Synergistic Immunomodulatory Effects of Interferon-β1b and the Phosphodiesterase Inhibitor Pentoxifylline in Patients with Relapsing-Remitting Multiple Sclerosis

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Subcutaneous application of interferon-β1b (IFN-β1b) is an established therapy for patients with relapsing-remitting multiple sclerosis (RRMS), but early side effects are still a major concern. In vitro studies with myelin basic protein (MBP)–specific T-cell lines revealed a synergistic suppressive effect of IFN-β1b and the phosphodiesterase inhibitor pentoxifylline (PTX) on proliferation and the production of tumor necrosis factor-α (TNF-α), lymphotoxin (LT), and interferon-γ (IFN-γ). In an initial, open labeled prospective trial, the cytokine messenger RNA (mRNA) expression of blood mononuclear cells from MS patients, receiving either IFN-β1b alone or in combination with oral PTX, was determined by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Patients treated with IFN-β1b alone reported more side effects during the first 3 months of treatment and had upregulated TNF-α as well as IFN-γ mRNA expression during the first month, which was not detected in patients receiving both drugs. A synergistic effect of both drugs was observed on the upregulation of interleukin (IL)-10 mRNA, which was accompanied by an increase in IL-10 serum levels. Both in vitro and in vivo data suggest that co-treatment of IFN-β1b with PTX is a promising approach to correct the disturbed cytokine balance in MS patients.

Weber F, Polak T, Günther G, Kubuschok B, Janovskaja J, Bitsch A, Poser S, Rieckmann P. Synergistic immunomodulatory effects of interferon-β1b and the phosphodiesterase inhibitor pentoxifylline in patients with relapsing-remitting multiple sclerosis. Ann Neurol 1998;44:27–34

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). There is increasing evidence that autoreactive T lymphocytes specific for myelin antigens, like myelin basic protein (MBP) or other locally expressed autoantigens, play an important role in the initiation of the immunopathological cascade.[1] Cytokines produced by CNS-invading cells, such as the potent proinflammatory cytokine IFN-γ, are mediators of the immune reaction; and some, such as TNF-α and LT, have effector functions, which are both myelinotoxic in vitro and enhance the inflammatory reaction.[2,3] In MS patients an upregulation of TNF-α, IFN-γ, and LT was detected in white blood cells before a relapse and the immunomodulatory cytokines IL-10 and transforming growth factor-β (TGF-β) were upregulated during remission.[4,5] Immunomodulatory treatment aimed at this imbalanced cytokine regulation in MS is under intensive investigation.

Several studies have established a role for interferon-β (IFN-β) in the treatment of relapsing-remitting MS (RRMS). In a large placebo-controlled, double-blind, multicenter trial, it was shown that alternate-day subcutaneous IFN-β1b (Betaseron, Berlex, Richmond, CA) significantly reduced the relapse rate, relapse severity, and progression of magnetic resonance imaging (MRI)–detected lesions in patients with RRMS.[6,7] In another trial, weekly intramuscular injection with IFN-β1a (Avonex) significantly delayed the time to sustained disease progression on the Expanded Disability Status Scale (EDSS), lowered the relapse rate, and reduced the extension of gadolinium-enhancing MRI brain lesions.[8]

Despite these beneficial actions of β-interferons on disease activity, initiation of IFN-β treatment results in a transient induction of TNF-α and IFN-γ,[9] which correlates with side effects and may trigger exacerbations.[10] It is therefore conceivable that the addition of an antiinflammatory drug may reduce the upregulation

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Received Sep 26, 1997, and in revised form Jan 26, 1998. Accepted for publication Jan 26, 1998.

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of proinflammatory cytokines and thereby decrease side effects during the initiation of IFN-β1b treatment. Pentoxifylline (PTX), a phosphodiesterase inhibitor, is a potent suppressor of TNF-α and IFN-γ production and can be administered orally at a concentration that has been shown to affect cytokine production in vivo.[11] Clinical observations demonstrated that co-treatment with PTX ameliorated side effects, which occurred after initiation of IFN-β therapy.[12] We therefore offered MS patients, to be started on IFN-β1b, an additional treatment with PTX and determined the cytokine mRNA expression of blood mononuclear cells during the first 3 months of treatment. To further investigate the immunomodulatory capacity of IFN-β1b and PTX alone or in combination on putative autoreactive T cells, we established an in-vitro system using human MBP-specific T-cell lines to determine the effect on antigen-specific proliferation and cytokine production.

Materials and Methods

Patients and Treatment

Patients with RRMS and a high relapse rate during the last 2 years who fulfilled the criteria for IFN-β1b treatment were recruited in this study. Informed consent was obtained from each individual. Patients were instructed for safe subcutaneous injections (needle exchange, cooling, alternating injection sites) and offered an additional treatment with PTX at a concentration of 800 mg twice daily. This high dose was chosen, because it was recently shown to affect cytokine production in vivo.[11] Twelve patients received IFN-β1b and PTX, whereas 8 patients were started on IFN-β1b alone. All patients were allowed to take ibuprofen or paracetamol to reduce side effects. Patients were given a diary to document all side effects, body temperature, and nonsteroidal antiphlogistics taken during the first 3 months of treatment. Clinical status was determined by physical examination at beginning, month 3, and month 6. IFN-β1b was administered at night starting with 2 MIU and increased by 2 MIU at each new injection until the final concentration of 8 MIU was reached. Ten of the 12 patients in the IFN-β1b/PTX group continued co-treatment after 3 months. Blood sampling for reverse transcriptase polymerase chain reaction (RT-PCR) was performed in the morning of specified injection days. Ten milliliters of EDTA blood was collected before the first injection of IFN-β1b (day 0) and at day 2, 4, 6, 8, 14, 28, 56, and 84.

RNA ISOLATION. RNA was isolated from 10^6 mononuclear blood cells separated by Ficoll gradient using guanidinethiocyanate solution and phenol-chloroform extraction according to standard methods. In brief, frozen cells were resuspended in 500 μL of lysis buffer, vortexed, and incubated on ice for 5 minutes. RNA was extracted twice with 500 μL of ice cold phenol-chloroform and precipitated in isopropanol at −20°C for 2 hours. An aliquot of the RNA was separated on a formaldehyde-denaturing agarose gel to check for purity and stability of RNA.

RT-PCR. Eight microliters of resuspended RNA was incubated for 10 minutes at 65°C with 2 μg random hexamer oligonucleotide primer (Boehringer, Mannheim, Germany), 1 μL dNTPs (20 mM each; Pharmacia, Freiburg, Germany), 1 μL RNAsin (Promega, Heidelberg, Germany), 4 μL 5× RT buffer (BRL), and 1.5 μL MMLV reverse transcriptase (200 units, BRL); 2.5 μL ddH_2O was mixed on ice, added to the RNA mix, and incubated at 38°C for 60 minutes. The enzyme was inactivated for 10 minutes at 95°C. The reaction mix was adjusted to 100 μL with ddH_2O.

The following PCR reaction mix was prepared, aliquoted, and stored at −20°C until use: 10 μL of cytokine primer (100 pmol each), 10 μL 10× PCR buffer (500 mM KCL, 100 mM Tris-HCL, ph 8.3, gelatin and 40 mmol $MgCl_2$), and 10 μL dNTP (Pharmacia, 2 mM each). After thawing 2 units of Taq polymerase (Boehringer), 60 μL dH_2O was added. The reaction mix was overlayed with 100 μL of mineral oil (Sigma), and 10 μL of cDNA was pipetted through the oil layer into the PCR mix. Cycles were for 45 seconds at 92°, 55°, and 72°C for each of the 35 cycles, with an initial denaturation time of 6 minutes and a final extension step of 5 minutes. The sets of primers shown in Table 1 were used.

All oligonucleotides used in this study were synthesized trityl off on a 381A DNA Synthesizer from Applied Biosystems (Foster City, CA) and column purified according to the instructions of the manufacturer (Applied Biosystems).

Amplified PCR products were visualized in ethidium bromide–stained 2% agarose gels (0.5 μg/mL ethidium bromide) run at 80 V in 0.5× TBE buffer for 1 hour. Southern blot hybridization was performed with ^{32}P-labeled internal oligonucleotide probes, and autoradiographies were quantitated using densitometric scanning. Semi-quantitative analysis of PCR products was performed as described recently.[5]

Values are given as relative units defined by density of each cytokine PCR product divided by the density of the corresponding β-actin PCR product × 1,000.

T-CELL LINES Human T-cell lines specific for human MBP (isolated according to Eylar and associates[13]) were generated from the blood of 2 patients with RRMS during a stable phase of disease and from a healthy donor.[14] T-cell lines were kept in RPMI 1640 medium supplemented with 2 mM

Table 1. Primer Sets Used for RT PCR

IL-4	
Upstream	CTTCCCCCTCTGTTCTTCCT
Downstream	TTCCTGTCGAGGGGTTTGAG
IL-10	
Upstream	ATGCCCCAAGCTGAGAACCAAGACCCA
Downstream	TCTCAAGGGGCTGGGTCAGCTATCCCA
IFN-γ	
Upstream	GAAATATACAAGTTATATCTTGGCT
Downstream	GATGCTCTTCGACCTCGAAACAG
TNF-α	
Upstream	CAGAGGGAAGAGTTCCCCAG
Downstream	CCTTGGTCTGGTAGGAGACG
LT	
Upstream	CCTCACACCTTCAGCTGCCC
Downstream	GAGAAACCATCCTGGAGGAA
b-Actin	
Upstream	ACGGGGTCACCCACACTGTGC
Downstream	CTAGAAGCATTGCGGTGGACGATG

L-glutamine, 10^3 U/mL penicillin, 10^3 U/mL streptomycin (Gibco, Eggenstein, Germany), 10% heat-inactivated human AB+ serum from local donors, and 50 U/mL IL-2 (Pharma Biotechnologie, Hannover, Germany) at 37°C in 5% CO_2. Re-stimulation was performed every 2 weeks by presenting MBP (30 μg/mL) on irradiated (50 Gy) autologous peripheral blood mononuclear cells. Cells were stained with anti-CD3-PE, anti-CD4-FITC, and anti-CD8-PE (all from Dianova, Hamburg, Germany) or anti CD8-tricolor (Medac, Hamburg, Germany) according to established protocols[15] and analyzed by FACScan.

PROLIFERATION ASSAYS OF T-CELL LINES. Three × 10^4 T cells were re-stimulated with MBP (30 μg/mL) and 2 × 10^5 irradiated, autologous PBMC in the presence of increasing concentrations of IFN-β1b (Betaseron, Schering, Berlin, Germany) and PTX (Rentyllin, Laupheim, Germany) in round-bottomed microwells. After 72 hours, cells were labeled with [^{3}H]thymidine (Amersham, Braunschweig, Germany) and harvested 18 hours later. [^{3}H]thymidine incorporation was measured with a scintillation counter (Packard, Frankfurt, Germany).

CYTOKINE ASSAYS OF T-CELL LINES AND SERUM. T cells were re-stimulated as described previously. After 24 and 48 hours, supernatants were collected, centrifuged (5,000 rpm, 5 minutes, Eppendorf centrifuge), and immediately stored in aliquots at −70°C. Cytokine concentrations were determined by commercially available double-sandwich enzyme-linked immunosorbent assays (ELISAs). To detect IL-10 in serum, samples from patients were separated immediately, filtered, and frozen at −70°C until usage. ELISAs for TNF-α, IFN-γ, IL-4, and IL-10 were purchased from Medgenix (Fleurus, Belgium), and an ELISA for LT was obtained from Bender MedSystems (Vienna, Austria). All ELISAs were performed according to the instructions of the manufacturers.

STATISTICS. Analysis was performed with Wilcoxon test or chi-quare test if appropriate. A p value of < 0.05 was regarded as significant.

Results

No serious adverse event occurred in either treatment group. Transient mild lymphopenia was observed in 4 patients (2 in each group). During the 6-month observation period, one relapse (optic neuritis) occurred in the IFN-β1b group and 2 patients demonstrated transient progression of paraparesis. All patients in the IFN-β1b/PTX treated group were clinically stable. As can be seen in Table 2, patients with combination treatment reported significantly less chills, myalgia, injection side reactions, and fever ($p < 0.01$; χ^2 test). The high dose of PTX was well tolerated by the patients, and typical side effects of this medication, such as headache, nausea, and gastric discomfort were equally distributed among both groups.

During the first 3 months the cytokine mRNA expression pattern of blood mononuclear cells was compared between groups for each time point. There was a marked upregulation of both TNF-α and IFN-γ mRNA during the first weeks of treatment in the IFN-β1b group, which was not observed in patients co-treated with IFN-β1b and PTX (Fig 1A, B). LT mRNA expression was overall low and did not change significantly during the first 3 months of treatment in either group. The same was observed for IL-4 (data not shown). Interestingly, we observed a steady increase of IL-10 mRNA in both treatment groups, although it was more pronounced in patients co-treated with IFN-β1b and PTX (see Fig 1C). IL-10 mRNA levels tended to remain elevated, whereas the rise in TNF-α and IFN-γ mRNA was transient. The upregulated IL-10 mRNA expression was accompanied by increased serum levels of this cytokine. PTX seems to have a synergistic effect, because patients who received both drugs had higher serum values than individuals treated with IFN-β1b alone (see Fig 1D).

Table 2. Early Side Effects of Subcutaneous IFN-β1b Treatment in Patients with Relapsing-Remitting MS during the First 3 Months of Treatment

Event	IFN-β1b (n = 8)[a]	IFN-β1b + PTX (n = 12)[a]
Temperature >38°C	30	8[b]
Chills	33	10[b]
Myalgia	35	9[b]
Injection site reaction	36	24[b]
Headache	10	14
Gastric discomfort	7	10
Nausea	20	15

[a]Numbers are given as percentages of reported side effects per injections during the first 3 months of treatment; 345 injections in the IFN-β1b group and 526 injections in the IFN-β1b + PTX group; χ^2 test.
[b]$p < 0.001$.

To further investigate the immunomodulatory effect of both drugs on autoreactive T cells in vitro, we established a system using human, MBP-specific T-cell lines generated from two MS patients and one healthy donor. FACS analysis demonstrated that these T-cell lines were predominantly CD4+ (>90%), whereas a small portion of CD8+ and CD4+/CD8+ cells occurred. After antigen-specific stimulation T cells proliferated and a maximum of TNF-α and IL-4 production was detected after 24 hours, whereas the secretion of LT, IFN-γ, and IL-10 peaked after 48 hours (data not shown). T cells produced mainly TNF-α and IFN-γ. LT, IL-4, and IL-10 were detectable in smaller quantities (Fig 2). This cytokine pattern was stable during consecutive re-stimulations. The MBP-specific T-cell line of a healthy donor showed an identical cytokine profile.

Stimulation of T cells in the presence of increasing concentrations of IFN-β1b and PTX—alone and in

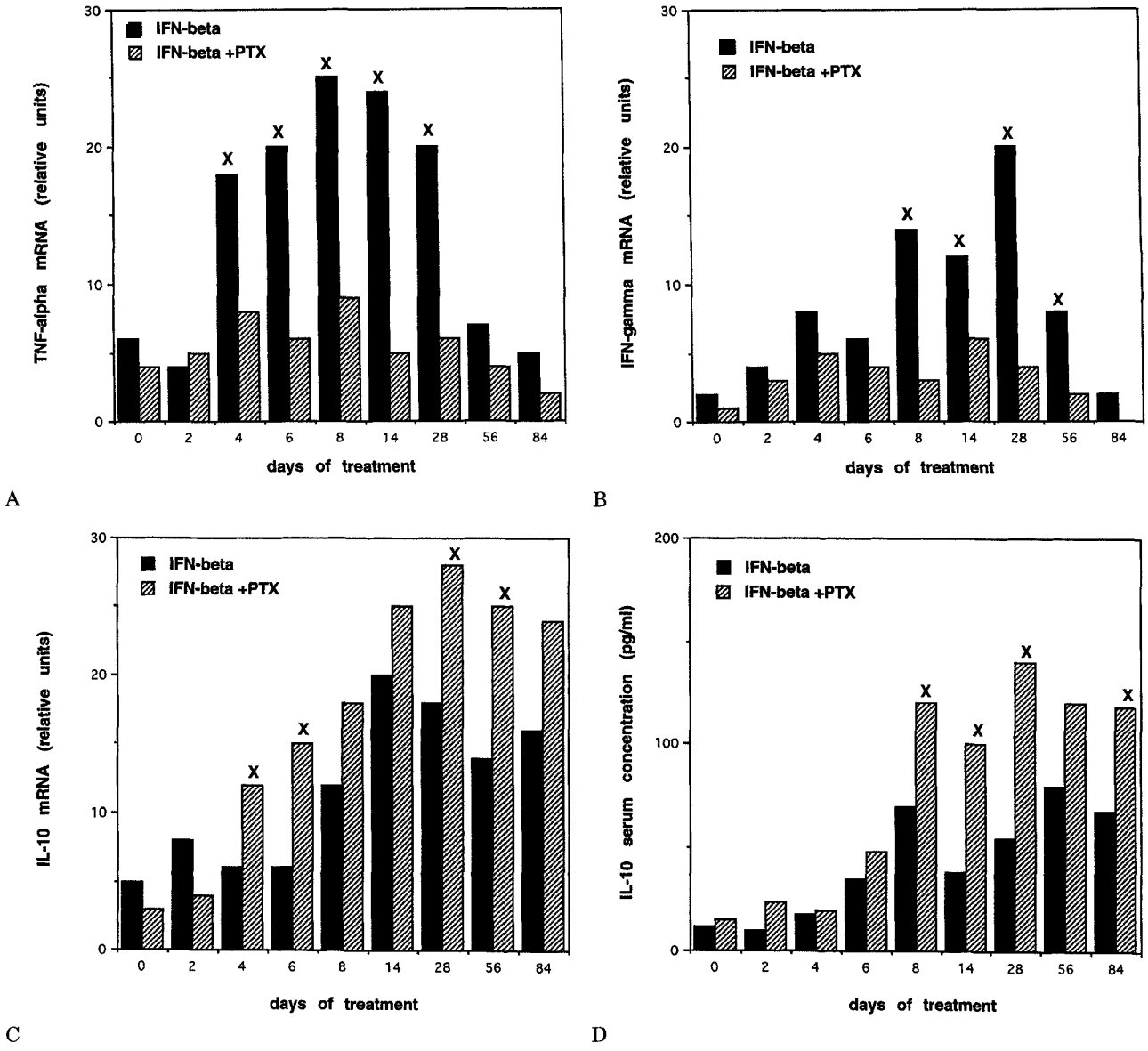

Fig 1. Cytokine mRNA expression measured by semi-quantitative RT-PCR in blood mononuclear cells (A–C) and IL-10 serum concentration (D) of patients with RRMS during the first 84 days of treatment with alternate-day subcutaneous IFN-β1b (n = 8) or a combination of alternate-day subcutaneous IFN-β1b and daily oral PTX (n = 12). Significant differences between both treatment modalities are indicated by an X (Wilcoxon test).

combination—demonstrated a dose-dependent suppression of proliferation and production of TNF-α, LT, and IFN-γ determined in culture supernatants by ELISA. There was no difference between two MBP-specific T-cell lines generated from 1 MS patient and one line from a healthy donor. Interestingly, the combination of IFN-β1b and PTX showed a synergistic, suppressive effect on proliferation and on production of TH1-cytokines (eg, TNF-α, LT, and IFN-γ) in all T-cell lines. The synergistic effect was most pronounced at the highest dose tested. Results are summarized in Figure 3A through D.

TH2 cytokines, however, demonstrated different results. There was no significant effect of both drugs—alone and in combination—on the production of IL-4 (see Fig 3E). IL-10, however, was suppressed by PTX in a dose-dependent manner, whereas IFN-β1b induced IL-10 in all T-cell lines (see Fig 3F). Combination of both drugs suppressed IL-10 at the highest concentration, but, as shown in Table 3, production of TH2 cytokines was less affected than production of TH1 cytokines.

Discussion

In experimental autoimmune encephalomyelitis (EAE), there is strong evidence that T cells reactive for myelin

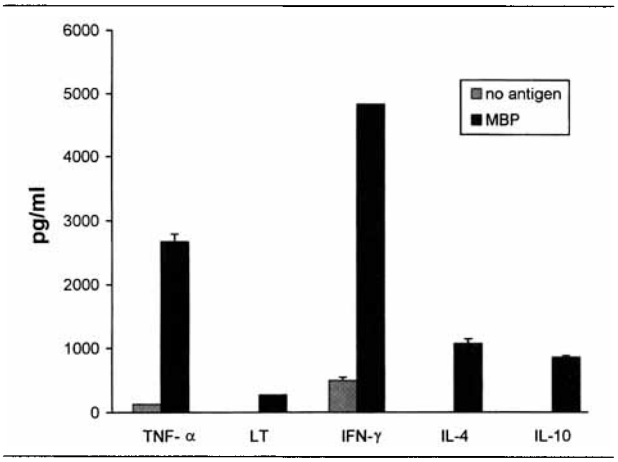

Fig 2. The peak cytokine secretion pattern of four MBP-specific T-cell lines of 2 MS patients and of one T-cell line of a healthy donor were determined by ELISA. Supernatants for TNF-α and IL-4 were collected after 24 hours, whereas supernatants for LT, IFN-γ, and IL-10 were collected 48 hours after re-stimulation with autologous antigen-presenting cells in the presence of MBP (30 μg/mL) and without antigen. One representative experiment is shown.

components or other proteins of the CNS are responsible for the induction of the autoimmune process.[16–19] In this model, encephalitogenicity of T cells correlates with the production of TNF-α, and encephalitogenic T cells are considered as TH1 cells.[20] In MS it was demonstrated that TH1 cytokines increase before relapse.[4,5] Furthermore, there is accumulating evidence that TH2 cytokines may limit the inflammatory process in MS and EAE. Treatment with IL-4 ameliorated EAE.[21] Application of recombinant IL-10, however, showed amelioration or worsening of EAE.[22,23] These contradictory results were best explained by Skias and associates, who demonstrated that physiological doses of IL-10 were inhibitory but that high doses of IL-10 made EAE worse.[24] In PBMCs of MS patients, IL-10 mRNA is low during relapse and serum IL-10 levels are reduced in comparison to healthy controls.[5,9,25]

Production of TH1 cytokines by human, MBP-specific T-cell lines was demonstrated by us and others.[15,26] Extending these observations to TH2 cytokines, the data presented here demonstrate a low but constant production of IL-4 and IL-10 by MBP-specific T-cell lines after antigen specific stimulation. Therefore, the cytokine spectrum of our T-cell lines is very similar to that reported by Hemmer and coworkers[27] and Hermans and associates[28] for T-cell clones.

To get further insight into the effect of IFN-β1b and PTX on putative autoaggressive T cells, we studied the immunomodulatory effect of both drugs on MBP-specific T-cell lines in vitro. IFN-β1b and PTX exhibited a dose-dependent suppressive effect on proliferation and production of TH1 cytokines (eg, TNF-α, LT, and IFN-γ), and the combination of both drugs showed a significant synergistic effect. The suppression of proliferation and IFN-γ by IFN-β1b in T-cell lines after antigen-specific activation is in agreement with a report of Milo and Panitch.[29] Pette and colleagues reported suppression of proliferation but a slight induction of TNF-α and IFN-γ in two MBP-specific clones generated from 2 MS patients under similar culture conditions.[30] Differences in the cytokine profile of the T cells used in both studies may be responsible for this. Pette and colleagues investigated two T-cell clones, which lack IL-10 production even in the presence of IFN-β, whereas the T-cell lines tested in our study showed a low but constant secretion of IL-10 after stimulation with MBP, which is known to inhibit the production of TNF-α.[22]

So far the effect of IFN-β1b and PTX on the production of TH2-cytokines by human MBP-specific T cells after antigen-specific activation has not been described. PTX did not affect the production of IL-4 but decreased the production of IL-10 in MBP-specific T-cell lines after antigen-specific stimulation. The missing effect on IL-4 production is in agreement with a report of Rott and colleagues, who tested T cells of rats expanded in the presence of IL-2 and Con A after stimulation with lectin.[31] Suppression of IL-10, however, was rather unexpected, because we showed previously that PTX upregulates the production of IL-10 in PBMCs from MS patients after stimulation with phytohemagglutinin.[11] Differences in cell populations and the mode of activation may explain the different findings.

IFN-β1b, however, induced IL-10 production in MBP-specific T-cell lines after antigen-specific stimulation. So far this has been described for antigen nonspecific activation of T cells with CD3 mAb, a combination of CD2 and CD28 mAb or PMA and ionomycine.[32] The combination of IFN-β1b and PTX suppressed production of IL-10 in MBP-specific T-cell lines, whereas there was no significant effect on the production of IL-4 (see Fig 3E, F). The production of IL-10, however, was less inhibited than the secretion of TH1 cytokines. Therefore, the net effect of treating MBP-specific T-cell lines with a combination of IFN-β1b and PTX results in a shift from a TH1 to a TH2 response (see Table 2).

To investigate the effect of both drugs on cytokine production in vivo, we determined the cytokine mRNA profile of PBMCs isolated from MS patients under treatment with IFN-β alone or in combination with PTX. Treatment with IFN-β1b alone increased TNF-α and IFN-γ mRNA in freshly isolated PBMC during the first weeks, which is in agreement with the findings of Bykosh and associates.[9] Upregulation of

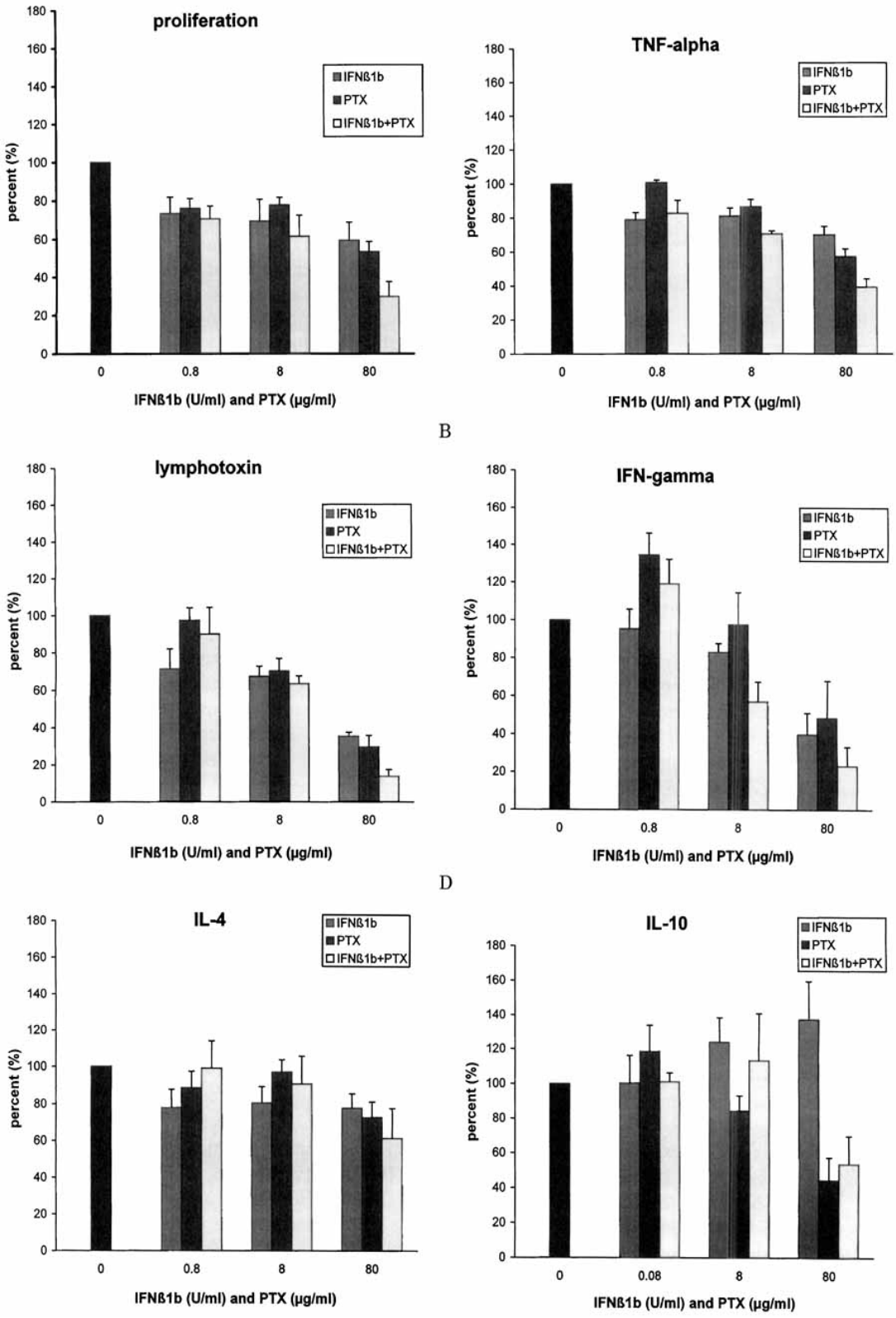

Fig 3. Dose-dependent effect of IFN-β1b and PTX—alone and in combination—on proliferation (A) and production of cytokines (B–F). Values represent mean ± SEM of two MBP-specific T-cell lines generated from a patient with MS and one line from a healthy donor. Results are given as percentage of the control value (ie, proliferation or cytokine production in the absence of IFN-β1b or PTX) and were reproduced in independent experiments.

Table 3. Combination of IFN-β1b and PTX Suppresses Predominantly TH1 Cytokines

T-Cell Line	IFN-β1b (U/mL) + PTX (μg/mL)	IL-4/ LT	IL-4/ IFN-γ	IL-10/ LT	IL-10/ IFN-γ
FHBP8	0.8	0.96	0.80	1.23	1.00
	8	0.98	0.95	0.95	0.92
	80	2.42	3.63	2.33	3.50
FHBP13	0.8	0.86	0.56	nd	nd
	8	1.37	1.39	2.10	2.04
	80	2.40	4.50	6.25	3.13
GZBP1	0.8	1.96	1.27	1.46	0.94
	8	1.93	3.18	2.30	3.79
	80	3.73	1.78	3.77	1.80

Two MBP-specific T-cell lines of the MS patient FH and one line of the healthy donor GZ are shown. Suppression of cytokines by the combination of IFN-β1b (U/mL) and PTX (μg/mL) was calculated as percentage of the control value, that is, cytokine production in the absence of IFN-β1b and PTX (1.0). Ratios of TH2-/TH1-cytokines are given.

these proinflammatory cytokines correlated with side effects. Addition of PTX, however, blocked the IFN-β–induced increase of TH1 cytokines and ameliorated side effects. Expression of IL-10 mRNA was increased by the combination of both drugs more than by therapy with IFN-β1b alone, whereas the production of IL-4 mRNA was not affected. Upregulation of IL-10 was confirmed by determination of IL-10 serum levels with ELISA. Together with the in-vitro data collected on the T-cell lines, these results suggest that, in the presence of PTX, IL-10 may be produced predominantly by other cell types than T cells; for example, monocytes produce IL-10, which can be further enhanced by cyclic adenosine monophosphate (AMP)–enhancing agents such as PTX.[33]

IFN-β exhibits its effect by binding to its receptor on the cell surface and subsequently requires the activation of two cytoplasmic tyrosine kinases JAK1 and TYK2.[34] Its antiproliferative action, and the modification of cytokine responses are well known but so far not elucidated in detail. PTX is a phosphodiesterase inhibitor that is known to inhibit proliferation and the production of TH1 cytokines by T cells.[31] For TNF-α, it was demonstrated that transcription is blocked by PTX,[35] and the induction of IL-10 in monocytes by PTX may be mediated by cyclic AMP–responsive elements present in the IL-10 promotor.[33] Although the molecular basis of action is not yet defined in detail, the described data support the hypothesis that both drugs interfere with different regulatory mechanisms within the cell.

Overall therapy with PTX was tolerated well without major side effects, and all patients in the IFN-β1b+PTX–treated group were clinically stable, whereas 2 patients worsened during treatment with IFN-β1b alone and 1 patient had a relapse. The data recorded on cytokine production of putative autoreactive T-cell lines and PBMCs of patients under treatment demonstrate that co-treatment with PTX enhances suppression of proinflammatory TH1 cytokines, which are upregulated before relapse[4,5] and during the initiation of IFN-β1b therapy.[9] The downregulation of TH1 cytokines and the rise of IL-10 serum levels indicates a shift from a TH1 to a TH2 profile. In addition, reduction of side effects occurring during the first weeks of IFN-β therapy improves quality of life and may increase compliance of patients.

In summary, data presented here indicate that co-treatment with the phosphodiesterase inhibitor PTX is a promising approach to correct the disturbed cytokine balance in patients with MS and to ameliorate early side effects of IFN-β therapy, but an extended study including more patients is warranted to assess the long-term effects of this combination therapy on the course of MS.

This work was supported by the Deutsche Multiple Sklerose Gesellschaft and the Gemeinnützige Hertie-Stiftung (GHS 315/94).

We thank Schering AG, Berlin, Germany, for providing IFN-β1b and Dr Rentschler Arzneimittel, Laupheim, Germany, for providing pentoxifylline.

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