Enhancement of Chemokine Expression by Interferon Beta Therapy in Patients With Multiple Sclerosis

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Background: Interferon beta has been approved for the treatment of multiple sclerosis (MS). It is believed that immunomodulatory rather than antiviral activity of interferon beta is responsible for disease amelioration. The impact of interferon beta on the chemotraction of immune cells has not been fully addressed.

Objective: To address the influence of interferon beta on the expression of chemokines and their receptors in a standardized setting.

Design: The expression of 14 chemokines and 14 chemokine receptor genes was determined by quantitative real-time polymerase chain reaction from fresh blood samples.

Setting: Outpatient units in Germany.

Patients: Untreated and interferon beta–treated patients with MS who tested positive and negative for neutralizing antibodies (NABs) were recruited from August 24, 2006, through December 15, 2006, for the initial study and from March 12, 2007, through April 2, 2007, for the validation study.

Main Outcome Measures: Gene expression and serum chemokine protein levels.

Results: CCL1, CCL2, CCL7, CXCL10, CXCL11, and CCR1 gene expression was strongly upregulated in interferon beta–treated, NAB-negative MS patients. In contrast, gene expression in interferon beta–treated, NAB-positive MS patients did not differ from untreated control donor individuals. Antibody titers inversely correlated with chemokine and chemokine receptor gene expression. Accordingly, serum chemokine protein levels of interferon beta–treated, NAB-negative MS patients were significantly higher than in untreated or interferon beta–treated, NAB-positive MS patients.

Conclusions: We demonstrate that interferon beta strongly upregulates a set of chemokines and CCR1 in peripheral immune cells. The peripheral upregulation of these chemokines may reduce the chemotraction of immune cells to the central nervous system and thus add to the therapeutic effects of interferon beta.

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Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that leads to demyelination and axonal damage. Histopathologic studies demonstrate that acute MS lesions in the CNS are characterized by activation of microglia and infiltration of mononuclear cells, primarily T lymphocytes, monocytes, macrophages, and occasionally B lymphocytes. The key players involved in the transmigration through the blood brain barrier are adhesion molecules and chemokines. Chemokines are a group of more than 50 structurally related low-molecular-weight molecules (approximately 8-14 kDa), mostly basic cytokines. On the basis of their structure, 4 subfamilies have been defined. CC chemokines are characterized by the presence of 2 adjacent cysteine residues near the N-terminus, whereas, in CXC chemokines, the corresponding cysteines are separated by 1 amino acid. The small group of XC chemokines contains only 2 conserved cysteines instead of 4. The CX3C group consists only of fraktalkine, which has 3 amino acids between the first 2 cysteines. The chemokines interact with a subset of 7 transmembrane, G protein–coupled surface receptors. Most of the chemokine receptors recognize more than 1 ligand, and several chemokines act on more than 1 receptor.

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Because chemokines control cell migration to sites of inflammation, it has been hypothesized that chemokines and their receptors are essential in the recruitment of pathogenic immune cells into the CNS. Although CC chemokines attract mononuclear cells to sites of chronic inflammation, at least some CXC chemokines attract polymorphonuclear cells to sites of acute inflammation. Therefore, many studies in human and animal models have investigated the role of chemokines and their receptors in brain, serum, and cerebrospinal fluid (CSF). In CNS tissue, the chemokines CCL2 (monocyte chemoattractant protein 1 [MCP1]), CCL4 (macrophage inflammatory protein 1β [MIP1β]), CCL5 (regulated on activation of normal T cells expressed and secreted [RANTES]), and CXCL10 (interferon-inducible protein of 10 kDa [IP-10]) were detected in the active demyelination of MS lesions. The related receptors CCR1, CCR2, CCR5, and CXCR3 are mainly expressed on T cells and monocytes and are also found in elevated levels in patients with MS lesions, which suggests that these cells are specifically attracted to sites of inflammation. More recently, it was shown that CXCL12 and CXCL13 levels were elevated in patients with active MS lesions, which suggests a role of B-cell guidance to the inflamed tissue. In CSF, CXCL10 was most consistently found to be upregulated and CCL2 to be downregulated. The expression of CXCL10 was reported to be higher in relapsing-remitting MS (RRMS) than in primary progressive MS. Since expression of this chemokine was higher in active than in stable MS, it may be involved in acute CNS inflammation. Although most T cells in the CSF express the complementary receptor CXCR3 even in noninflammatory conditions, the number of CXCR3+ CD4+ T cells in CSF seems to correlate with disease activity in MS. On the basis of their expression in the inflammatory lesion and their role in immune cell migration, chemokines and their receptors have become attractive therapeutic targets in MS.

Interferon beta is the most widely used immunomodulatory drug in the treatment of MS. Interferon beta, a cytokine produced by cells of the innate immune system, is essential to the control of viral infections. In the CNS, microglia and astrocytes produce interferon beta. Although interferon beta reduces relapse rates and activity as seen on magnetic resonance imaging (MRI) and seems to slow progression, its mode of action in MS is still only incompletely understood. Although interferon beta seems to have an effect on immune cell migration, the differential effect on the various chemokines and their receptors has not been fully investigated. We studied RNA transcript expression of 14 chemokines and 14 chemokine receptors in the blood of untreated and interferon beta–treated MS patients. The interferon beta–induced upregulation of gene transcripts was validated in patients who developed neutralizing antibodies against interferon beta.

### METHODS

#### PATIENTS

Seventeen RRMS patients given interferon beta-1a (Avonex; Biogen Idec, Research Triangle Park, North Carolina) intramuscularly once weekly, 9 age-matched untreated RRMS patients, and 8 age-matched healthy control donor individuals were enrolled in the initial study from August 24, 2006, through December 15, 2006. These patients were from the outpatient units of the MS Practice Study Group in Ulm, Germany. Patient characteristics are given in Table 1. For the validation study, an additional group of interferon beta–treated (interferon beta-1a; Avonex) RRMS patients was recruited from March 12, 2007, through April 2, 2007. This second cohort of patients comprised 4 MS patients who developed neutralizing antibodies (NABs) during the treatment compared with 7 MS patients who did not. The study was approved by the ethics committee of the University of Düsseldorf and the Technical University of München.

#### SAMPLE COLLECTION

Fresh blood samples were collected 12 hours after administration of interferon beta. To protect RNA, 5 mL of blood was collected directly in specialized RNA-protecting tubes (PAXgene, PreAnalytiX; Qiagen GmbH, Hilden, Germany). Serum samples were collected in parallel. After centrifugation at 1900g for 10 minutes, the serum was aliquoted and stored at −20°C.

#### RNA PREPARATION

High-throughput RNA preparation was performed (PAXgene 96 Blood RNA Kit; Qiagen GmbH). The quality of the RNA was analyzed with a bioanalyzer (Biacore; GE Healthcare, Chalfont St Giles, England). The purity and concentration were determined with photometer (Nanodrop ND1000, PEQLAB Biotechnology GmbH, Erlanger, Germany) analysis. RNA was immediately reverse transcribed to complementary DNA (cDNA) by a high-capacity cDNA reverse transcription kit (Applied Biosystems Inc, Foster City, California).

#### QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

The quantitative gene expression analysis was performed using low-density macroarrays from Applied Biosystems Inc. The arrays contained primer pairs and probes (TaqMan gene expression assays; Applied Biosystems Inc) for 14 different chemokine and 14 different chemokine receptor genes; GAPDH and 18S were used as endogenous controls, each preloaded in duplicates on a 384-well microfluidic card. The format enabled us to run an individual sample in parallel against the selected TaqMan expression assays. A sample of cDNA diluted in 50 µL of water and 50 µL of master mix (Applied Biosystems Inc) was mixed and added to a fill port of the card (200 ng of reverse-
Antibodies to interferon beta were determined by a capture enzyme-linked immunosorbent assay (cELISA), as previously described. Antibody titers were considered positive when they exceeded 5 SDs of the median of untreated patients and controls. The binding capacity of each serum sample was calculated relative to the binding capacity of a serum standard calibrator. The values are expressed as percentage of binding relative to positive standard samples. The biological activity of the antibodies was determined by the in vivo induction of MxA after interferon beta injection. The RNA expression level of MxA was determined 12 hours after interferon beta injection in each blood sample by quantitative real-time polymerase chain reaction (PCR). The MxA was normalized to GAPDH expression, and the relative expression compared with standard samples was calculated. The values are expressed as percentage of MxA induction relative to the MxA induction of newly treated MS patients. A value of 100% corresponds to a normal MxA induction after interferon beta therapy, and 0% corresponds to MxA levels observed in untreated patients and healthy control donor individuals. Patients were considered to have high titers of NABs when antibodies were detected by cELISA and the MxA response was reduced to the level of untreated MS patients.

**Table 2. Quantitative Expression Analysis of Chemokines and Chemokine Receptors in Nontreated Compared With Interferon Beta-1a–Treated Multiple Sclerosis Patients**

<table>
<thead>
<tr>
<th>Gene chemokine</th>
<th>CCL1</th>
<th>Mean (Range)</th>
<th>Interferon Beta-1a Treated, Mean (Range)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated</td>
<td>1.3</td>
<td>(0.3-3.8)</td>
<td>27.8 (1.2-112.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CCL2</td>
<td>0.9</td>
<td>(0.3-1.3)</td>
<td>110.9 (3.0-454.1)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CCL5</td>
<td>1.3</td>
<td>(0.8-1.7)</td>
<td>1.2 (0.8-1.8)</td>
<td>.51</td>
</tr>
<tr>
<td>CCL7</td>
<td>1.0</td>
<td>(0.4-1.9)</td>
<td>85.3 (0.4-462.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CCL11</td>
<td>1.1</td>
<td>(0.7-2.8)</td>
<td>1.3 (0.6-2.8)</td>
<td>.19</td>
</tr>
<tr>
<td>CCL17</td>
<td>0.7</td>
<td>(0.4-1.6)</td>
<td>0.8 (0.3-1.6)</td>
<td>.26</td>
</tr>
<tr>
<td>CCL21</td>
<td>0.6</td>
<td>(0.3-1.3)</td>
<td>0.6 (0.2-1.3)</td>
<td>.98</td>
</tr>
<tr>
<td>CXCL3</td>
<td>1.6</td>
<td>(0.2-2.8)</td>
<td>1.7 (0.2-3.4)</td>
<td>.84</td>
</tr>
<tr>
<td>CXCL5</td>
<td>1.0</td>
<td>(0.5-2.7)</td>
<td>1.1 (0.7-1.9)</td>
<td>.32</td>
</tr>
<tr>
<td>CXCL9</td>
<td>1.9</td>
<td>(0.2-4.5)</td>
<td>4.7 (1.0-12.4)</td>
<td>&lt;.007</td>
</tr>
<tr>
<td>CXCL10</td>
<td>1.8</td>
<td>(0.2-4.0)</td>
<td>155.1 (1.0-965.7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CXCL11</td>
<td>2.2</td>
<td>(0.9-9.8)</td>
<td>135.5 (3.9-667.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CXCL12</td>
<td>4.5</td>
<td>(0.2-16.2)</td>
<td>14.4 (0.5-50.1)</td>
<td>.02</td>
</tr>
<tr>
<td>CXCL13</td>
<td>1.4</td>
<td>(0.4-2.2)</td>
<td>3.9 (0.6-10.8)</td>
<td>.10</td>
</tr>
</tbody>
</table>

**FLOW CYTOMETRY–BASED CELL SORTING**

Peripheral blood mononuclear cells (PBMCs) isolated from 2 mL of peripheral blood were resuspended in 100 µL of phosphate-buffered saline and 5% fetal calf serum and stained with 10 µL of anti-CD19 allophycocyanin (B cells), anti-CD56 phycoerythrin (natural killer cells), and anti-CD3 fluorescein isothiocyanate (T cells) and incubated for 10 minutes at 4°C. After washing with phosphate-buffered saline and 5% fetal calf serum, sorting of 4 different immune subsets was performed on a cell sorter (FACS Aria; Becton, Dickinson and Company, Franklin Lakes, New Jersey). Monocytes were sorted according to forward and sideward scatter properties. All antibodies were purchased from Becton, Dickinson and Company. From each cell subset, 5000 cells were sorted. The RNA was immediately isolated and reverse transcribed to cDNA.

**NONPARAMETRIC ANALYSIS**

The Wilcoxon rank sum test was used to compare the expression between untreated and interferon beta–treated patients. The parametric test was used to compare the chemokine expression between groups. To adjust for multiple testing, the false discovery rate was calculated for each comparison. An expression was considered statistically significant when the P value was lower or equal to the false discovery rate for the analyzed gene. The Spearman rank correlation was applied to determine the relation between anti–interferon beta antibodies and the gene expression and between the MxA induction and the gene expression.
The aim of the study was to determine the influence of interferon beta on the expression of chemokine and chemokine receptor genes in vivo. Using a low-density macroarray for quantitative real-time PCR, we determined the expression of 14 chemokines and 14 chemokine receptors in blood samples of 8 untreated MS patients, 17 interferon beta–treated MS patients, and 9 healthy control donor individuals. Patient characteristics are given in Table 1. To compare the untreated and interferon beta–treated MS patients, the expression of each gene was normalized to the endogenous control gene GAPDH, and the n-fold expression difference compared with the gene expression in healthy control donor individuals was determined in each patient (Table 2). Overall, no significant expression difference was observed between untreated MS patients and healthy control donor individuals. The expression of most genes was also not influenced by the administration of interferon beta. By contrast, 5 chemokines, CCL1, CCL2, CCL7, CXCL10, and CXCL11, and 1 chemokine receptor, CCR1, were strongly upregulated in the blood of interferon beta–treated MS patients compared with untreated MS patients. The chemokines CXCL9 and CXCL12 were moderately upregulated (Figure 1). None of the chemokine or chemokine receptor genes was significantly downregulated in interferon beta–treated MS patients compared with untreated MS patients.

INFLUENCE OF NEUTRALIZING ANTIBODIES ON CHEMOKINE EXPRESSION

It is well known that up to 30% of interferon beta–treated MS patients develop NABs to interferon beta.26,27 These antibodies interfere with the therapeutic activity of interferon beta. To validate the impact of interferon beta on chemokine and chemokine receptor gene induction, we performed gene expression analysis in a second independent group of MS patients treated with interferon beta. The group included patients who had developed NABs and patients who were NAB negative. Four patients had high titers of interferon beta–specific antibodies and completely lost their interferon beta–induced MxA response. Seven patients were NAB negative and showed the expected strong upregulation of MxA gene transcripts in the peripheral blood (Figure 2A). When we determined chemokine transcript levels in those patients, we found that NAB-positive patients had expression levels of CCL1, CCL2, CCL7, CXCL9, CXCL10, CXCL11, CXCL12, and CCR1 comparable to untreated MS patients and controls (Figure 2B). By contrast, NAB-negative patients showed upregulation of all 8 genes comparable to the first patient group (Figure 1 and Figure 2B). In 1 patient, who developed NABs during interferon beta treatment, we had the opportunity to analyze the expression of chemokine and chemokine receptor genes before and after the patient had developed NABs. As expected, the expression of CCL1, CCL2, CCL7, CXCL9, CXCL10, CXCL11, and CXCL12 decreased once NABs had developed (Figure 2C and D). By contrast, interferon beta–dependent upregulation of chemokine genes did not change over time in patients who remained NAB negative (data not shown). Next, we analyzed the relation between NAB titer and the regulation of chemokine and chemokine receptor gene expression in interferon beta–treated patients. Induction of chemokine and chemokine receptor gene transcripts correlated strongly with transcripts of the interferon beta response gene MxA (R=0.82). An inverse correlation was observed between chemokine and chemokine receptor gene expression in peripheral immune cells and the level of interferon beta–specific antibody (R=−0.67).

Figure 1. Expression of CCL1, CCL2, CCL7, CXCL9, CXCL10, CXCL11, CXCL12, and CCR1 in untreated and interferon beta–treated patients with multiple sclerosis (MS). Shown are all chemokines (A) and chemokine receptor gene transcripts (B), which were significantly upregulated in patients treated with interferon beta (see also Table 2). The expression level of each gene was normalized by the housekeeping gene GAPDH (ΔCT). The normalized values of untreated and interferon beta–treated MS patients were calibrated with normalized values of healthy control donor individuals. Therefore, all gene transcriptions are expressed as n-fold changes relative to healthy control individuals (ΔΔCT) and transformed to log_{10} for presentation. Each dot represents 1 patient. nt indicates untreated MS patients; IFN, interferon beta-1a–treated MS patients.
Next, we investigated whether the increased expression of chemokine RNA in the blood results in higher chemokine protein serum levels. As shown for the CC chemokine CCL2, interferon beta–treated patients (mean, 340 pg/mL) had significantly higher chemokine levels compared with untreated MS patients (mean, 103 pg/mL) (Figure 3A). We also determined the serum concentration of CXCL11 and found a significant upregulation in interferon beta–treated (mean, 317 pg/mL) compared with untreated MS patients (mean, 57 pg/mL) for a CXC chemokine (Figure 3B). We also determined the serum level in NAB-positive patients for both chemokines. By contrast, chemokine levels in the serum of patients with NABs did not differ from the levels in untreated patients (Figure 3A and B). Similar to the RNA transcripts, we found a strong positive correlation between MxA gene upregulation and the protein concentration in serum as shown for CXCL11 (Figure 3C).

INTERFERON BETA–INDUCED CHEMOKINE LEVELS IN SERUM

To identify the cellular source of chemokine upregulation in response to interferon beta, we analyzed RNA expression in different immune subsets isolated from the PBMCs of 2 interferon beta–treated patients compared with 1 untreated MS patient (Figure 4). As exemplified for CXCL11, we demonstrated that interferon beta mainly upregulates chemokine expression by monocytes, whereas little to no changes were observed in other immune cell subsets.

COMMENT

Interferon beta is the most widely used immunomodulatory drug in the treatment of MS that exerts a significant impact on relapse rate and inflammatory MRI activity. However, the detailed mechanism of the way...
that interferon beta acts in MS is still unclear. One possible mode of interferon beta action may be an effect on the expression of chemokines and their receptors and thereby modulates the migration of immune cells to brain parenchyma. Several studies have investigated the influence of interferon beta in vitro and in vivo with a focus on selected chemokines or chemokine receptors. However, conflicting results have been reported.

We aimed to analyze the impact of interferon beta on the expression of a set of 14 chemokines and 14 chemokine receptors by quantitative real-time PCR in a standardized setting. Blood was taken 12 hours after the last interferon beta injection, and the messenger RNA expression level was determined directly ex vivo in fresh whole blood samples. We demonstrate that interferon beta selectively upregulates CCL1, CCL2, CCL7, CXCL10, and CXCL11. The chemokines CXCL9 and CXCL12 were moderately upregulated. The impact on chemokine receptor expression was less pronounced, with CCR1 being the only significantly upregulated gene. We confirmed our findings in a second group of interferon beta–treated patients, including patients who developed NABs during their treatment with interferon beta. Although the same chemokine induction was seen in interferon beta–treated, NAB-negative patients, the effect was completely abolished in treated NAB-positive patients. Antibody titers to interferon beta inversely correlated with chemokine induction, a result that further supports the specific effect of interferon beta on those chemokines.

Interestingly, upregulation of chemokine genes, as exemplified for CXCL11, was strongest in monocytes compared with all other peripheral immune subsets, although all were exposed to interferon beta.

The results of our study were somewhat surprising. The chemokines that we found to be upregulated in response to interferon beta specifically bind to CXCR3 and CCR5, which are expressed on activated memory T cells; CCR1, CCR2, CCR3, and CCR5, which are expressed on mono-
express the corresponding receptor bind the chemokine and undergo rapid cytoskeletal rearrangement followed by migration toward a higher concentration of the chemokines. At higher concentrations, some chemokines can spontaneously form oligomers or heteromers, which strongly enhance leukocyte migration and activation.34,35 As observed in our study, chemokines are strongly upregulated in the periphery, which may indeed decrease or even reverse the gradient between the inflammatory lesion and the peripheral immune compartment. This may prevent cell migration to the lesion or even drive cells back from the lesion to the periphery. In detail, all 3 CXCR3+ agonists, CXCL9, CXCL10, and CXCL11, may prevent the attraction of CXCR3+ T cells, CCL7 of CCR1+ monocytes, and CCL2 together with CCL7 of CCR2+ monocytes, and CXCL12 of CXCR4+ B cells. Because the CCR8 receptor is expressed on a subset of memory CD4+ T cells that comprise Th2 effector T cells and also regulatory T cells, the upregulation of CCL1 could lead to enhanced migration of regulatory T cells to the inflammatory site, which may regulate the inflammatory response in the CNS.46-47

As an additional concept, the exposure to high chemokine concentrations may desensitize the chemokine receptors in cells of the immune system. The exposure to high concentrations of chemokines often results in the rapid attenuation and desensitization of the receptor by receptor phosphorylation followed by uncoupling of the receptor from heterotrimERIC G proteins and internalization of the receptor.48-50 Internalization leads to the reduction of surface expression of the receptor, which leaves the cell unresponsive to further stimulus without significant changes in RNA level. Elevated chemokine concentrations in serum, as observed in our study, could thus prolong the unresponsiveness of the cell until the chemokines are consumed.

Both hypotheses are supported by several studies30-32 that demonstrated a reduced surface expression of CCR5 and CXCR3 on the PBMCs of interferon beta–treated MS patients. This concept is also supported by the analysis of serum CXCL10 and CCL2 after interferon beta administration. When blood was taken 6 hours after the last interferon injection, levels of both chemokines were elevated but decreased to normal within 24 hours.33 In a study with sampling 9 to 12 hours after the last injection, plasma levels of CCL3, CCL4, CCL5, and CXCL9 were unaltered, but the CXCL10 level was increased after interferon beta therapy.34 In line with our results, CCL2 serum concentration significantly increased as shown by others.35 In vitro studies in which PBMCs exposed to interferon beta were analyzed for early interferon beta responsive genes by cDNA microarray analysis support our findings. The analysis revealed that the RNA expression of CXCR3 ligands CXCL9, CXCL10, and CXCL11 and CCR2 ligands CCL2 and CCL8 was upregulated after 3-hour exposure to interferon beta in culture.36 Although we did not observe a downregulation of any chemokine, others have shown reduced serum levels for CXCL837 and reduced CXCL1 RNA expression ex vivo.38 Neither chemokine was analyzed in our study.

In summary, our in vivo data provide strong evidence that interferon beta has an influence on the expression of particular chemokines and receptors. The induction of chemokine expression in the periphery may affect migration of immune cells to the inflammatory lesions. This may explain the rapid onset of the anti-inflammatory activity of interferon beta in treated patients. Additional studies are warranted to determine whether this effect is central to the therapeutic efficacy of interferon beta.

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