation of blood-born molecules into white matter tissue of the CNS (Raine, 1991), can be measured by enhanced permeability to gadolinium (GD) on Magnetic Resonance Imaging (MRI) (Kermode *et al*, 1990).

Interferon-beta (rIFN-β) treatment of RR MS patients dramatically reduces Gd-enhancing lesions on MRI (Stone *et al*, 1995; Jacobs *et al*, 1996; PRISMS Study Group, 1998) suggesting that such treatment may inhibit BBB opening. While the mechanism for this has not been determined, recent studies (Defazio *et al*, 1998; Miller *et al*, 1996; Calabresi *et al*, 1997a,b; Soilu Hanninen *et al*, 1995) suggest that changes in cell adhesion molecules may be involved.

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rIFN-β-1b downregulates the TNF-α-induced expression of the ICAM-1 on human brain microvascular endhotelial cells (BMECs) (Defazio *et al*, 1998). A recent report (Calabresi *et al*, 1997b) shows a slight increase of serum circulating ICAM-1 (sICAM-1) and a significant increase of serum circulating vascular cell adhesion molecule-1. (sVCAM-1) levels which correlates with a decrease in the number of Gd-enhancing lesions on MRI during rIFN-β-1b treatment.

In the present study we examined both the changes of serum sICAM-1 levels over a 12 months period in 36 RR MS patients undergoing chronic rIFN-β-1b treatment, and the changes of the TNF-α-induced membrane-bound ICAM-1 (mICAM-1) expression on cultured rat BMECs due to rINF-β-1b. The rIFN-β-1b increased serum levels of sICAM-1 and decreased TNF-α-induced ICAM-1 expression on BMECs. Both these effects may act in reducing BBB damage in rIFN-β-1b-treated MS.

Results

rIFN-β effect on sICAM-1 serum levels

sICAM-1 serum levels at baseline (mean ± s.d. 238 ± 39 ng/ml; median 241 range 161 − 306) significantly increased in the 1st (mean ± s.d. 261 ± 33 ng/ml, median 254 range 199 − 325; $P < 0.001$) and in the 2nd months of treatment (mean ± s.d. 279 ± 39 ng/ml, median 277 range 221 − 346; 1st *versus* 2nd $P < 0.05$), and then persisted at higher levels than the baseline values at the 3rd (mean ± s.d. 270 ± 38 ng/ml, median 270 range 186 − 366; $P < 0.05$), the 9th (mean ± s.d. 278 ± 65 ng/ml, median 270 range 148 − 418; $P < 0.05$) and the 12th (mean ± s.d. 258 ± 38 ng/ml, median 253 range 191 − 350; $P = 0.05$) months. The serum sICAM-1 curve is reported in Figure 1A.

The integrated area under the curve of Expanded Disability Status Score normalised to entry baseline (ΔEDSS AUC) of the 2nd semester (mean ± s.d. −1.06 ± 2.98) of treatment resulted significantly ($P < 0.05$) lower than those of the 1st (mean ± s.d. −0.33 ± 2.19) (Figure 1B).

The percentage of patients with active (Gd+) scans at MRI significantly decreased from baseline values (62%) in the 1st (33%) semester of treatment and remained stable in the 2nd (29%) (baseline *versus* 1st and 2nd semesters $P < 0.05$). Seventeen patients experienced relapses, for a total of 26 relapses during the whole treatment period. The baseline frequency (44%) of patients with relapses decreased significantly ($P < 0.05$) in the 2nd (25%) semester of treatment.

rIFN-β effect on cultured BMEC mICAM expression

Confluent rat BMECs from passage 1 culture constitutively expressed at low level basally mICAM 1 molecules. TNF-α (100 u/ml for 24 h) significantly upregulated mICAM 1 expression.

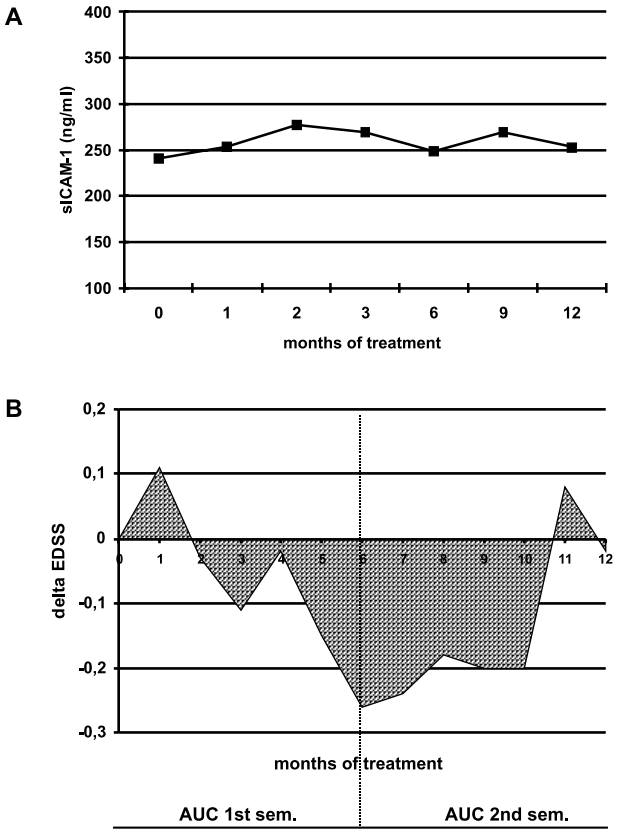

Figure 1 (**A**,**B**) Longitudinal evaluation of serum sICAM-1 (median) (**A**) and the ΔEDSS AUC (mean) (**B**) during 12 months of rIFN-β-1b treatment in 36 RR MS patients. (see text for statistics).

Culture exposure to 2000 u/ml rIFN-β-1b for 24 h failed to modify either basal mICAM 1 expression or, under simultaneous treatment, TNF-α-induced mICAM 1 expression. (Figure 2A). When co-incubation with rIFN-β-1b (2000 u/ml) and TNF-α (100 u/ml) for 24 h was preceded by 48 h treatment with rIFN-β-1b (2000 u/ml), a significant suppression of mICAM 1 elevation was achieved. Under the same treatment protocol, 500 − 1000 u/ml rIFN-β-1b failed to antagonise TNF-α effect. Treatment of BMECs with 2000 u/ml rIFN-β-1b for overall 72 h did not affect basal mICAM 1 expression (Figure 2B).

Discussion

In vivo data demonstrated that 1 year treatment with rIFN-β-1b increased serum sICAM-1 levels Calabresi *et al* (1997b) reported a slight increase of serum sICAM-1 and a significant increase of serum sVCAM-1 levels during rIFN-β-1b treatment which negatively correlated with the number of contrast-enhancing lesions at the MRI. Accordingly we found that sICAM-1 increase paralleled a period of disease improvement, as demonstrated by a significant

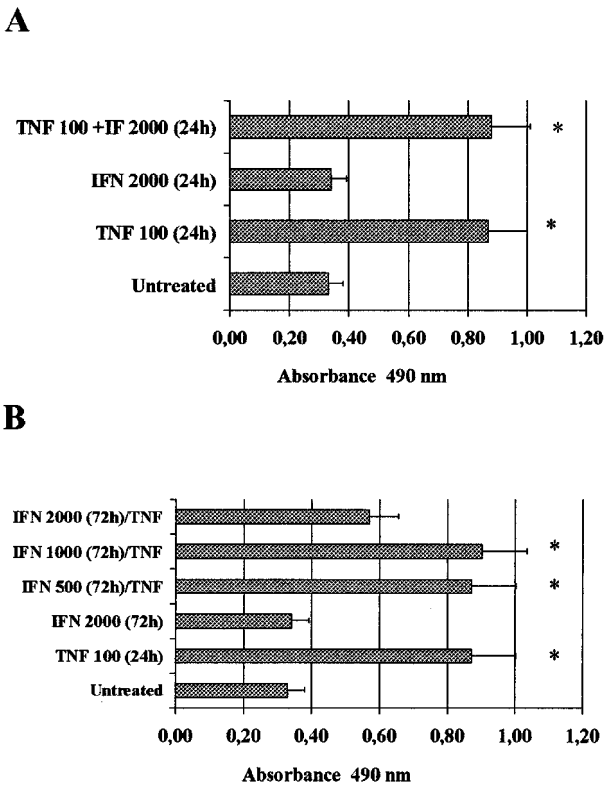

Figure 2 (**A**,**B**) Expression of ICAM 1 molecules on brain microvascular endothelial cells cultures was measured by ELISA following incubation with: (**A**) 100 u/ml TNF-α (TNF) for 24 h and/or 2000 u/ml rIFN-β-1b (IF) for 24 h; (**B**) 100 u/ml TNF for 24 h, 2000 u/ml rIFN-β-1b for overall 72 h, or rIFN-β-1b (500–2000 u/ml) for 48 h followed by a combined treatment with rIFN-β-1b and TNF for another 24 h. One-way ANOVA: (**A**) F=15.3, $P<0.0001$; Newman-Keuls test, *different from untreated and rIFN-β-1b 2000, $P<0.05$. (**B**) F=11.7, $P<0.0001$; Newman-Keuls test, *different from untreated, rIFN-β-1b 2000 (48 h)/TNF+rIFN-β-1b 2000 (24 h) and rIFN-β-1b 2000 (48 h)/rIFN-β-1b 2000 (24 h), $P<0.05$.

reduction in the number of patients experienced relapses and with Gd+MRI scans and, more importantly, by a decrease of the integrated ΔEDSS AUC, a more powerful and clinically meaningful than conventional methods of assessing small disability changes (Liu *et al*, 1998). The source and mechanism(s) responsible for the increase of serum ICAM-1 during chronic rIFN-β treatment remain unclear. Irrespective of the mechanisms involved, the rise of serum ICAM-1 may be relevant to effect of rIFN-β-1b on BBB permeability. In fact, serum circulating ICAM-1 seems to have a role of protection towards the BBB by reducing the T-cell traffic likely by a competitive mechanism with the endothelium-bound form (Trojano *et al*, 1996; Rieckmann *et al*, 1995).

In vitro data showed that TNF-α, at a concentration similar to the mean concentration found in the CSF of patients with active RR MS and BBB damage (Sharief and Thompson, 1992), upregulated mICAM 1 expression on cultured BMECs, whereas 24–72 h exposure to a concentration of IFN-β-1b (2000 u/ml) comparable to that reached in serum following the systemic administration of 8 MIU in MS patients (The IFN-β MS Study Group, 1993) failed to affect basal mICAM 1 expression. However when BMEC cultures were pretreated with rIFN-β-1b for 48 h prior to incubation with rIFN-β-1b and TNF-α for 24 h, rIFN-β-1b antagonised ICAM 1 upregulation due to TNF-α. Interestingly, a similar treatment schedule but a different dosage (1000 u/ml) was required for rIFN-β-1a antagonisation of TNF-α-induced ICAM-1 expression on the same cell culture (Defazio *et al*, 1998). The *in vitro* modulation of TNF-α-induced mICAM-1 expression by rIFN-β-1b may be relevant to the *in vivo* therapeutic effect of rIFN-β-1b in MS. In fact, disruption of the BBB occurs early in the course of MS (Kermode *et al*, 1990) and increased BMEC ICAM-1 expression may be at least partially responsible for the increased BBB. If similar changes are induced *in situ* on BMECs by TNF-α locally secreted by active T-cells, then the presence of high circulating levels of rIFN-β-1b can counteract the TNF-α effect and decrease BBB permeability. It is not known whether the mechanisms underlying the downregulation of TNF-α-induced ICAM-1 expression on BMECs and the increase sICAM-1 serum levels by rIFN-β-1b are reciprocally linked. rIFN-β might induce a proteolytic cleavage of the membrane bound molecules generating soluble ICAM-1 or, alternatively an increased production by microvascular endothelia (Kallmann *et al*, 1998) of circulating sICAM-1. Since the expression of membrane-bound ICAM-1 and sICAM-1 are differentially regulated in some cell types (Rokhlin and Cohen, 1996) and given that sICAM-1 has been postulated to arise from alternatively spliced mRNA transcripts (King *et al*, 1995) the chronic rIFN-β-1b treatment might favour the production of the free circulating form of ICAM-1 in endothelial cells. In conclusion, the effects of rIFN-β-1b on both sICAM-1 and mICAM-1 reported herein might almost partially explain the results of recent clinical trials (Stone *et al*, 1995; Jacobs *et al*, 1996) indicating an improvement of the BBB damage in rIFN-β-1b-treated MS. An understanding of new mechanisms by which rIFN-β acts may improve the use of this drug and lead to a better definition of the immuno-pathological processes underlying both the clinical and the MRI MS activities.

Materials and methods

sICAM-1 analysis in serum of rIFN-β-1b-treated MS patients

Patients Thirty-six (10 men and 26 women) definite (Poser *et al*, 1983) RR MS patients were selected for the study since were clinically inactive and steroid free in the 30 days preceding the entry

into the study, presented at least two exacerbations during the last 2 years, aged between 18 and 50 years and had a Kurtzke expanded disability status scale (EDSS) (Kurtzke, 1983) score ranging between 1 and 3.5. The patients had a mean disease duration of 5.51 ± 3.2 years, a mean age of 26.82 ± 4.96 years and a mean EDSS score of 2.39 ± 0.85. During the treatment period 17 patients experienced relapses, for a total of 26 relapses. rIFN-β-1b (Betaferon, Farmades) was administered s.c. every other day at a 8 MIU dosage for 12 months. Clinical relapses during the rIFN-β-1b therapy were treated with Methylprednisolone i.v. 1 g/day for 5 days. Clinical assessments including scoring for EDSS of treated MS patients were performed every 12 weeks and at the time of exacerbations. For each patient the ΔEDSS, obtained by subtracting the EDSS value at baseline from that observed at each follow-up, was calculated. The integrated area under the ΔEDSS-time curve (ΔEDSS AUC) (trapezoidal integration by Matlab) was then calculated (Liu *et al*, 1988) for each of the four trimesters of treatment.

MRI examination MRI of brain and spinal cord was performed in all MS patients before and every 6 months during rIFN-β-1b treatment, on a 1,5 TESLA scanner (Siemens Magnetom). T1-weighted spin echo (TR 2200 msec, 80 msec) and T2-weighted spin echo (TR 600 msec, 15 msec) images were obtained, with a 256×256 matrix and a 5 mm slice tickness. Gd-DTPA was given intravenously in a dose of 0.2 mmol/kg. Presence of Gd-DTPA-enhancing lesions were determined by an experienced neuroradiologist, blind with regard to clinical data, about 15–20 min after contrast injection.

Serum sampling Peripheral blood samples were obtained prior to treatment (baseline), monthly in the first 3 months of the treatment, and quarterly thereafter. The samples were collected in ethylenediamine tetra-acetic acid test tubes, kept in ice until the centrifugation at 3000 r.p.m. for 10 min and then aliquoted and stored at -80°C.

sICAM-1 analysis sICAM-1 serum levels were evaluated by dual antibody solid phase enzyme-linked immunoassay (ELISA) according to the manufacturer's instructions (Medgenix Diagnostics SA, Fleurus, Belgium) (Trojano *et al*, 1996). Absorbances were read within three hours (BIORAD 3550 Microplate reader) at 450 nm, and concentration calculated upon a linear-linear standard curve by appropriate software (BIORAD). The detection limit for sICAM was 0.3 ng/ml. The mean values for intra-assay and inter-assay variabilities ranged between 5 and 10%.

mICAM-1 analysis in rIF-β-1b and TNF- treated cultured rat BMECs BMECs were isolated and cultured according to the method described by Carson and Haudenschild (1986) with some modifications as previously reported (Defazio *et al*, 1998). The endothelial nature of cultured cells was assessed by immunocytochemical staining for factor VIII-related antigen (Defazio *et al*, 1998). Approximately 96% of cells in confluent passage 1 cultures had fibroblast-like appearance, and showed typical granular cytoplasmic immunofluorescence with Factor VII antiserum. Human recombinant rIFN-β-1b (gently supplied by Farmades spa, Rome, Italy) was stored as a powder at +4°C. Immediately before use, it was diluted into growth medium to a final concentration of 500–2000 u/ml. Human recombinant TNF-α (Sigma, EC50 0.024 ng/ml) was aliquoted into growth medium and stored at -70°C until use. Prior to each experiment, it was thawed and diluted in growth medium to 100 u/ml, a concentration similar to the mean TNF-α concentration found in the CSF of patients with RR MS and BBB damage (Sharief and Thompson, 1992). BMECs were exposed to TNF-α, rIFN-β-1b or to a combination of rIFN-β-1b and TNF-α for 24 h, a time at which upregulation of ICAM 1 elicited by TNF-α reach maximal levels (Wong and Dorovini-Zis, 1992). ICAM 1 expression was subsequently assessed. In separate experiments coincubation with rIFN-β-1b and TNF-α for 24 h was preceded by 48 h incubation with rIFN-β-1b alone.

Membrane bound ICAM 1 expression on BMEC monolayers grown in 96 wells was assessed by ELISA as reported (Defazio *et al*, 1998). Cells were sequentially incubated (2 h at room temperature) with 1 : 20 mouse anti-rat ICAM 1 (Seikagaku, Japan) and with 1 : 3000 peroxidase conjugated rabbit anti mouse lgG (H+L) (Jackson Immunoresearch). Primary antibody control was an irrelevant isotype-matched mAb. Absorbance was measured at 490 nm on a ELISA Microtiter Plate Reader (BIO-RAD). Background of the isotype control (ranging from 0.095 to 0.123 in different experiments) was subtracted by data presented in Figure 2. Values were expressed as mean and standard deviation of triplicate cultures from a representative experiment repeated twice.

Statistical analysis The nonparametric Friedman test (a two-way analysis on ranks) followed by the Dunn's *post-hoc* test were used to test multiple comparison between longitudinal evaluations of serum sICAM-1 and of the ΔEDSS AUCs during the treatment. Fisher's exact test was used to test the differences between the frequencies of patients with relapses and with Gd+MRI scans before and during the treatment. One-way ANOVA followed by a *post hoc* Newman-Keuls test was used to compare the means of mICAM-1 expression on cultured BMECs in the different experiments. P values < 0.05 were considered significant.

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