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Neutralizing antibodies against IFN-β in multiple sclerosis: antagonization of IFN-β mediated suppression of MMPs

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Summary

Neutralizing antibodies (NAb) against interferon-β (IFN-β) develop in about a third of treated multiple sclerosis patients and are believed to reduce therapeutic efficacy of IFN-β on clinical and MRI measures. The expression of the interferon acute-response protein, myxovirus resistance protein A (MxA) is a sensitive measure of the biological activity of therapeutically applied IFN-β and of its reduced bioavailability due to NAb. However, MxA may not be operative in the pathogenesis of multiple sclerosis or the therapeutic effect of IFN-β. Instead, matrix metalloproteinases (MMPs) are increased in brain tissue, CSF and blood circulation of multiple sclerosis patients and function as effector molecules in several steps of multiple sclerosis pathogenesis. One of the molecular mechanisms by which IFN-β exerts its beneficial effect in multiple sclerosis is reduction of MMP-9 expression and increase of its endogenous tissue inhibitor, TIMP-1. Quantitative PCR measurements of MMP-2 and MMP-9, TIMP-1 and TIMP-2, and MxA were performed in peripheral mononuclear cells from clinically stable multiple sclerosis patients with relapsing remitting disease course after short-term and long-term treatment with IFN-β. IFN-β therapy down-regulated the expression of MMP-9 and abolished that of MMP-2 in long-term, but not short-term treated multiple sclerosis, while levels of MxA were increased in both instances. The presence of NAb reversed these effects, i.e. led to reduced MxA and increased MMP-2/MMP-9 expression levels compared with NAb– patients. In contrast, expression of TIMPs in peripheral blood mononuclear cells remained unaffected by IFN-β therapy and the presence of NAb. While MxA is able to detect the biological action and reduced bioavailability of IFN-β on the basis of single injections, only MMP-9 shows quantitative correlation with the NAb titre. Together with evidence that an imbalance between MMP and TIMP expression is a crucial pathogenetic feature in multiple sclerosis, these findings support the concept of a significant role of NAb in reducing the therapeutic efficacy of IFN-β.

Keywords: multiple sclerosis; IFN-β; neutralizing antibodies; MMP; MxA

Abbreviations: CPE = cytopathic effect; C_T = cycle threshold; GAPDH = glyceraldehyde phosphate dehydrogenase; IFN-β = interferon-β; im = intramuscular; LU = laboratory units; MMP = matrix metalloproteinase; MxA = myxovirus resistance protein A; NAb = neutralizing antibodies; PBMC = peripheral blood mononuclear cells; RR = relapsing remitting; sc = subcutaneous; SP = secondary progressive; TIMP = tissue inhibitor of metalloproteinases.

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Introduction

Interferon-β (IFN-β) is the first drug established as an effective treatment for patients with relapsing remitting (RR) and secondary progressive (SP) multiple sclerosis (IFNB Multiple Sclerosis Study Group and University of British Columbia Multiple Sclerosis/MRI Analysis Group, 1995; Jacobs *et al*., 1996; European Study Group on Interferon β-1b in Secondary Progressive Multiple Sclerosis, 1998; PRISMS Study Group and University of British Columbia Multiple

Sclerosis/MRI Analysis Group, 2001). As IFN-β does not restore pre-existing structural damage and functional impairment, but rather acts by attenuating damaging effects of inflammatory processes, continuous administration is required to maintain therapeutic effects. Such long-term therapy leads to the development of binding antibodies (BAb) and neutralizing antibodies (NAb) in up to 80% of patients (for a review, see Giovannoni *et al*., 2002). A number of reports have addressed the biological significance of the latter on clinical and MRI measures; most of these suggest decreased treatment efficacy of IFN-β (IFNB Multiple Sclerosis Study Group and University of British Columbia Multiple Sclerosis/MRI Analysis Group, 1996; Rudick *et al*., 1998; PRISMS Study Group and University of British Columbia Multiple Sclerosis/MRI Analysis Group, 2001; Polman *et al*., 2003). Interferon-induced proteins such as neopterin, β_2-microglobulin, 2′-5′ oligoadenylate synthetase and myxovirus resistance proteins (MxA and MxB) show an acute up-regulation after IFN-injection and can serve as a measure of its biological activity (Chiang *et al*., 1993; Witt *et al*., 1993; Williams *et al*., 1998). Persistent NAb were shown to attenuate the induction of MxA on the protein (Deisenhammer *et al*., 1999; Vallittu *et al*., 2002) and transcriptional level (Bertolotto *et al*., 2003) in peripheral blood mononuclear cells (PBMC) from multiple sclerosis patients.

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent endoproteinases that act as effector molecules in several steps of multiple sclerosis pathogenesis (for a review, see Kieseier *et al*., 1999; Leppert *et al*., 2001). The subfamily of gelatinases (MMP-2 and MMP-9) mediates the opening of the blood–brain–barrier and the extravasation of immune cells into the brain parenchyma in animal and *in vitro* models (Gijbels *et al*., 1994; Leppert *et al*., 1996; Stüve *et al*., 1996). Other features of MMPs related to multiple sclerosis pathogenesis are their ability to regulate the activity of several cytokines and their receptors, and of adhesion molecules (Schönbeck *et al*., 1998; for a review, see Leppert *et al*., 2001) and to lyse myelin components thus contributing to epitope spreading through the release of immunogenic degradation products (Proost *et al*., 1993). In relapsing remitting (RR) and secondary progressive (SP) multiple sclerosis, levels of MMP-9 are permanently increased in brain tissue, CSF and serum (Leppert *et al*., 1998; Lee *et al*., 1999; Lindberg *et al*., 2001; Waubant *et al*., 1999, 2003). IFN-β decreases the transcriptional and protein expression of MMP-2 and MMP-9 in T-cells and monocytic cells, with the functional effect of reduced capacity to cross a model blood–brain–barrier (Leppert *et al*., 1996; Stüve *et al*., 1996; Galboiz *et al*., 2001, 2002; Schmidt *et al*., 2001; Nelissen *et al*., 2002). In multiple sclerosis patients, IFN-β therapy down-regulates increased serum levels of MMP-9 and increases those of its endogenous tissue inhibitor TIMP-1 (Waubant *et al*., 1999, 2003), thus correcting excessive proteolytic activity as a molecular mechanism of its therapeutic effect. In return, we hypothesized that reduced bioavailability of IFN-β due to NAb may not only result in decreased MxA synthesis, but also in attenuation of IFN-β mediated suppression of MMP-2 and MMP-9.

The purpose of this study was to: (i) test for the effect of short-term (12 h after injection) and long-term (>3 months) treatment of IFN-β on the transcriptional regulation of MMP-2 and MMP-9, and their respective endogenous inhibitors TIMP-2 and TIMP-1; (ii) determine whether the occurrence of NAb affects expression levels of MMPs/TIMPs; and (iii) correlate the expression of MMPs/TIMPs with those of MxA and NAb titres.

Methods

Patients and control subjects

This study was approved by the Regional Ethical Committee of Piedmont. Informed consent was obtained from each patient before beginning the study.

One hundred and four clinically stable patients (70 females, 34 males) with definite RR multiple sclerosis according to the McDonald criteria (McDonald *et al*., 2001) were retrospectively included in this study. Thirty-nine patients had never been treated with IFN-β before entering this study (treatment-naive patients) and 65 patients (long-term treated patients) were on treatment with recombinant IFN-β [11 patients were treated with 30 μg intramuscular (im) IFN-β1a (Avonex®) (Biogen; Cambridge, MA, USA) once a week (IFN-$\beta1a_{im}$); 47 were treated with IFN-β1a (Rebif®) (Serono, Geneva, Switzerland), either with 22 μg ($n = 31$) or 44 μg ($n = 16$) subcutaneous (sc) three times a week (IFN-$\beta1a_{sc}$); and seven had been treated with IFN-β1b (Betaferon®) (Schering; Berlin, Germany) for at least 3 months (mean ± SD = 24 ± 20 months, range 3–84)]. Of the treatment-naive patients, 16 received IFN-$\beta1a_{im}$, 10 IFN-$\beta1a_{sc22\mu g}$, eight IFN-$\beta1a_{sc44\mu g}$ and five IFN-β1b.

The expanded disability status scale (EDSS) score ranged from 0 to 6 points (mean ± SD = 2 ± 1.4). Patients had: (i) no immunosuppressive drug therapy; (ii) no switch of IFN-β type; and (iii) no glucocorticosteroid therapy <30 days prior to study. The control group included 23 healthy volunteers (13 females, 10 males) who did not display symptoms of viral infection at least 2 weeks before and after blood donation.

NAb evaluation

Both long-term treated and treatment-naive patients were evaluated for the presence of NAb at study entry (baseline). In long-term treated patients, longitudinal measurements of NAb were performed in serum samples collected at least 36 h after the last IFN-β injection every 3 months until gene expression analysis. However, the last NAb measurement and gene expression analysis were made from the same blood sample, i.e. 12 h after the last injection. NAb were measured with the cytopathic effect (CPE) assay as previously described (Bertolotto *et al*., 2003); to insure maximal ligation with presumed NAb, measurements were performed with the same type of IFN-β as used in individual patients for therapy (Antonelli *et al*., 1998). Briefly, a mean concentration of 7×10^4 cells in DMEM medium 2% FCS (1 : 2.5 up to 1 : 5120) per 100 μl of the human lung carcinoma cell line A549 (ECACC, Salisbury, UK) were plated on 96-flat well tissue culture plates and incubated overnight to form a confluent monolayer. Serum samples were diluted (1:2.5 up to

1:5120), mixed with one of the three IFN-β preparations to a final concentration of 10 IU/ml, and then incubated for 1 h. Serum-IFN-β mixture (100 µl) was then incubated with A549 cells for 24 h. Cells were then infected with the cytopathic encephalomyocarditis murine virus at a concentration of 5–10 virus particles/cell. After 24 h incubation, viable cells were fixed and stained with crystal violet in 20% ethanol. Excess dye was eluted with 33% acetic acid and absorbance was measured in a densitometer at 620 nm. Controls for viral activity, cellular viability and titration of the IFN-β preparation were run in parallel in each assay. According to World Health Organization recommendations, data from neutralization assays are reported as the reciprocal of the highest dilution of serum inducing 50% neutralization (i.e. neutralizing 10 U/ml of IFN activity to an apparent 1 U/ml of activity). The neutralization titre of a serum sample was calculated according to Kawade's formula (Kawade *et al*., 1986) and expressed in laboratory units (LUs). A level of ⩾20LU is generally considered the threshold of positivity (Bertolotto *et al*., 2003). Three categories of patients were identified based on the CPE assay: (i) patients in whom no NAb were detected during the whole period of follow-up (NAb–); (ii) patients tested positive for NAb in ⩾2 consecutive samples were declared persistent NAb+ (pNAb+); and (iii) patients who had single positivity at the time point of gene expression analysis were declared isolated NAb+ (iNAb+).

Gene expression analysis

In treatment-naive patients, blood samples were taken prior to the first and after the second (7 days + 12 h) IFN-β injection (mean±SD 12.1 ± 1.2 h, range 10–13 h). For long-term treated patients, blood specimens were obtained 12.3 ± 1.6 h (range 9–14 h) after the last IFN-β injection. PBMC were separated from edetic anticoagulated whole blood by Ficoll-Paque (Pharmacia; Uppsala, Sweden) centrifugation and subjected to RNA extraction using RNAwiz, following the manufacturer's instructions (Ambion; Austin, TX, USA). Total RNA (50 ng/µl final concentration) was first incubated with 0.5 µg of random hexamer at 70°C for 2 min and then reverse transcribed at 37°C for 1 h in reaction mixture containing a final concentration of 500 µM of each deoxynucleotide triphosphate, 1 U/µl of Moloney murine leukaemia virus reverse transcriptase and 1 U/µl of RNAase inhibitor (RNAsin) (both from Promega Corporation; Madison, WI, USA). cDNA was used as a template for the real-time PCR analysis based on the 5′-nuclease assay with the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, PE Europe B.V., Rotkreuz, Switzerland). Real-time PCR primers and probes were designed using primer-Express™ software (Applied Biosystems). Expression of MMP-2, MMP-9, TIMP-1, TIMP-2 and MxA was analysed and transcriptional expression was normalized using the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) as reference in order to avoid differences due to possible RNA degradation/contamination or different reverse transcription efficiency. The relative expression levels of targets were calculated by the comparative cycle threshold (C_T) method provided by Applied Biosystems. Targets were considered detectable with C_T values <40. Expression levels of targets with C_T values >35 were not described as relative expression, due to the semi-quantitative character of C_T values in this range. Comparison of baseline expression levels of MxA in multiple sclerosis treatment-naive patients and controls was performed using competitive PCR results as previously described (Bertolotto *et al*., 2003).

Statistical analysis

Primary data are expressed as median (range) unless otherwise stated. Data were analysed using non-parametric statistical tests except for the linear regression analysis of the correlation of expression levels of MxA, MMP-9 and TIMP-1 as a function of treatment duration. Spearman rank correlation of expression levels between different genes, of intra-individual gene expression at different time points, and of MxA/MMP-9 expression levels with NAb titres were calculated. Expression levels of genes at different time points or between different treatment regimens, and according categorical differences of NAb status, were compared using the Mann–Whitney *U* test. The incidence of NAb development was compared between different treatment groups using the Fisher's exact test. *P* values <0.05 were considered significant.

Results

NAb status and treatment schemes

At baseline, both treatment-naive and long-term treated patients scored negative for NAb (data not shown). In the course of long-term IFN-β treatment, NAb were detectable in 26% (17 out of 65) of patients: 22% (14 out of 65) had pNAb+ and 3% (5 out of 65) had iNAb+ (at the time point of blood sampling for gene expression analysis). Of the 17 long-term IFN-β treated patients who had developed NAb at the time point of gene expression analysis, four were treated with IFN-β1b and 13 were treated with IFN-β1a$_{sc}$ (22 µg/injection: 7 out of 13; 44 µg/injection: 6 out of 13), whereas no NAb were detected in patients treated with IFN-β1a$_{im}$. The duration of treatment was significantly longer with IFN-β1b [58 months (15–73 months)] compared with the various types and dosages of IFN-β1a [sc$_{22}$: 20 months (3–84 months); sc$_{44}$: 15 months (3–68 months): im: 18 months (5–60 months); all $P < 0.016$], whereas the duration of treatment between the three groups of patients who received IFN-β1a was not statistically different (all $P \geqslant 0.36$).

Short-term effects of IFN-β injection on MxA and MMP/TIMP gene expression

Figure 1A shows that low amounts of MxA [relative expression compared with GAPDH: 0.08930 (0.02121–2.29740)] are constitutively expressed in all multiple sclerosis patients; the expression levels are similar to those in 23 healthy control subjects as measured by competitive PCR (Bertolotto *et al*., 2003) (data not shown; $P = 0.62$). The injection of IFN-β in treatment-naive patients led to a >20-fold increase of median MxA expression [1.9185 (0.1756–4.4840)] 12 h later ($P < 0.0001$). However, individual changes were highly variable, i.e. baseline MxA expression levels were not predictive for their degree of induction ($P = 0.99$, $r < 0.001$). Three patients had exceptionally high MxA levels at baseline who scored around the median value observed after IFN-β injection; of these, one patient showed no, and two others only a slight increase (1.4- and 1.8-fold, respectively) of MxA mRNA on IFN-β injection (Fig. 2). A

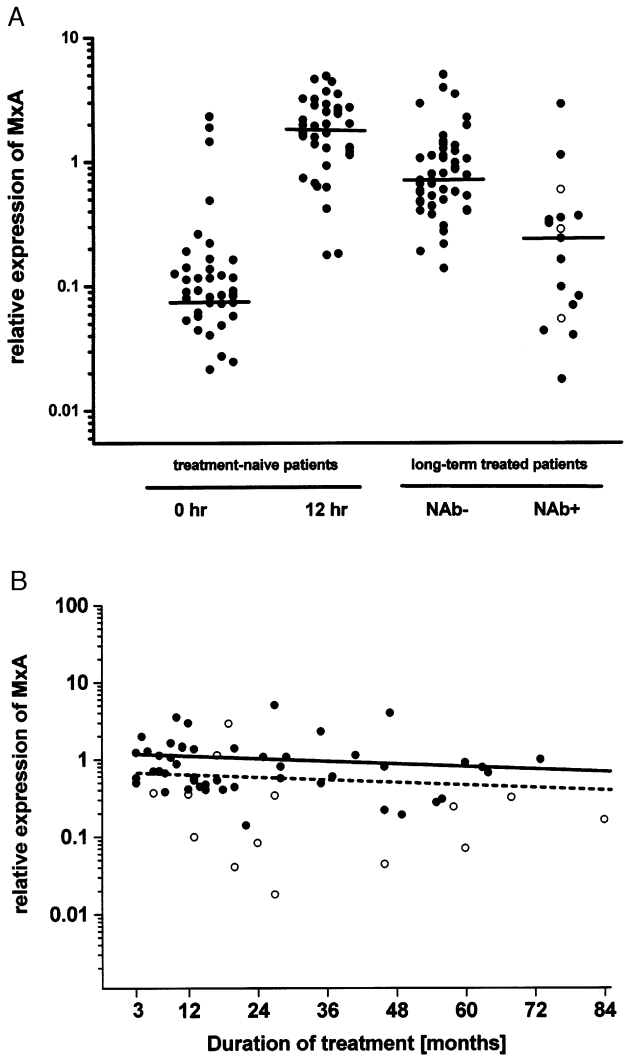

Fig. 1 (**A**) Comparison of MxA mRNA levels in treatment-naive patients before first and after second injection, and long-term IFN-β treated (filled circles = pNAb+; open circles = iNAb+) multiple sclerosis patients. Horizontal bars indicate medians. (**B**) MxA RNA levels in NAb– (closed circles) and pNAb+ (open circles) patients as a function of IFN-β treatment time. Results of statistical evaluation of linear regression analysis for patients without (straight line) and with (dashed line) NAb were $P_{\text{NAb}-} = 0.30$; $r = 0.15$ and $P_{\text{pNAb}+} = 0.47$; $r = 0.22$.

retrospective evaluation of case file histories of these patients did not reveal indications of intercurrent viral infections or other diseases, and the percentage of patients with such high expression of MxA was similar to that found earlier (Bertolotto *et al*., 2001, 2003). Transcripts for MMP-9 [0.0046 (0.0005–0.1756)], TIMP-1 [0.3220 (0.1713–6.5889)] and TIMP-2 [0.0581 (0.0052–1.1728)] were present in all treatment-naive patients (Fig. 3A,C and D). In contrast, MMP-2 expression levels were not detectable in 18% (7 out of 39) of samples and too low in the remainder for relative expression to be quantified. Therefore, MMP-2 expression was quantified on the basis of C_T values only (Fig. 3B). Other

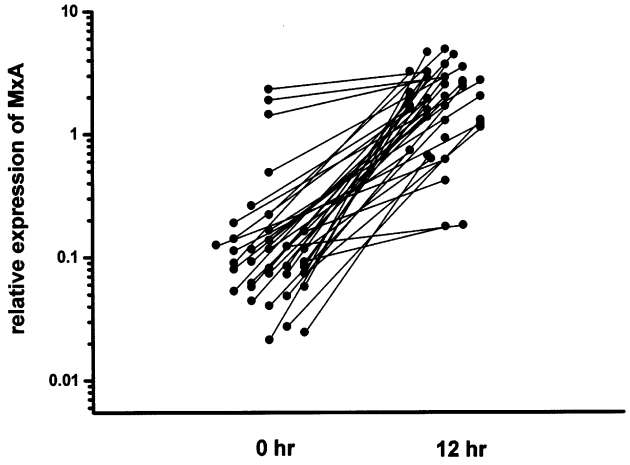

Fig. 2 Change of MxA expression levels in individual treatment-naive patients as shown in Fig. 1A.

than for MxA, the transcriptional expression of MMP-2 and MMP-9 and their respective inhibitors (TIMP-2 and TIMP-1) did not show significant acute phase changes after IFN-β injection. The inducibility of MxA and MMP/TIMP expression did not differ after treatment with either types or dosage of IFN-β (data not shown).

Long-term effects of IFN-β treatment on MxA and MMP/TIMP gene expression

In long-term IFN-β treated, NAb– patients, the median MxA expression level was more than 8-fold higher than the baseline value [0.7341 LU (0.1373–4.9588LU), $P < 0.0001$] (Fig. 1A), but less than a third compared with that observed in treatment-naive patients after the second injection ($P < 0.0001$). The attenuation of MxA up-regulation observed here in the first 3–6 months of chronic IFN-β treatment has been demonstrated by others, both on the transcriptional and the protein level (Vallittu *et al*., 2002; Gniadek *et al*., 2003). Conversely, in the later course of IFN-β treatment (months 3–84), MxA levels remained stable, i.e. they showed no change as a function of treatment duration (Fig. 1B). In parallel, long-term IFN-β therapy led to a 5-fold decrease of MMP-9 transcripts [0.0008 (not detectable–0.0146)] compared with untreated patients ($P < 0.0001$) and was below detection limit in 14.5% (7 out of 48) of samples (Fig. 3A). However, no significant correlation between MxA and MMP-9 expression levels on an individual basis could be established (data not shown).

The suppressive effect of IFN-β was even more pronounced for MMP-2, where all 48 samples from NAb– patients scored below detection threshold ($P < 0.0001$ for comparison with treatment-naive patients at baseline) (Fig. 3B). In contrast, transcriptional expression of both TIMPs remained unchanged under long-term IFN-β treatment (Fig. 3C and D).

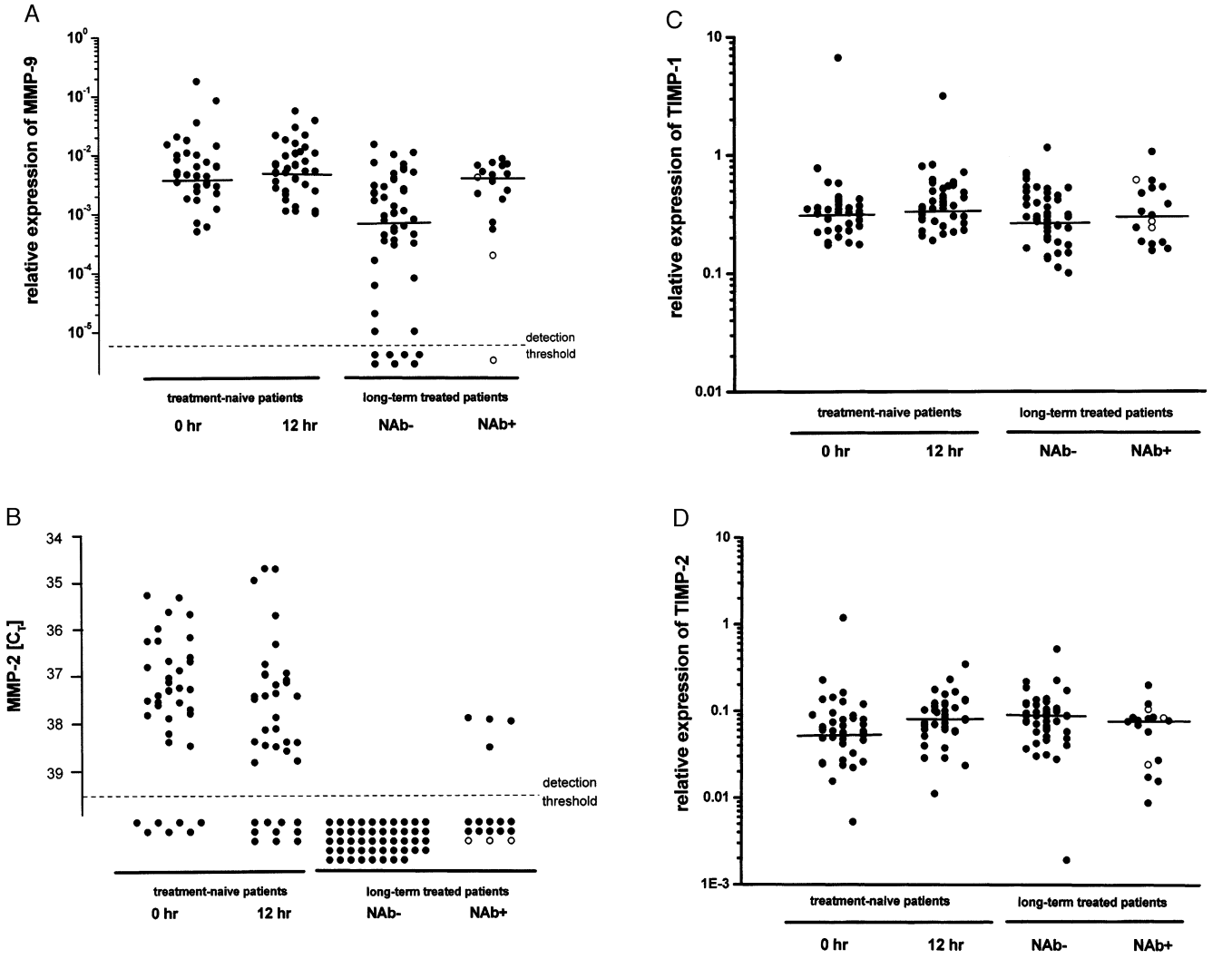

Fig. 3 Comparison of (**A**) MMP-9, (**B**) MMP-2, (**C**) TIMP-1 and (**D**) TIMP-2 mRNA levels in treatment-naive and long-term IFN-β treated (filled circles = pNAb+; open circles = iNAb+) multiple sclerosis patients. Horizontal bars indicate medians of all patients per group. Respective targets were considered not being detectable with C_T values ⩾40. Note that levels of MMP-2 (**B**) are depicted as a C_T value, as relative expression normalized with GAPDH is not quantitative with low expressions of the target gene.

To exclude the possibility of delayed pharmacodynamic effects of IFN-β, expression levels of MMPs/TIMPs were analysed as a function of time. As for MxA, transcriptional expression of MMP-9 in NAb– patients was independent of treatment duration (Fig. 4A). Hence, for both genes the effect of long-term IFN-β therapy was established in the first 3 months of treatment. Similarly, TIMP-1 (Fig. 4B) and TIMP-2 (data not shown) expression levels remained stable under long-term IFN-β therapy.

There was no difference between the various types and dosages of IFN-β in up-regulating MxA expression (data not shown). In contrast, suppression of MMP-9 expression was less pronounced in patients treated with IFN-β1a$_{im}$ [0.0037 (not detectable–0.0146)] compared with those on IFN-β1b [0.0004 (not detectable–0.0026)] (P = 0.049) or IFN-β1a$_{sc22}$ [0.0006 (not detectable–0.0047)] (P = 0.0012), while a tendency for such a difference was seen for IFN-β1a$_{sc44}$ [0.0016 (not detectable–0.0099)] (P = 0.09). On the other hand, differences in MMP-9 expression levels between the two dosages of IFN-β1a$_{sc}$ and IFN-β1b were not significant (data not shown). Accordingly, expression levels of TIMP-1 and TIMP-2 were independent of types and dosages of IFN-β.

Effects of NAb on MxA, MMP/TIMP expression

Analysis stratified by NAb status

In long-term treated patients with NAb+ (pNAb+ and iNAb+), median MxA expression levels were 3.1× [0.2384 (0.0177–2.8679)] lower than those of NAb– patients (P < 0.0001) (Fig. 1A). However, two pNAb+ patients showed MxA expression in the range of NAb– patients. Although median MxA expression in NAb+ patients was still

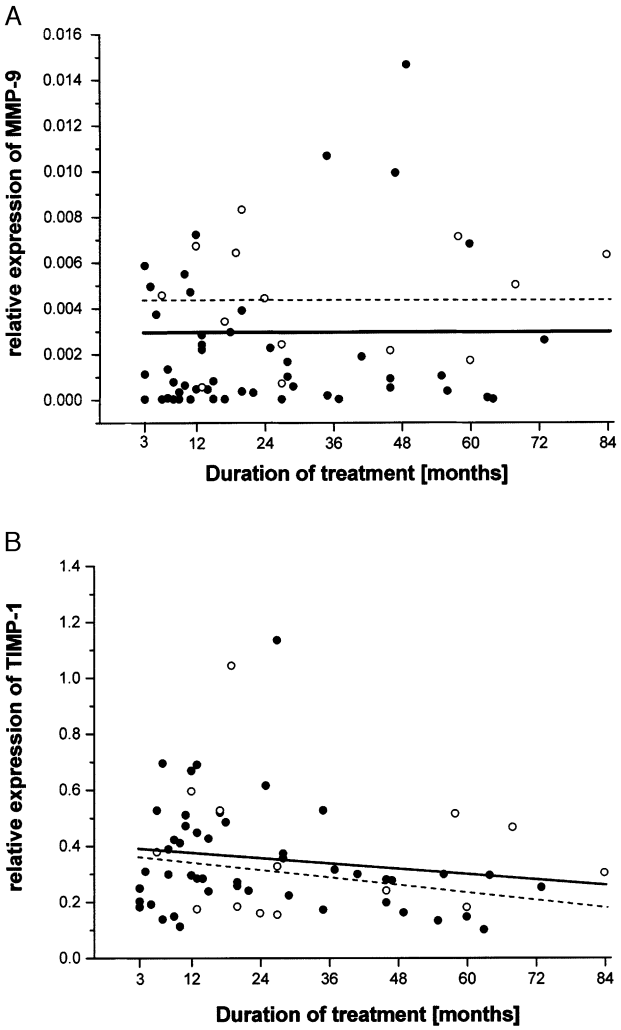

Fig. 4 (**A**) MMP-9, (**B**) TIMP-1 RNA levels in NAb– (filled circles) and NAb+ (open circles) patients in function of IFN-β treatment time. For calculation of regression analysis, values for MMP-9 (see Fig. 3A) below the detection threshold were not counted. Results of statistical evaluation of linear regression analysis for patients without (straight line) and with (dashed line) NAb were $P_{NAb-} = 0.91$; $r = 0.016$ and $P_{pNAb+} = 0.62$; $r = 0.146$ for MMP-9, and $P_{NAb-} = 0.055$; $r = 0.279$ and $P_{pNAb+} = 0.79$; $r = 0.077$ for TIMP-1. Note different scale of *y*-axis for respective targets.

2.7× higher than in treatment-naive patients at baseline (T_0), the statistical comparison failed to show a significant difference between the two groups ($P = 0.15$). Accordingly, the presence of NAb+ correlated with higher levels of MMP-9 [0.00399 (not detectable–0.0829)] compared with NAb– samples ($P = 0.0184$) and reached similar values as untreated multiple sclerosis patients ($P = 0.14$) (Fig. 3A). Moreover, MMP-2 was detectable in 24% (4 out of 17) NAb+ patients compared with 0% in NAb– patients ($P < 0.0001$). However, this number was still lower in untreated patients [82% (32 out of 39)] ($P = 0.0001$) (Fig. 3B).

As for NAb– patients, pNAb+ did not change the transcriptional expression of MxA (Fig. 1B), MMP-9 (Fig. 4A), TIMP-1 (Fig. 4B) and TIMP-2 (data not shown) as a function of treatment duration or the types of IFN-β used (data not shown). Such analysis was not possible for MMP-2 due to the lack of quantitative data.

Correlation of NAb titres with MxA and MMP-9 expression levels

Earlier work has shown that NAb titres <45 LU have variable impact on the transcriptional expression of MxA, whereas titres >45 LU led to a complete suppression as measured by competitive PCR (Bertolotto *et al*., 2003). Accordingly, real-time PCR used here demonstrates that in presence of NAb (with the exception of two outliers), MxA levels ranged around the median value of treatment-naive patients at baseline and transcriptional induction by IFN-β was abrogated. Fig. 5A demonstrates that this effect occurs independently of the titre of NAb (be it above or below 45 LU). In contrast, there is a tendency for a correlation of MMP-9 expression levels and the whole range of NAb titres ($P_{(n = 17)} = 0.066$; $r = 0.46$). Restricting the analysis to titres >45LU this correlation becomes highly significant ($P_{(n = 12)} = 0.011$, $r = 0.77$) (Fig. 5B).

Discussion

There is ongoing controversy about the impact of NAb against IFN-β on its therapeutic effect in multiple sclerosis (Giovannoni *et al*., 2002; Polman *et al*., 2003). In large cohorts, up to a third of multiple sclerosis patients receiving IFN-β develop NAb, mostly after 6–18 months of treatment. Over 90% of those are reported to convert back to seronegativity after 8.5 years of treatment (Rice *et al*., 1999). However, the presence of NAb may be long lasting. In NAb+ patients observed for up to 67 months of IFN-β treatment, seroconversion is more likely to occur with NAb titres <200 LU, whereas titres above this threshold are associated with persistent seropositivity; in fact, 62% of NAb+ patients remained seropositive during this time period (Capobianco *et al*., 2003). In the PRISMS-4 trial (IFN-β1a_{sc}), negative effects of NAb on clinical parameters were not obvious during the first 2 years of treatment whereas, after 4 years, NAb+ patients had a 62% higher per year relapse rate and an almost 5× higher increase in T2 lesion load in MRI (PRISMS Study Group and University of British Columbia Multiple Sclerosis/MRI Analysis Group, 2001). Similar results were observed with IFN-β1b (Polman *et al*., 2003) and IFN-β1a_{im} (Rudick *et al*., 1998) on these measures.

The current standard method to determine NAb in serum is indirect, i.e. via their *in vitro* capacity to antagonise the antiviral activity of IFN-β in a CPE assay. This test is technically difficult and there is no industrial standard with regard to the definition of seropositivity *per se* (detection of NAb in a single versus two consecutive samples) and the cut off level of titre considered biologically relevant (Giovannoni

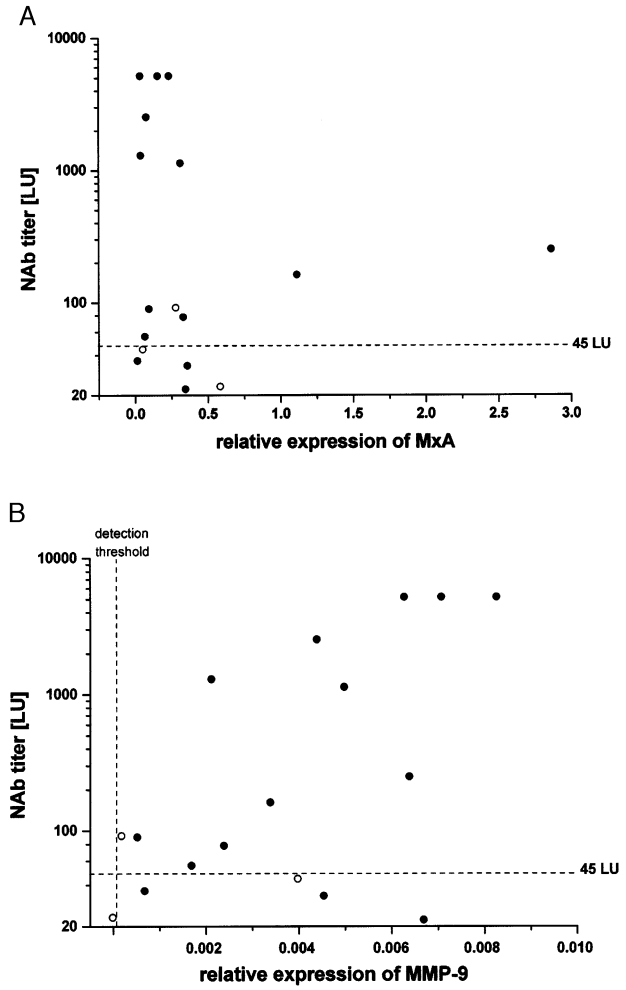

Fig. 5 Correlation of NAb titres with expression levels of MxA (**A**) and MMP-9 (**B**) in patients positive for NAb (filled circles = pNAb+; open circles = iNAb+) at the time point of gene expression analysis. For MMP-9 and NAb titres, statistical evaluation of correlation resulted in $P_{(n=17)} = 0.066$; $r = 0.46$ (all patients) and $P_{(n = 12)} = 0.011$; $r = 0.77$ (patients with titres >45LU), respectively.

et al., 2002). The measurement of transcriptional or protein expression of IFN acute-response proteins such as neopterin, β-2-microglobulin and Mx proteins offers an alternative measure for IFN-β bioavailability which overcomes some of the disadvantages of the CPE assay (Vallittu *et al*., 2002; Bertolotto *et al*., 2003). However, there is no evidence for a functional role of these molecules in multiple sclerosis and data showing a direct correlation of acute-response protein induction by IFN-β with the long-term clinical course of multiple sclerosis patients with or without NAb has been published. Instead, MMPs are pre-eminent effector molecules in several crucial steps of multiple sclerosis pathogenesis (Kieseier *et al*., 1999; Leppert *et al*., 2001). A specific feature of multiple sclerosis, as opposed to other neuro-inflammatory disorders such as infectious meningitis (Leppert *et al*., 2001), is the induction of MMPs in the CNS and the peripheral blood circulation without compensatory up-regulation of their endogenous tissue inhibitors, TIMPs, leading to a persistent imbalance towards excessive proteolytic activity (Lee *et al*., 1999; Waubant *et al*., 1999, 2003; Lindberg *et al*., 2001). Moreover, intercurrent increase of MMP-9, or of the MMP-9/TIMP-1 ratio in serum of RR and SP multiple sclerosis predicts upcoming subclinical disease activity as detected by the development of new gadolinium-enhancing lesions in MRI (Waubant *et al*., 1999, 2003). There is accumulating evidence that a large part of the beneficial effect of IFN-β in multiple sclerosis results from its capacity to suppress the expression of MMPs (for a review, see Yong *et al*., 2002) and, in parallel, to up-regulate TIMP-1 (Waubant *et al*., 1999, 2003). Interestingly, the pathophysiological link between MMPs and IFN-β in multiple sclerosis is not unidirectional. A recent study demonstrated that MMP-9 exerts proteolytic activity on IFN-β; thus the inherently increased concentrations of MMP-9 in serum, CSF and brain tissue of multiple sclerosis patients destroy the bioactivity of endogenous and therapeutically administrated IFN-β (Nelissen *et al*., 2003).

Here we show that IFN-β leads to an inverse transcriptional regulation of MxA and of MMP-2 and MMP-9 in RR multiple sclerosis patients. Conversely, the presence of NAb against IFN-β are associated with an attenuated induction of MxA and reduced suppression of MMPs. Based on these results, we propose increased MMP activity as one mechanism by which NAb abrogate the effect of IFN-β in multiple sclerosis. The response patterns of MxA and MMP-9 differ considerably; MxA is very sensitive to detect decreased bioavailability of IFN-β due to NAb on the basis of single measurements after a single injection, but lacks a dynamic response in function of NAb titres. This agrees with the results of other studies where no correlation of NAb titres with expression levels of MxA (Deisenhammer *et al*., 1999; Bertolotto *et al*., 2003) or with other IFN acute-response proteins (neopterin, β_2-microglobulin) (Rudick *et al*., 1998) was demonstrated. On the other hand, the finding that NAb titres correlate positively with MMP-9 expression suggests that the quantitative extent of the immune response of multiple sclerosis patient against therapeutically applied IFN-β may be of biological relevance.

With the caveat that treatment groups were not randomized and had different treatment durations, quantitative differences of MMP-9 suppression in NAb– patients suggest that IFN-β1a_{sc} and IFN-β1b exert a slightly higher biological activity compared with IFN-β1a_{im}. Although the kinetics of MMP-9 suppression by the various preparations of IFN-β are not known, it is conceivable that the higher total amount or the shorter dosage intervals of the former types of IFN-β may be responsible for this result. It is unclear, however, whether these differences are clinically relevant, vis-à-vis the fact that the less suppressive activity of IFN-β1a_{im} may be counterbalanced by a far lower incidence of NAb induction observed in all therapeutic trials with the current formulation (for a review, see Giovannoni *et al*., 2002) and in longitudinal comparisons between the different types of IFN-β (Bertolotto *et al*., 2002), as well as in the present study cohort.

At first glance, the present findings seem to partly contradict earlier work where IFN-β was shown to increase serum protein levels of TIMP-1 after 4 months of treatment (Waubant *et al*., 2003). Moreover, high amounts of MMP-2 are constitutively expressed in CSF and serum, and are widely believed not to vary in the course of multiple sclerosis, specifically during IFN-β therapy (Trojano *et al*., 1999; Waubant *et al*., 2003). However, protein levels in serum are representative of contributions from various cellular compartments, whereas mRNA measurements as used here allow the quantification of gene usage in a specific cell type. Besides PBMC, endothelial cells and other cells of the vascular lining contribute to the production of MMPs and TIMPs (Bugno *et al*., 1999; Nelissen *et al*., 2002; Taraboletti *et al*., 2002). Quantitatively, leukocytes produce predominantly MMP-9, while the amounts of MMP-2 are relatively scarce as shown for transcripts (present results), as well as in the protein level in PBMC (Johnatty *et al*., 1997), and may be absent in resting T-cells (Leppert *et al*., 1996) and monocytes (Xie *et al*., 1994). In contrast, endothelial cells produce predominantly MMP-2, representing probably the predominant source in serum, while they contribute only little MMP-9 (Nelissen *et al*., 2002). Thus, quantitatively subtle up-regulation of MMP-2 expression in the PBMC compartment of multiple sclerosis patients may easily escape detection in serum measurements. Conversely, IFN-β treatment efficiently reduces MMP-2 transcription in PBMC of multiple sclerosis patients as observed here and by others (Galboiz *et al*., 2001); this is further corroborated by the changes on the protein level in experimental autoimmune encephalitis (Schmidt *et al*., 2001), and *in vitro* (Leppert *et al*., 1996).

Along with present results, others have found that mRNA levels of TIMP-1 and TIMP-2 from PBMC of IFN-β treated multiple sclerosis patients are unchanged or rather decreased, respectively (Lichtinghagen *et al*., 1999; Galboiz *et al*., 2001). We therefore conclude that the up-regulation of TIMP-1 protein in serum of IFN-β treated patients does not originate from PBMC, but derives most probably from endothelial cells. IFN-β does not modulate expression of MMP-2 and MMP-9 in endothelial cells (Nelissen *et al*., 2002) *in vitro*, but the effects on TIMP-1 regulation and the impact of NAb have not so far been investigated. However, there is indirect and partial evidence that IFN-β up-regulates TIMP-1 production in endothelial cells. In multiple sclerosis patients, the decreased production of the anti-inflammatory cytokine IL-10 by PBMC is corrected by IFN-β (Chabot *et al*., 2000; Ozenci *et al*., 2000), and this cytokine increases the production of TIMP-1 and decreases that of MMP-2 and MMP-9 in endothelial cells (Stearns *et al*., 1999).

Our study has some obvious limitations, as it was not designed to investigate whether NAb-induced changes of MxA and MMPs relate to decreased therapeutic effect of IFN-β and, hence, a more severe clinical course. Secondly, the interpretation of single measurements of these markers in individual patients may be of little value considering the wide range of expression levels and the overlap with NAb– patients. A small study, which attempted to delineate the impact of NAb on the protein levels of MMP-9 (Trojano *et al*., 1999), showed that pNAb+ multiple sclerosis patients had higher MMP-9 serum levels during an observation time of 6–18 months of IFN-β therapy and a greater increase of clinical disability as measured by Expanded Disability Status Score compared with NAb– patients. However, the NAb+ cohort consisted of only five patients and MMP-9 measurements were performed by semi-quantitative zymography, which detects only selected species of MMP-9 (TIMP-1-free, pro- and active MMP-9). Moreover, increased MMP-9 expression in IFN-β treated multiple sclerosis patients is not specific for the presence of NAb, but has also been observed immediately before and during the occurrence of new gadolinium-enhancing lesions (Waubant *et al*., 2003). Such increased subclinical disease activity may explain why, in some patients, IFN-β treatment was not effective in lowering MMP-9 expression levels compared with controls, despite the absence of NAb. This could also be the case for MxA, as high expression levels due to intercurrent viral infection could interfere with the suppression by NAb activity. Hence, the simultaneous measurement of both targets in a longitudinal manner may be compulsory for a meaningful interpretation of NAb titres for clinical diagnostic purposes. Prospective studies are needed to determine whether measurements of MMPs, in combination with MxA and other IFN-response proteins, would provide a statistically robust array of markers to allow predicting the degree of clinical response to IFN-β on an individual basis, and to indicate decreased treatment effect due to NAb.

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