Interferon receptor expression in multiple sclerosis patients

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Abstract

To determine the gene expression of IFNAR1, IFNAR2 and MxA protein and the association with IFNβ treatment response in MS patients.

MS patients treated with IFNβ had a significant decrease in IFNAR1 and IFNAR2 expression, and a significant increase in MxA compared to non-treated patients and healthy controls. Also, those patients who had a good response to treatment had a significant decrease in IFNAR1 and IFNAR2 expression compared to non-responders, non-treated patients and healthy controls.

IFNβ influences the expression of its receptors, and is greater in patients who respond to IFNβ treatment. This down-regulation could be indicative of the response to IFNβ.

Keywords: IFNAR; Multiple sclerosis; Interferon beta; MxA

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) (Polman and Uitdehaag, 2000). The disease is characterised by different patterns of lesions, involving the myelin sheath or oligodendrocyte destruction, but axonal lesion also occurs from onset, correlating with long-term irreversible disability (Polman and Uitdehaag, 2000; Trapp et al., 1999). Immune factors are involved in the pathogenesis of MS, suggesting that treatment should be directed at re-establishing the immune alterations. Interferon beta (IFNβ) is a cytokine that possesses immunomodulatory activity (Pestka et al., 1987). The biological activity of IFNβ takes place by interaction with high-affinity cell-surface receptors. All type I interferons (alpha, beta and omega) share the same surface

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receptor, composed of two subunits, IFNAR1 and IFNAR2, each formed by an extracellular transmembrane and intracytoplasmic domains (Oritani et al., 2001). These two subunits are not pre-associated on the cell surface, but interaction with the ligand of one subunit leads to binding of the other, which in turn activates the intracellular signalling cascade (Croze et al., 1996). The different type I interferons have different cellular effects upon interaction with the same receptor, as their binding sites differ (Platanias et al., 1996). All type I IFNs activate receptor-associated tyrosine kinases, Jak1 and Jak2, which in turn activate a series of latent, cytoplasmic transcriptional activator proteins known as Stat (Joe and Lau, 2002). These all result in the induction of several proteins responsible for the biological function of interferons, such as the myxovirus resistance protein A (MxA) (von Wussow et al., 1990).

Two forms of recombinant IFNβ (IFNβ-1a and IFNβ-1b) have been approved for the treatment of relapsing-remitting MS by the FDA. The main difference is that IFNβ-1b is a non-glycosylated recombinant product in which serine is

substituted for cysteine at position 17 (Runkel et al., 1998). These differences have repercussions on the immunological response (Fernández et al., 2001), but not on their efficacy in MS patients (The IFN-β Multiple Sclerosis Study Group, 1993; PRIMS, 1998; PRISMS study group, 2001). However, a percentage of MS patients are considered suboptimal or non-responders to this treatment. One of the explanations for this lack of response could be the presence of neutralising antibodies against IFNβ (NABs), (Redlich et al., 1991) the clinical significance of which remains controversial. Some authors found an association between NABs and lack of clinical response (Pachner et al., 2003; Rudick et al., 1998; The IFN-β Multiple Sclerosis Study Group, 1996; Sorensen et al., 2003) after at least 18 months of therapy (Vartanian et al., 2004), whereas others found no definitive association (Fernández et al., 2001; Antonelli et al., 1998; Mayorga et al., 1999), most probably due to low sample sizes. Another reason might be a low IFN receptor expression in patients, which would hinder the biological activity of IFNβ, especially if we consider the low number of IFNAR on the cell surface compared to receptors for other substances (Novick et al., 1994).

The purpose of this study was to determine the relative gene expression of the two subunits of type I IFN receptor as well as the MxA protein in peripheral blood cells from MS patients, with and without IFNβ treatment, using Real Time-PCR, and to analyse the association between these expressions and the response to IFNβ treatment.

2. Material and methods

2.1. Subjects

2.1.1. Patients

The study included Caucasian MS patients from Carlos Haya Hospital cohort, over a three-year period (2003-2005) treated with IFNβ-1a (Avonex, Biogen, Cambridge, MA and Rebif, Serono SA Laboratorios, Madrid, Spain) or IFNβ-1b (Betaferon, Schering AG, Berlin, Germany) and patients who had not yet started therapy. The clinical data were monitored throughout the treatment, and the annual relapse rate, expressed as the total number of relapses in a year per group with respect to the total number of patients in that group, was established. Exacerbations and Expanded Disability Status Scale (EDSS) scores were recorded by the same neurologist evaluator. Relapses were defined according to the Poser criteria (Poser et al., 1983). Patients treated with IFNβ were classified as suboptimal responders or non-responders if they had one or more relapses or an increase in sustained progression of 0.5 or more points on the EDSS after one year of treatment compared with the year prior to IFNβ therapy.

Demographic and clinical characteristics comprised sex, age, age at disease onset, disease duration, MS type according to Lublin's classification (Lublin and Reingold, 1996), treatment onset, relapse rate, EDSS score (Kurtzke, 1983), time of progression for the secondary-progressive (SP) patients, type of IFNβ preparation, time interval between disease onset and treatment, and time interval between starting treatment and sample collection.

2.1.2. Healthy control group

This consisted of 30 age- and sex-matched healthy subjects.

2.2. Sample collection

For each subject, 15 ml of peripheral blood was collected 24 h after the last IFNβ injection; 5 ml in an EDTA tube (Vacutainer, Becton Dickinson, Meylan Cedex, France) was processed immediately for separation of peripheral blood mononuclear cells (PBMC) and 10 ml in clot activator tubes (Vacutainer) to obtain serum for the antibody determinations, stored at − 20 °C till use.

2.3. RNA extraction and cDNA synthesis

PBMC were isolated on a Ficoll-Hypaque density gradient (Sigma, St Louis, MO) and total RNA was extracted as described (Chomczynski and Sacchi, 1987). Complementary DNA (cDNA) was obtained using 20 U MMLV (Roche Diagnostics, Penzberg, Germany), 1 mmol/L each of the dNTPs (Boehringer Mannheim), 10 U of RNase inhibitor (Roche Diagnostics), 4 μg hexamer primer (Roche Diagnostics), 10× PCR buffer (Boehringer Mannheim), and $MgCl_2$ (5 nmol/L) in a final volume of 20 μL. The reverse transcription was performed at 42 °C for 60 min followed by 70 °C for 15 min with a thermocycler (Perkin-Elmer Corp, Irvine, CA). The cDNA was stored at − 80 °C until use.

2.4. Real time-PCR

This was carried out using 10 mM of specific oligonucleotide primer pair sequences for IFNAR1, IFNAR2, MxA and PBGD (porphobilinogen deaminase) synthesised by Proligo Primers and Probes (Proligo, Paris, France): for IFNAR1 5′-AGAAGTACATTTAGAAGCTG-3′ and 5′-AGTGCTGCTTTAACTTT-3′, for IFNAR2 5′-CACCAGAGTTTGAGATTGT-3′ and 5-′AAGGGAGACTTTATTACTGCT-3′, for MxA 5′ ACCTACAGCTGGCTCCTGAA-3′ and 5′-GCACTCAAGTCGTCAGTCCA-3′, and for PBGD 5′-TCCAAGCGGAGCCATGTCTG-3′ and 5′-AGAATCTTGTCCCCTGTGGTGGA-3′. PBGD was used to normalise PCR products. For the LightCycler reaction, FastStart DNA master SYBR Green I kit was used following the manufacturer's indications (Roche Diagnostic). The experimental run protocol used for all the gene expressions analysed was the same, apart from the annealing temperature, which was 52 °C for IFNAR1 and IFNAR2, and 60 °C for PBGD and MxA. The amplified products were confirmed by specific melting curves (IFNAR1, 78.7 °C; IFNAR2, 79.8 °C; MxA and PBGD, 88 °C) verified in each run. Quantification was

performed by crossing-point extrapolation into a standard curve with known cDNA concentrations. The results were finally presented as the ratio of each subunit expression with respect to PBGD expression: IFNAR1/PBGD, IFNAR2/PBGD and MxA/PBGD.

2.5. Determination of neutralising antibodies (NAB) to IFN-β

The assay was made as previously described (Mayorga et al., 1999). Briefly, plates (Nunc, Roskilde, Denmark) were seeded with a human epidermal cell line (HEP-2) at 1.5×10^5 cells/ml incubated for 24 h. Previously inactivated sera were mixed at two-fold serial dilutions (ranging from 1:5 to 1:640) with 100 IU/ml of IFNβ-1b or 20 IU/ml of IFNβ-1a (differences in the IFNβ concentrations used are due to the different specific activity of the two IFN-β preparations) and incubated for 1 h and then 100 μl/well was added to the monolayer culture. A virus control (without interferon) and a cell control (without interferon or virus) were added to each plate. The plates were incubated overnight and then all infected with vesicular stomatitis virus, Indiana strain, except for the cell control wells. After incubating the plates for 24 h, the contents were discarded and 100 μl of crystal violet stain (Merck, Darmstadt, Germany) was added to each well. After 15 min the plates were rinsed and washed with water. Then 100 μl of acetic acid 33% was added and read at 630 nm in a spectrophotometer. NAB levels were expressed as the reciprocal of the dilution serum producing 50% inhibition of the IFN-β action in the virus cytopathic effect.

2.6. Statistical analysis

Descriptive statistics included mean and standard deviation for quantitative variables and relative frequency for qualitative variables. Kruskal-Wallis test was applied to compare quantitative non-related variables, and Pearson chi-square for qualitative variables. Spearman test was used to analyse the correlation between quantitative variables. All *P* values represented two-tailed tests, with values b 0.05 considered statistically significant. The statistical analysis was performed using the SPSS program, version 11.5.

3. Results

The study included 250 MS patients, 84 men (mean age, 41.4 ± 9.14 years), and 166 women (mean age, 39.3 ± 11.2 years). One hundred and eighty-two patients (73.76%) had RRMS and 63 (26.24%) SPMS. The patients were classified into two groups according to treatment: 219 treated with IFNβ and 31 not treated. Significant differences were found in evolution time ($P = 0.003$) and sex ($P = 0.028$) comparing treated versus non-treated MS patients by chi-square analysis. Nevertheless, no significant differences in age ($P = 0.327$) or MS clinical form ($P = 0.157$) were found by the Student *t* test.

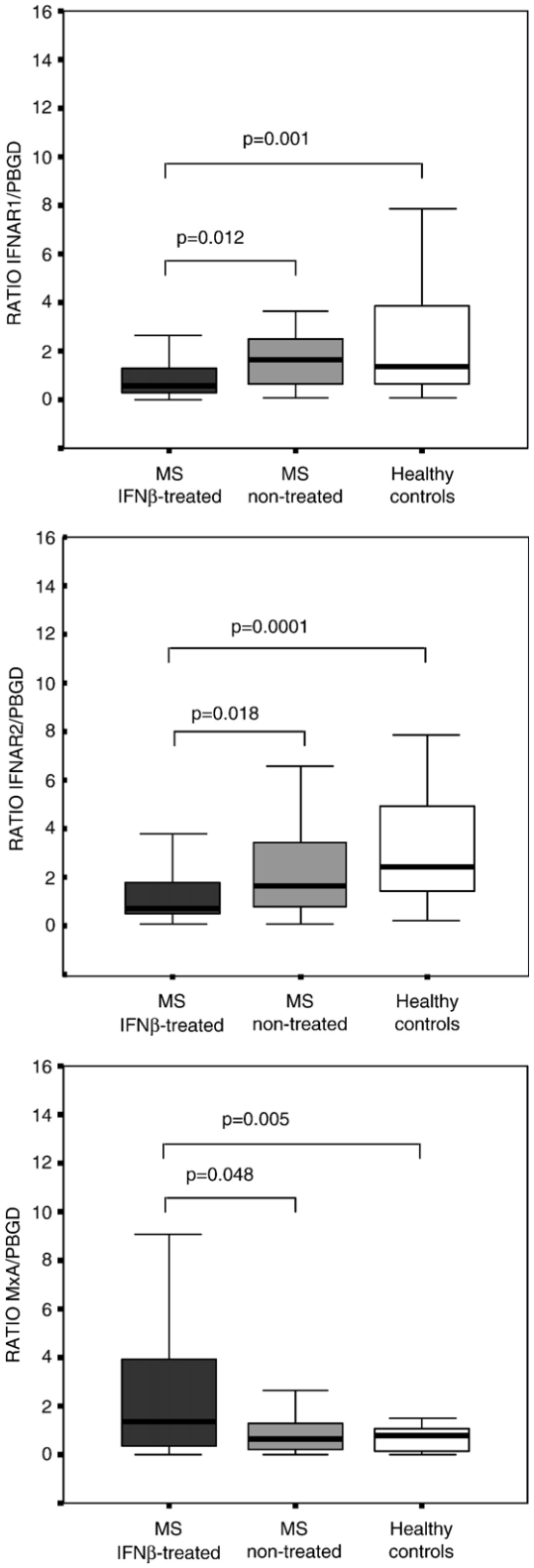

Fig. 1. Box plots of the IFNAR1, IFNAR2 and MxA mRNA expression in MS patients treated with IFNβ, non-treated MS patients and healthy controls. Differences (*) are considered significant with a *P* b 0.05.

3.1. Influence of IFNβ treatment on IFNAR1, IFNAR2 and MxA expression

Because the expression of IFNAR1, IFNAR2 and MxA did not follow a normal distribution, we used a non-parametrical test. We found no significant differences in the expression of IFNAR1, IFNAR2 and MxA in non-treated MS patients compared to healthy controls, although a pronounced decrease in IFNAR2 expression was detected in non-treated MS patients (Fig. 1).

Comparison of the three gene expression levels in IFNβ treated patients showed that IFNAR1 expression significantly decreased in MS patients treated with IFNβ (median:0.54, IR:0.30-1.1) when compared to non-treated patients (median:1.56, IR:0.51-2.56) or healthy controls (median:1.36, IR:0.64-3.99), $P = 0.012$ and $P = 0.001$, respectively. IFNAR2 expression levels were also significantly decreased in those MS patients treated with IFNβ (median:0.76, IR:0.48-1.89) compared to non-treated patients (median:1.65, IR:0.76-3.49) or healthy controls (median:2.49, IR25:1.43-IR75:5.1), $P = 0.018$ and $P = 0.0001$, respectively (Fig. 1).

Measurement of MxA protein expression showed a significant increase in patients treated with IFNβ (median:1.31, IR:0.37-3.99) compared to non-treated patients (median:0.64, IR:0.017-1.34) or healthy controls (median:0.79, IR:0.064-1.101), $P = 0.048$ and $P = 0.005$, respectively (Fig. 1).

The MS patients were treated with three different IFNβ molecules (59 treated with IFNβ-1b (Betaferon), 61 with IFNβ-1a (Avonex), and 99 with IFNβ-1a (Rebif)). We found a significantly higher disease duration in patients treated with Betaferon compared to Avonex ($P = 0.001$) and Rebif ($P = 0.001$) but no differences in sex or age for any of the groups included. Comparisons of the expression of the two IFNAR subunits and the MxA in MS patients receiving each IFNβ treatment showed no significant differences.

3.2. Association of IFNAR1, IFNAR2 and MxA expression with the response to IFNβ treatment in MS patients

Of the 219 MS patients treated with IFNβ, 147 (67.2%) were considered as responders to the treatment and 72 (32.8%) as non-responders according to the criteria indicated in Material and methods. No significant differences in sex, age, EDSS or relapse rate at the beginning of treatment, disease duration prior to treatment, or time between starting treatment and sample collection were found between both groups (responders and non-responders) of treated patients. Although we found that the relapsing-remitting form was slightly more frequent in the responder group (77.4%) than in the non-responders (63.6%), the difference was not significant ($P = 0.82$) (Table 1).

Comparisons with all the groups included in the study showed significant differences by Kruskal-Wallis test for the gene expression of the two subunits of the IFNβ receptor (IFNAR1 and IFNAR2) and the inducible protein MxA, ($P = 0.001$, $P = 0.001$ and $P = 0.012$, respectively). Two-by-two comparisons for IFNAR1 showed a significant decrease in MS patients considered as responders (median:0.51, IR:0.31-1.02) compared to non-responders (median:0.91, IR:0.33-1.01) ($P = 0.014$), MS non-treated (median:1.56, IR:0.51-2.56) ($P = 0.003$) and healthy controls (median:1.36, IR:0.64-3.99) ($P = 0.001$). For IFNAR2, we again found a significant decrease in responders (median:0.71, IR:0.49-1.61) compared to non-responders (median:1.04, IR:0.46-3.26) ($P = 0.042$), MS non-treated (median: 1.65, IR:0.76-3.49) ($P = 0.004$) and healthy controls (median:2.49, IR:1.43-5.1) ($P = 0.0001$). In this case, a significant decrease was also found in non-responders compared to healthy controls ($P = 0.002$). The MxA expression showed significant increases in responders (median: 1.23, IR:0.39-3.7) and non-responders (median:1.91, IR:0.34-4.09) only compared to healthy controls (median:0.79, IR:0.64-1.1) ($P = 0.009$ and $P = 0.003$, respectively). These data are shown in Fig. 2. Correlation analysis by Spearman test showed a significant negative association between IFNAR2 expression level and the response to treatment ($P = 0.026$).

3.3. IFNAR1, IFNAR2 and MxA expression in PBMC depending on the presence of NABs

The percentage of NABs and the clinical form are shown in Table 1. We determined the presence of NABs in the 219 treated patients included in the study and detected NABs in 28 patients (12.7%), whereas no presence of NABs was detected in 191 patients (87.2%). In these two groups (presence or absence of NABs), we analysed the association with the expression of IFNAR1, IFNAR2 and MxA, but found no significant differences.

Table 1
Clinical data of the MS patients included in the study and the percentage of NABs positivity in the different groups

MS patients		Age (years)	Evolution time mean±SD	Treatment duration mean±SD	Positive NABs	Clinical form (%)	
						RR	PS
Non-treated $n=31$ (12.4%)		37.44±11,76	8.07±7.36	–	0	85.7	14.3
IFNβ Treated $n=219$ (87.6%)	Responders $n=147$ (67.2%)	40.29±11.0	12.31±7.73	3.34±2.20	12.3	77.4	22.6
	Non-responders $n=72$ (32.8%)	39.54±9.49	11.72±6.92	4.20±2.19	13.1	63.4	36.6

Evolution time: time interval between disease onset and sample collection (years).
Treatment duration: time interval between onset of treatment and sample collection (years).

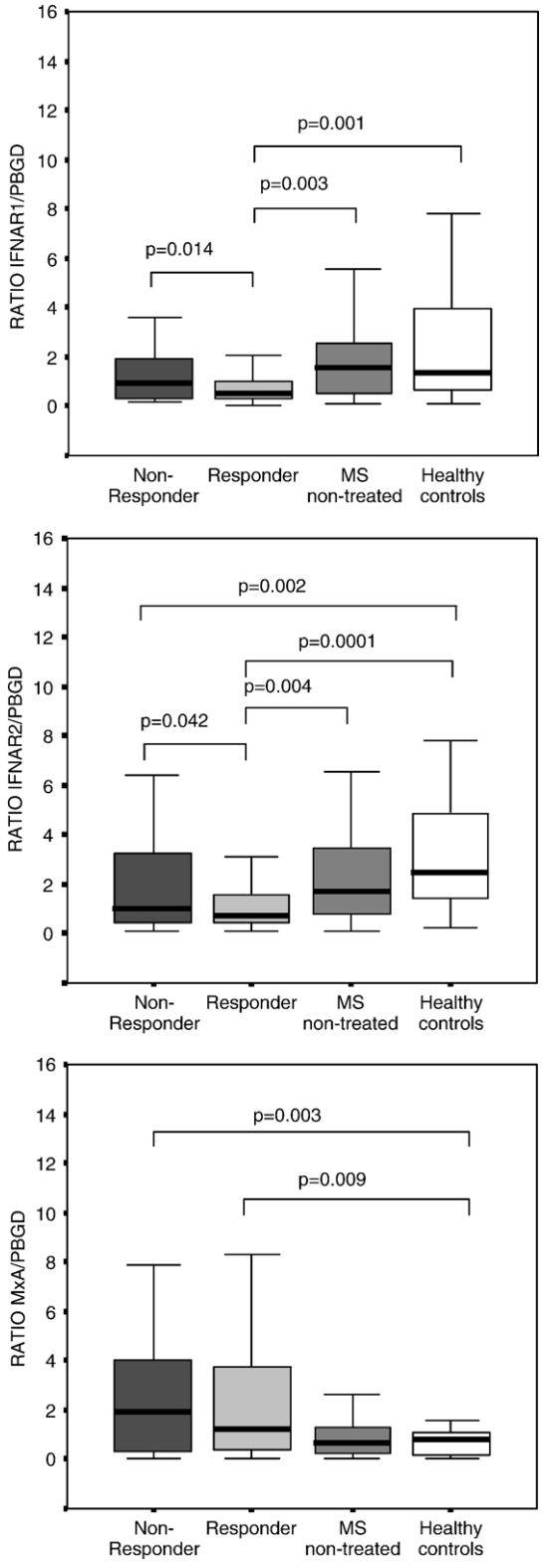

Fig. 2. Box plots of the IFNAR1, IFNAR2 and MxA mRNA expression in MS patients, classified as responders or non-responders to IFNβ, non-treated MS patients and healthy controls. Differences (*) are considered significant with a $P \leq 0.05$.

4. Discussion

IFNβ treatment has important benefits in MS, decreasing the relapse rate and the lesions measured by MRI, and probably also slowing the accumulation of disability (Polman and Uitdehaag, 2000; Weinshenker et al., 1989). There is, however, a high degree of variability in the response, and about 30% of patients fail to respond, or respond suboptimally to IFNβ therapy. No consensus exists regarding the definition of response to IFNβ treatment and no surrogate clinical marker of response has yet been found; consequently, criteria for evaluation of IFNβ efficacy are still a matter of debate. We decided to base our criteria for response on a deviation from the reported natural history of the disease. The reported natural history of MS, in several series of patients (Weinshenker et al., 1989; Fernández et al., 2005), sets a mean annual relapse rate of one relapse per year and a mean progression index of 0.3-0.5 points per year. Taking this into account, the cut-off point for suboptimal response was fixed for the non-responders at an increase after one year of treatment of one or more relapses or progression of 0.5 or more points on the EDSS score, compared to the year before the start of IFNβ treatment. These agree with the conclusions of two recent international consensus meetings about therapeutic responses (Cohen et al., 2004; Freedman et al., 2004).

One possible limitation of our study is related to the longer disease evolution of the treated patients compared to the non-treated patients. Although this was significantly different ($P = 0.003$), it did not seem to have important implications in our results since no association between disease duration and IFNAR1 or IFNAR2 expression levels was found in MS patients, whether treated or not.

The reasons for this lack of response remain unclear, despite important efforts (Fernández et al., 2001; The IFN-β Multiple Sclerosis Study Group, 1996; Sorensen et al., 2003; Vartanian et al., 2004; Antonelli et al., 1998; Mayorga et al., 1999). One reason could be the development of NABs, which are associated with reduced clinical efficacy of IFNβ (Perini et al., 2004). Nevertheless, we now know that these antibodies are responsible for only a small percentage of the lack of therapeutic response, so other reasons must underlie this lack of response. On the other hand, development of NABs does not seem to influence the expression of IFNAR1 or IFNAR2. Other studies have found a lower expression of MxA in NAB-positive patients compared with NAB-negative patients, demonstrating that NABs abolish interferon activity (Bertolotto et al., 2003). We did not find these results in our study, maybe because the samples were collected 24 h after the last IFNβ injection instead of 12 h, when the maximum expression of this protein is observed, or simply, due to small sample size.

As mentioned, IFNβ exerts its biological responses via a cell-surface receptor comprising two subunits, IFNAR1 and IFNAR2. A lower production of type I interferon receptors in

response, since even in healthy subjects, IFNAR1 and IFNAR2 are expressed at very low levels on the majority of T cell subsets (Novick et al., 1994; Pogue et al., 2004), providing a short safety margin for response. In this study we examined the mRNA expression of these subunits in MS patients and the influence of IFNβ on these levels. We found that non-treated MS patients did not differ significantly from healthy controls with respect to IFNAR1, IFNAR2 and MxA expression. On the contrary, IFNβ-treated MS patients showed a significant decrease in the expression of both subunits, with a parallel increase in the expression of MxA when compared to healthy controls and non-treated MS patients. This is in accordance with the physiological pathway of IFNβ activity, where a down-regulation and degradation of these subunits occur after IFNβ-IFNAR interaction (Branca and Baglioni, 1982; Ito et al., 2004). Receptor down-regulation is thought to be caused by post-translational events, such as internalisation and degradation of protein (Constantinescu et al., 1995).

On the other hand, we found that MxA expression was higher in IFNβ-treated patients compared with non-treated patients and healthy controls, and the MxA expression in non-treated patients did not differ significantly from control subjects, as has been reported (Kracke et al., 2000). This increased expression of MxA is probably a consequence of an appropriate interaction between IFNβ and its receptor, as well as the subsequent activation of the signalling cascade.

Regarding the influence of the receptor expression on the clinical effects of IFN therapy, in chronic hepatitis C, a higher decrease in IFNAR expression has been found in those patients who respond better to treatment compared to those who respond poorly (Morita et al., 1998; Fukuda et al., 1997). In our study, although the disease is different, we found similar results, with a strong decrease in the expression of the two IFNAR subunits in patients classified as responders compared to non-responders, indicating a better IFNβ-IFNAR interaction in responders, in whom exposure to exogenous IFNβ leads to the down-regulation of IFNAR expression. This mechanism tends to fail in non-responders, probably due to a primary defect in production of IFNAR transcripts (Massirer et al., 2004). Surprisingly, in our series, MxA expression did not differ significantly between responders and non-responders. This is apparently in disagreement with previous results from other authors, where MxA values in responders were significantly higher than in non-responders (Kracke et al., 2000). These differences, as commented previously, are probably attributable to differences in time of determination or sample size.

Different type I IFNs exert different biological effects, which depend on the type of interaction with the two IFNAR subunits (Platanias et al., 1996). In the case of IFNβ, the main interaction takes place with IFNAR2 (Croze et al., 1996), although IFNAR1 is also necessary. In patients with chronic hepatitis, the most important finding is that IFNAR2 down-regulation correlates well with the clinical response to IFN-alpha therapy. To this extent, we found that although both IFNAR1 and IFNAR2 expression significantly decreased in responders, only IFNAR2 expression correlated with the response to IFNβ treatment (Spearman test). This confirms the importance of IFNAR2 expression in the response to IFNβ treatment in MS patients as well as the fact that IFNAR2 expression is more highly regulated than IFNAR1 expression by IFNβ.

In summary, this study shows that treatment of MS patients with IFNβ influences the expression of its receptor, inducing a down-regulation in the expression of the two subunits of the receptor and that this regulation is more important in patients who respond to IFNβ treatment, possibly because it binds more efficiently to its receptor. These findings suggest that modulation of the expression of the two IFNAR subunits may be one of the main mechanisms for regulation of cell responsiveness to IFNβ. Further studies are necessary to clarify whether the response to treatment is influenced by changes in the level of the type I IFN receptor depending on the pathological alteration of gene expression, or on its ligand-receptor interaction (affinity, specificity, etc). Whatever the situation, studies to detect these receptors might help to predict the treatment response in MS patients.

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