T-Cell Interferon Gamma Receptor Binding in Interferon Beta-1b–Treated Patients With Multiple Sclerosis

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Objective: To investigate the effects of interferon beta treatment on T-cell interferon gamma binding (which is a possible marker for T-cell–dependent immune function) in patients with multiple sclerosis (MS).

Design: Assay interferon gamma binding on T lymphocytes from patients with stable relapsing-remitting MS before, 3 months after, and 6 months after initiating interferon beta-1b treatment.

Setting: The study was performed on ambulatory patients in a tertiary care center, where patients were diagnosed as having definite MS.

Patients: Eighteen patients with clinically definite, stable, relapsing-remitting MS (13 women and 5 men; mean age [± SD] 32.6 ± 7.1 years) were selected consecutively. Clinical status was defined according to the Kurtzke Expanded Disability Status Scale. All patients were treated with $8 \times 10^6$ IU interferon beta-1b subcutaneously every other day. Eighteen age- and sex-matched healthy subjects with no family history of neuropsychiatric disorders formed the control group.

Results: T lymphocytes from untreated patients with MS had significantly smaller amounts of interferon gamma receptors than those from control subjects (638 ± 7 [SE] vs 707 ± 11 [SE] receptors per cell). After 3 months of interferon beta-1b treatment, they showed a significant increase in interferon gamma binding (681 ± 9 [SE] receptors per cell). After 6 months, T-cell interferon gamma maximal receptor values were even higher (700 ± 7 [SE] receptors per cell), only slightly lower than those of control subjects.

Conclusion: Given that reduced interferon gamma binding might be related to lymphocyte activation, our data seem to demonstrate that the major effect of interferon beta-1b treatment is a decrease in T-cell activation.

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MULTIPLE sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). Current opinion holds that immunemediated factors are likely to be involved in MS pathogenesis.\textsuperscript{1,2} Cytokines represent important modulators of cell-to-cell interactions, even in the CNS\textsuperscript{3,4}; the unbalanced immune response in patients with MS might depend on derangement of a cytokine network. Among cytokines, interferons play an important role; they are classically defined as having antiviral activity. In addition, they are known to be potent antiproliferative and immunomodulatory agents. Two interferon types have been identified: type I, including interferon alfa and interferon beta, produced during viral or bacterial infection; and type II, interferon gamma, which is produced primarily by T lymphocytes on mitogen or antigen stimulation. The immune effects of interferon gamma include promoting T- and B-cell proliferation, generating cytotoxic T-lymphocyte activity, inducing high-affinity interleukin (IL) 2 receptor (IL-2R) expression, modulating antibody, IL-1, and tumor necrosis factor production, and increasing expression of major histocompatibility complex (MHC) molecules on the surface of many cell types.\textsuperscript{5}

In the CNS of patients with MS, interferon gamma is closely associated with active plaques on glial elements. Interferon gamma is able to induce expression of MHC class II molecules on glial cells;\textsuperscript{6} antigen presentation by MHC positive astrocytes could enhance the local immune response, thus facilitating continuous growth of lesions. Significantly higher cerebrospinal fluid interferon gamma levels have been found in patients with MS than in subjects with other neurological

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SUBJECTS, MATERIALS, AND METHODS

SUBJECTS

Eighteen patients with clinically definite, stable RR MS were consecutively selected at the Department of Neurosciences, Section of Neurology, University of Pisa in Italy. The RR course was defined as the occurrence of clear episodes of disease worsening over 24 to 48 hours with full or partial recovery.18 Clinical status was defined according to the Kurtzke Expanded Disability Status Scale.20 There were 5 men and 13 women, aged 24 to 46 years (mean ± SD age, 32.6 ± 7.1 years). Disease onset ages ranged from 16 to 41 years; disease duration, 1 to 11 years; and Kurtzke Expanded Disability Status Scale scores, 1 to 3.5 (Table). T-cell interferon gamma binding was measured in all patients before the initiation of alternate-day subcutaneous treatment with 8 × 106 IU interferon beta-1b30 and after 3 months and 6 months during therapy (on-treatment). Serum IL-2 and soluble IL-2R levels were used as markers for circulating activated T cells: for IL-2 and soluble IL-2R immunoenzymatic assays, commercial kits (Genzyme Srl, Milan, Italy) were used. Patients were monitored for adverse effects during the 6-month period, including systemic symptoms, local injection site reaction, and changes in peripheral total leukocyte count and lymphocyte count.

At the time of the first observation and blood sampling, none of the patients had received corticosteroids for at least 1 month, or immunosuppressive drugs for at least 6 months; during interferon beta-1b treatment none of them received immunosuppressive medications. No subject showed any symptoms or signs of concurrent infection either before or during interferon beta-1b therapy.

The control population consisted of 18 age- and sex-matched healthy subjects selected among blood donors or laboratory personnel, aged 18 to 51 years (mean age ± SD, 31.7 ± 9.6 years).

METHODS

Separation of T Cells From Peripheral Blood

Venipuncture was performed on all subjects after informed consent between 7 AM and 8 AM to avoid circadian variations of lymphocyte subsets. Blood was centrifuged with Ficoll-Paque (Amersham Pharmacia Biotech Italia, Milan, Italy) at 400g for 30 minutes at room temperature. Buffy coats of mononuclear cells were spun at 800g for 10 minutes at 4°C to get cell pellets. After cell counting with a Neubauer hemocytometer, mononuclear cells in Dulbecco modified minimal Eagle medium (Sigma Aldrich Srl, Milan) with 10% fetal calf serum (Bio-Whittaker International PBI Spa, Milan) were plated overnight in Petri dishes at 4°C to separate peripheral blood lymphocytes from macrophages. Peripheral blood lymphocytes were incubated in Dulbecco modified minimal Eagle medium with 10% fetal calf serum at 4°C for 2 hours in mouse anti-human IgG (Sigma)–coated Petri dishes. Such a pan-binding procedure was repeated 3 times so that we got 97% pure T-cell suspensions. T lymphocytes were identified morphologically and as Leu4+ cells by flow cytometry (Figure 1). No significant differences were observed among various subject groups.

T-Cell Interferon Gamma Binding

We radioactively labeled recombinant human (RH) interferon gamma with iodine 125 (Genzyme) (specific antiviral activity: 1 × 107 U/µg of protein) according to the method previously described.20 In standard binding assays, 6 × 106 T cells from each subject were incubated in duplicate at 4°C in 750 µL Dulbecco modified minimal Eagle medium with HEPES buffer (10 mmol/L, pH 7.4) and 10% fetal calf serum for 2 hours with different amounts of 125I-RH interferon gamma (0.1-0.5 ng). In competitive binding experiments, increasing amounts of unlabeled RH interferon gamma were added to standard binding assays. Non-specific binding was determined by adding in duplicate a 100-fold excess of unlabeled RH interferon gamma. Radioactivity was counted in a gamma counter (Beckman Italia Spa, Milan) with 50% efficiency. Final results about binding parameters were achieved through the Scatchard equation, using McPherson’s Ligand program (version 3.1, 1985), which determines the maximal receptor number (Bmax) and the dissociation constant (Kd). A molecular weight of 34 kd, corresponding to the dimeric form of RH interferon gamma, was used for calculations. Statistical evaluation was performed using the 2-tailed Student t test and analysis of variance, according to the number (≥2) of subject groups to be compared.

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sociation constant in the picomolar to nanomolar range, expressed in various human tissues, including peripheral blood mononuclear cells.\textsuperscript{19-21} Freshly isolated normal human T cells activated in vitro with phytohemagglutinin, concanavalin A, or phorbol myristate acetate have been reported to express fewer interferon gamma receptors than resting lymphocytes.\textsuperscript{22} A functional interferon gamma receptor requires at least 2 components: one is a ligand-binding molecule, and the other is a signal-transducing, species-specific factor encoded by a gene on chromosome 21.\textsuperscript{23,24} A membrane proximal region is required for ligand processing and Janus kinase binding.\textsuperscript{25} The binding of interferon gamma to its receptor, through the Janus kinase activation, induces phosphorylation of Stat1 (a member of a family of transcription factors) causing its dimerization and moving to the nucleus, where Stat1 binds the gamma-activated sequence in interferon gamma–responsive genes.\textsuperscript{25}

We measured interferon gamma receptor binding on T cells from untreated patients with MS,\textsuperscript{26} finding significantly lower numbers of interferon gamma receptors on lymphocytes from patients than on those from age-matched healthy control subjects: the lowest values were observed in patients with RR MS during relapses (407 ± 25 [SE] receptors per cell).

The aim of the present work has been to assay peripheral blood T-cell interferon gamma binding in interferon beta-1b–treated patients with RR MS as compared with healthy control subjects, and to study the in vivo effects of interferon beta-1b on T-cell activation, which seems to be linked to a down-regulation of interferon gamma receptors.

### RESULTS

We found that T cells from patients with MS and healthy control subjects constitutively express high-affinity interferon gamma receptors. The binding of \textsuperscript{125}I–RH interferon gamma was specific: only unlabeled RH interferon gamma significantly inhibited the binding, while the same amounts (100 ng) of RH interferon alfa or RH interferon beta were ineffective. A representative experiment set of competitive binding of \textsuperscript{125}I–RH interferon gamma and unlabeled RH interferon gamma to T cells is shown in Figure 2. Enriched T-cell suspensions were incubated with \textsuperscript{125}I–RH interferon gamma (0.5 ng) and different amounts of unlabeled RH interferon gamma. Saturation binding experiments resulted in similar findings.

Differences in Kd values were not found between patients with MS and healthy control subjects (1.1 ± 0.06 [SE] vs 1.0 ± 0.04 [SE] nmol/L), or among pretreatment and on-treatment groups. On the contrary, highly significant (P<.001) differences in B\textsubscript{max} values were observed between untreated patients and control subjects.

### Clinical Features of Patients

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*EDSS indicates Kurtzke Expanded Disability Status Scale.\textsuperscript{27}

Figure 1. Cell suspensions of 100 µL (either from freshly isolated mononuclear cells or from purified lymphocyte cell cultures [see “Subjects, Materials, and Methods” section]), were incubated in the dark for 20 minutes with 5 µL of fluorescein-isothiocyanate–conjugated monoclonal antibodies against CD3 (a marker for total T cells) (anti-Leu4; Becton Dickinson Italia Spa, Milan, Italy). Samples were counted through a flow cytometer (Epics XL-MCL; Coulter Electronics Ltd, Luton, England) equipped with a single 15-mW argon ion laser (excitation wavelength of 488 nm in combination with a 530-nm bandpass filter). Fluorescein-isothiocyanate–conjugated mouse IgG was used to evaluate nonspecific fluorescence. To exclude monocytes, other leukocytes, and debris, gates were selected on lymphoid cells, as determined by forward and right-angle scatter. Overlaid histograms of CD3+ cells in freshly isolated (1) and purified (2) cell cultures from a patient with multiple sclerosis (top) and a control subject (bottom).
Figure 4: 638 ± 7 (SE) vs 707 ± 11 (SE) receptors per cell. Interferon beta-1b therapy was associated with an increase in T-cell interferon gamma receptor number: \( B_{\text{max}} \) values were significantly \( P < .001 \) higher 3 months after initiation of treatment (681 ± 9 [SE] receptors per cell). At 6 months, \( B_{\text{max}} \) values increased further (700 ± 7 [SE] receptors per cell), although not significantly compared with 3-month values. There was a statistically significant \( P < .005 \) difference in T-cell interferon gamma receptor \( B_{\text{max}} \) values between patients with MS 3 months after initiation of interferon beta-1b therapy and control subjects, whereas patients’ 6-month values did not differ from those of control subjects. Linear regression analysis performed on groups of patients with MS resulted in no significant correlations between serum IL-2 levels and interferon gamma receptor \( B_{\text{max}} \) values, or between soluble IL-2R levels and \( ^{125}\text{I} \)-RH interferon gamma binding (data not shown).

In separate in vitro experiments with T lymphocytes from healthy control subjects (Figure 5), we observed a significantly increased \( P < .001 \) expression of high-affinity interferon gamma receptor (Kd: 0.9 ± 0.06 [SE] nmol/L) on concanavalin A–stimulated T cells treated with interferon beta-1b (500 U/mL), as compared with untreated control T lymphocytes activated with concanavalin A (5 ng/mL) (698 ± 16 vs 311 ± 15 [SE] receptors per cell). Interferon gamma \( B_{\text{max}} \) values of interferon beta-1b–treated T cells resembled those of freshly isolated unstimulated T lymphocytes from healthy control donors (708 ± 13 [SE] receptors per cell).

Multiple sclerosis is an inflammatory disease involving the white matter of the CNS. Although there is a localized immune response within the CNS, disease-related immune changes are also found in peripheral blood lymphocytes. \(^{26,34-36}\) Interferon gamma is a cytokine with pleiotropic effects; in particular, it is able to modulate the immune network both in the CNS and systemically. The initial event in the action of interferon gamma is the bind-
ing to specific receptors found on different cell types, including peripheral blood lymphocytes. The binding of interferon gamma to its receptor plays an obligate role in T-lymphocyte activation.

In the present work, we have found that untreated patients with MS have a significantly reduced number of T-cell interferon gamma receptors compared with healthy control subjects, thus confirming our previous findings. Since activated lymphocytes have fewer interferon gamma receptors than resting ones have, these data give further support for the presence of a systemic T-cell activation in MS.

In the present work, we did not find significant correlations between serum IL-2 levels and interferon gamma receptor Bmax values (P = .09) or between soluble IL-2R and 125I–RH interferon gamma binding (P = .07). On the contrary, many research groups have reported an association between IL-2R expression (and/or IL-2 production) and T-cell activation in MS. In our previous research work, we too found a significant (P < .01) negative correlation between serum IL-2 levels and T-cell interferon gamma binding in patients with relapsing and evolutive MS (but not in stable patients); moreover, by reexamining in a stable phase 6 patients with MS with relapses showing a significant (P < .05) negative correlation between serum IL-2 and interferon gamma receptor values, we did not observe any such significant correlation in the stable phase (P = .2). Therefore, the lack of a significant correlation between serum IL-2 levels and lymphocyte interferon gamma binding might depend on the fact that in the present work we studied patients with clinically definite, stable RR MS.

Interferon beta-1b is associated with an increase in the interferon gamma receptor number on T cells from patients with MS 3 months and even 6 months after the initiation of treatment. Interferon beta is an immunomodulatory cytokine, and its beneficial effect in MS might depend on its ability to regulate the 3 phases of immune response: it might lessen activation, blunt assault, or increase deactivation, or it might cause some combination of these 3. Interferon beta counteracts the protean immune-augmenting effects of interferon gamma, in particular interferon gamma–enhanced MHC expression on the surface of macrophages and astrocytes acting as antigen-presenting cells, an agent such as interferon beta, which lessens the capacity for antigen presentation to T cells, is expected to attenuate T-cell responses. Moreover, interferon beta reduces the release of interferon gamma and tumor necrosis factor α from cultured mononuclear cells and promptly restores the deficient suppressor function in patients with MS, at least in vitro.

An interferon beta-1b treatment–dependent increase in the number of T-cell interferon gamma receptors might be due, at least in part, to the antagonistic action of interferon beta-1b on interferon gamma, which is able to down-regulate its own receptors, as shown by the effects of anti–interferon gamma monoclonal antibodies in preventing a large portion of the phytomagglutinin-induced decrease of interferon gamma Bmax values. Given that less activated lymphocytes seem to have increased interferon gamma bind-

ing, the interferon beta-1b up-regulating effect on T-cell interferon gamma receptors might represent a favorable immunomodulatory mechanism: the T-cell–activated state (as marked by decreased interferon gamma binding) is progressively reduced during treatment (as shown by increased T-cell interferon gamma receptor numbers approaching more and more those of control subjects).

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