Inhibition of Endogenous Interferon Beta by Neutralizing Antibodies Against Recombinant Interferon Beta

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Objective: To determine if neutralizing antibodies (NAbs) against interferon beta from patients with multiple sclerosis (MS) cross-react with other type 1 interferons, especially endogenous interferon beta, and thus might impede the immune systems of affected patients.

Design: Masked serum samples from MS patients were challenged in vitro against recombinant interferon beta-1a and interferon beta-1b, as well as human leukocyte interferon and fibroblast interferon, the latter representing endogenous interferon. The neutralizing capacity of serum samples on these type 1 interferons was assessed using a luciferase reporter gene assay. Randomly selected samples were titrated to further delineate the cross-reactivity of antibodies.

Setting: University medical center in Düsseldorf, Germany.

Patients: We randomly selected 150 samples from interferon beta–treated MS patients who had previously been tested for the presence of binding antibodies and NAbs.

Main Outcome Measures: Neutralization of interferon beta bioactivity and cross-reactivity of anti–interferon beta antibodies.

Results: Antibody-mediated neutralization of interferon beta bioactivity in vitro against recombinant interferon beta was observed in all serum samples that had previously tested positive for binding antibodies and NAbs. A neutralizing pattern comparable to that of recombinant interferon beta was observed when endogenous interferon was assessed, reflecting cross-reactivity of NAbs. No differences in neutralization between recombinant and endogenous interferon were observed with respect to the interferon beta preparation used for treatment. Furthermore, no neutralization of other type 1 interferons by NAbs could be detected.

Conclusions: A proportion of MS patients who are treated with recombinant interferon beta develop NAbs that also neutralize endogenous interferon. Because NAbs at high titers can persist for years, these antibodies may impede the immune system in affected MS patients regardless of their current treatment regimen.

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In the presence of NAbS, physiologically required interferon levels may be met by exogenous interferon if patients continue to receive therapy. In such cases, endogenous interferon production and activity may appear less relevant because they are then replaced therapeutically by exogenous IFN. We have previously observed higher in vivo basal interferon responses in interferon-treated patients compared with untreated MS patients or healthy subjects.6 In addition, upregulation of interferon receptors has been described in NAb-positive patients.12 Both observations suggest a modulation of cytokine responses during continuous interferon therapy. However, if treatment with exogenous interferon is discontinued, NAb-mediated inhibition of endogenous interferon may theoretically become relevant. Cross-reactivity of NAbS with endogenous type 1 interferons might occur and thus might affect the immune systems as well as the clinical course of affected patients. To our knowledge, these aspects have never been studied.

In this study we aim to determine how anti–interferon beta antibodies cross-react with type 1 interferon produced by human fibroblasts, representing endogenous interferon beta, as well as interferon produced by human leukocytes, known as endogenous interferon alfa and interferon omega, 2 other type 1 interferons.

### METHODS

#### PATIENTS AND ASSESSMENT OF BAbS AND NAbS

Serum samples of 150 interferon beta–treated MS patients were retrospectively and randomly selected from the German reference laboratory for NAb testing (Düsseldorf). Initial sample selection was observer masked, and all selected samples were coded throughout the experiment. Patients gave informed consent, and the study was approved by the local ethics committee.

Serum samples were collected at least 12 hours after the last interferon beta injection and stored at -20°C until the time of analysis. The samples were first screened for the presence of BAbS by a capture enzyme-linked immunosorbent assay (ELISA), as described elsewhere.13 The BAb-positive samples were further tested for the presence of NAbS by a luciferase reporter gene assay, as recently described.14

#### GENERATING NONRECOMBINANT, ENDogenous interferon beta

Fibroblasts were cultured and used to produce natural, nonrecombinant interferon beta following published methods3 with added modifications.15 In brief, human dermal fibroblasts (PromoCell GmbH, Heidelberg, Germany) were seeded at a density of 5×10^4 cells/mL in 12-well cell culture plates in a complete medium (Dulbecco's modified eagle medium [DMEM]; PAA Laboratories GmbH, Pasching, Austria), supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Grand Island, New York). At 90% confluency, cells were primed by incubating them with 5 U/mL of human leukocyte interferon (PBL InterferonSource, Piscataway, New Jersey) for 16 hours. Primed cells were washed thoroughly with serum-free DMEM and stimulated with synthetic toll-like receptor 3 ligands (polyinosinic-polycytidylic acid; InvivoGen, San Diego, California) at 5 µg/mL, reconstituted in serum-free DMEM, by incubating for 2 hours. After stimulation, cells were washed thoroughly and superinduction was performed: first by incubating with 50 pg/mL of cycloheximide (Sigma-Aldrich GmbH, Steinheim, Germany) for 2 hours, and second by incubating with 1 pg/mL of actinomycin D (InvivoGen) for 1 hour. Finally, cells were washed and replenished with 800 µL of serum-free medium per each well and incubated for 24 hours to let the fibroblast interferon accumulate in the supernatant. All incubations were done in 5% carbon dioxide flow at 37°C. Supernatant with crude fibroblast interferons was concentrated by filtering through 20-µL centrifugal concentrators (Vivaspin; Sartorius AG, Gottingen, Germany), and the final crude interferon beta concentration was measured using an interferon beta–specific (non–cross-reactive to human interferon alfa, interferon gamma, mouse interferon beta, or rat interferon beta) ELISA kit (PBL InterferonSource) according to the manufacturer's protocol. Aliquots of supernatant samples with interferon beta were temporarily stored at 4°C until further processing.

#### CROSS-REACTIVITY OF ANTI–INTERFERON BETA ANTIBODIES AGAINST DIFFERENT INTERFERON ANTIGENS

Antibody cross-reactivity was assessed in vitro by comparing fixed amounts of patients' serum samples with fixed doses of recombinant interferon beta-1a (Avonex; Biogen Idec, Cambridge, Massachusetts), interferon beta-1b (Betaseron; Bayer Schering, Leverkusen, Germany), human fibroblast interferon (as described previously), and human leukocyte interferon (consisting of interferon alfa and interferon omega; PBL InterferonSource), as follows: 5 µL of patients' serum was incubated with 95 µL of recombinant interferon beta-1a (10 lab units [LU]/mL, approximately 20 IU/mL), interferon beta-1b (20 IU/mL), leukocyte interferon (125 IU/mL), and fibroblast interferon (diluted 10 times) separately for 1 hour in 96-well polystyrene microtiter plates. A parallel control was run for each patient's serum sample by incubating the serum without any interferon.

The magnitude of in vitro neutralization was measured following a method described by Farrel et al.14 In brief, HT-1080 fibrosarcoma cells were stably transfected with the firefly luciferase gene, which is linked to the interferon-stimulated responsive element. When interferon beta binds to and stimulates interferon receptors, this reporter cassette is activated, coexpressing luciferase as well. The luciferase activity can thus be quantified by adding a substrate (Steady-Glo luciferase assay solution; Promega, Madison, Wisconsin) and measuring the resultant chemiluminescence signal by a luminometer (Tecan GeniosPro microtiter plate reader; Tecan Group Ltd, Männedorf, Switzerland). These transfected fibrosarcoma cells were seeded in 96-well microtiter plates at 4×10^3 cells per well in complete DMEM without antibiotics or antimycotics. After overnight incubation the supernatant was decanted, the serum samples were incubated with different interferons, and the controls were transferred to fibrosarcoma cell plates. After 4 to 5 hours of incubation, 50 µL of Steady-Glo luciferase assay solution was added to each well and luminescence activity was measured in a Tecan-Geniospro microplate reader (Tecan Group Ltd, Männedorf, Switzerland). Results were expressed as luminescent counts per second. Thus, the final luminescent activity reflects the degree of in vitro neutralization of anti–interferon beta antibodies against tested interferon antigens. Serum samples were analyzed in duplicates and in parallel against the different interferons.

In a pilot experiment, luminescent activity was determined in a series of different interferon dilutions to determine a reference bioactivity level for 10 LU/mL of interferon beta-1a and an equivalent bioactivity level for fibroblast interferon and leukocyte interferon. In addition, interferon beta concentrations were also assessed using ELISA at 10 LU/mL-equivalent dilution points.

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TITRATION OF SERUM AGAINST DIFFERENT INTERFERON BETA ANTIGENS USING MODIFIED NAb ASSAYS

From the 150 serum samples investigated in this study, 30 samples were reselected and titrated using the luciferase reporter gene assay. Antigens of 3 different interferon beta preparations were used: interferon beta-1a, interferon beta-1b, and fibroblast interferon. For each serum sample tested, 3 NAb titers were determined. The raw NAb titer was calculated from the reciprocal value of the serum dilution point, when a 10-fold reduction from 10 LU/mL or equivalent interferon was reached. Thereby, a direct comparison of antigen-specific NAb titers for each serum sample was possible and enabled us to further assess differences in antibody cross-reactivity and affinity between respective antigens, including endogenous interferon beta.

STATISTICAL ANALYSIS

Spearman rank correlations and multiple comparisons using the Kruskal-Wallis test with post hoc analysis were performed when appropriate, using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego).

RESULTS

SAMPLES, BAbs, AND NAbs

Of the 150 interferon beta–treated MS patients, 29 (19.3%) were treated intramuscularly with interferon beta-1a (Avonex; Biogen Idec), 73 (48.7%) were treated subcutaneously with interferon beta-1a (Rebif; Merck Serono, Geneva, Switzerland), and 48 (32.0%) were treated subcutaneously with interferon beta-1b (Betaferon; Bayer Schering). Ninety-five samples (63.3%) were positive for BAbs as well as NAbs, 30 (20.0%) tested positive only for BAbs, and 25 (16.7%) did not contain BAbs or NAbs (Table).

BIOACTIVITY AND INTERFERON BETA CONCENTRATION

To render the different serum samples studied comparable to each other, a reference bioactivity level was defined at 10 LU/mL of interferon beta-1a (approximately 20 IU/mL), and equivalent bioactivity for fibroblast interferon and leukocyte interferon was determined accordingly. The concentrated crude fibroblast interferon beta supernatant was serially diluted, and a 10-fold dilution was found to yield a bioactivity level similar to 10 LU/mL of interferon beta-1a (Figure 1). Interferon beta concentrations were similar between interferon beta-1a and fibroblast interferon, whereas leukocyte interferon did not contain any detectable amount of interferon beta, as expected. Bioactivity level and protein content were higher with interferon beta-1b at an equivalent dose (ie, 20 IU/mL) than with interferon beta-1a.

CROSS-REACTIVITY OF ANTI–INTERFERON BETA ANTIBODIES AGAINST DIFFERENT INTERFERON ANTIGENS

Antibody-mediated neutralization or loss of interferon beta bioactivity in vitro against recombinant interferon beta was observed in all serum samples that tested positive for BAbs and NAbs (Figure 2). All serum samples with relevant antibody levels (ie, moderate or high levels of BAbs and positive for NAbs), revealed interferon bioactivity at baseline level, indicating complete neutralization of all interferon beta antigens. A similar pattern of neutralizing capacity was noted in the same samples when compared with fibroblast interferon (Figure 2). There was no significant neutralization of other type 1 interferons, such as leukocyte-derived interferon alfa or interferon omega, by anti–interferon beta NAbs. In all control samples, baseline interferon bioactivity levels re-
NAb-positive [NAb](Betaferon; Bayer Schering, Leverkusen, Germany) are shown with respective interferon beta concentrations, as measured using enzyme-linked immunosorbent assay. Supernatant with fibroblast interferon, recombinant interferon beta-1a (Avonex; Biogen Idec, Cambridge, Massachusetts), and recombinant interferon beta-1b (Betaferon; Bayer Schering, Leverkusen, Germany) are shown with different binding antibody (BAb) and neutralizing antibody (NAb) levels (x-axis) and their in vitro neutralizing capacities (y-axis). Statistically significant bioactivity levels between consecutive antibody categories are shown. Different BAb concentrations as measured by enzyme-linked immunosorbent assay are denoted as negative (BAb−), low (BAb+), moderate (BAb++), and high (BAb+++). The Spearman correlations for this range of antibodies (samples with bioactivity levels ≥1000 luminescent counts per second) revealed a stronger correlation between interferon beta-1a and fibroblast interferon (Spearman r=0.86; P<.001) than between interferon beta-1b and fibroblast interferon (r=0.57; P<.001), suggesting closer similarity in the antibody-binding pattern between the 2 first interferons.

When studying the cross-reactivity of anti–interferon beta antibodies, an interesting correlation between endogenous, fibroblast-derived interferon and both recombinant interferon beta-1a (Avonex) and interferon beta-1b (Betaferon) was found (Figure 3A and B). The Spearman correlations for this range of antibodies (samples with bioactivity levels ≥1000 luminescent counts per second) revealed a stronger correlation between interferon beta-1a and fibroblast interferon (r=0.86; P<.001) than between interferon beta-1b and fibroblast interferon (r=0.57; P<.001), suggesting closer similarity in the antibody-binding pattern between the 2 first interferons.

No statistically significant antibody cross-reactivity was found with regard to the treated interferon beta preparation administered to patients. The NAb that have developed in patients treated intramuscularly or subcutaneously with interferon beta-1a or subcutaneously with interferon beta-1b cross-react with and neutralize endogenous interferon beta.

**COMPARISON OF NAb TITERS CALCULATED FROM DIFFERENT NAb ASSAYS**

To further corroborate our observation, we conducted end point titrations on 30 samples with various BAb seropositivity. We tested 9 samples each from patients treated with Avonex or Rebif, as well as 12 samples each from patients treated with Betaferon. All NAb titers calculated from interferon beta-1a and fibroblast interferon assays were closely comparable, irrespective of the treated interferon preparations used therapeutically, further confirming the ubiquitous nature of cross-reactivity of the NAb (Spearman r=0.98; Figure 4). This also supports the similar antigen-
In this study, we report the degree and pattern of cross-reactivity of anti–interferon beta antibodies with different concentrations and neutralizing capacities against type 1 interferon antigens, with special reference to naturally occurring, endogenous interferon beta. These antibodies, which patients developed against systemically injected recombinant interferon beta, appear to be binding to and neutralizing natural interferon beta. Our findings corroborate the hypothesis that endogenous interferon beta activity could be disturbed in patients with NAbs. At higher antibody concentrations, a complete inhibition of endogenous interferon signaling was observed, further supporting the notion that NAbs critically impede physiological interferon beta signaling pathways.

The antibody-binding pattern between endogenous and recombinant interferon beta appeared very similar (Figure 3A and B), with greater similarities noted between interferon beta-1a and fibroblast interferon. Titration of serum samples to further delineate neutralizing capacities revealed comparable titers between endogenous and recombinant interferon beta, further supporting the close cross-reactivity of antibodies and suggesting the similar affinity profiles of antibodies against these 2 interferons. The sharing of common antigenic epitopes, possibly owing to the structural similarity between interferon beta-1a and fibroblast interferon, might explain this observation. Interestingly, cross-reactivity of antibodies to interferon beta-1b and fibroblast interferon was somewhat different compared with that of interferon beta-1a in the low to moderate range of antibody levels. Interferon beta-1b is a nonglycosylated protein and tends to form aggregates in solutions as a result of less solubility in water. This property may confound the antibody binding process on the interferon beta-1b molecule and account for these differences. However, the overall comparison still supports the ubiquitous nature of antibody cross-reactivity.

Anti–interferon beta antibody cross-reactivity was independent of the recombinant interferon beta preparation used for treatment in the individual patient. This may imply that antibodies are generated against common epitopes shared among the 2 different recombinant interferon beta molecules during the initial stage of a humoral immune response mounted against the injected drugs. Even though interferon beta-1b is structurally dif-
fferent from interferon beta-1a, and therefore the immuno-
genic peptide segments may be different from those of interferon beta-1a, we were unable to demonstrate meaningful differences in the neutralizing capacity of NAbs against both proteins.

More important, we did not observe relevant antibody cross-reactivity with other type 1 interferons. Neither low nor high titers of NAbs were able to interfere with the functionally relevant interaction between type 1–specific interferon receptors and interferon alfa or interferon omega, revealing similar luminescent activities regardless of the NAb titer in the serum samples studied. This clearly underlines the fact that the neutralizing specificity of NAbs is restricted to interferon beta and does not interfere with other type 1 interferons.

We believe that our findings are clinically meaningful. Immunomodulatory treatment of MS with interferon beta is associated with the development of NAbs. Their frequencies vary among the different drugs available. Especially high-titer NAbs tend to persist for years.18 The presence of memory B cells and plasma cells along with the boosting effect of endogenous interferon beta tend to maintain this antibody response, which is similar to that of a successful vaccination.19 Therapeutically, to our knowledge no treatment strategy has been found to date to remove NAbs efficiently; neither immunosuppressive regimens nor anti–B-cell-driven approaches, as such with the anti-CD20 antibody rituximab, exhibited a lowering effect on NAb titers.

In patients being treated with recombinant interferon beta, physiologically required interferon levels in the presence of NAbs may be compensated for by exogenous recombinant interferon beta. Alternatively, recombinant interferon beta saturates NAbs, and consequently endogenous interferon beta may maintain its physiological role. If treatment with an interferon beta preparation is discontinued, endogenous interferon beta activity will still be neutralized by NAbs. Thus, cross-reactivity of NAbs with endogenous type 1 interferons and its long-term effect on the immune system, as well as the clinical course of the disease, may have important consequences; however, to our knowledge, this has not been investigated to date. It would therefore be interesting to measure serum levels of endogenous interferon beta in such patients. This would technically be rather challenging, especially in patients treated with interferon beta. Theoretically, iatrogenic aberrations in immune function could be expected to result and to be sustained long-term in these patients. At present, no clinical evidence indicates that NAb-positive patients have a higher risk of contracting viral infections or exhibit other manifestations of compromised immunity. However, data on systematic long-term follow-up of larger numbers of NAb-positive patients are scarce or do not exist. Such a systematic follow-up is difficult outside the context of a clinical trial, particularly because many patients may be prescribed a different treatment once NAbs cause nonresponsiveness to interferon beta treatment. Shapiro et al20 reported that serum antibodies could inhibit interferon beta–mediated chemokine secretion by glia cells in vitro, supporting our concept that NAbs modulate endogenous interferon beta. Because NAbs can persist for years, even in the absence of exogenous interferon beta, these antibodies may unduly affect the immune system in affected MS patients far beyond discontinuation of their treatment with interferon beta.

Our present data underline the fact that NAbs have the potential to interfere with endogenous interferon beta and thus could compromise immune regulatory responses in the immune system. Further studies are warranted to shed light on this relevant issue.

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