Potential for Interferon Beta–Induced Serum Antibodies in Multiple Sclerosis to Inhibit Endogenous Interferon-Regulated Chemokine/Cytokine Responses Within the Central Nervous System

Aaron M. Shapiro, BSc; Carolyn S. Jack, BSc; Yves Lapierre, MD; Nathalie Arbour, PhD; Amit Bar-Or, MD; Jack P. Antel, MD

Background: A proportion of patients with multiple sclerosis (MS) receiving systemic interferon beta therapy will develop serum neutralizing antibodies (NAbs) that can reduce the activity of the drug. Interferon-β (IFN-β) is produced by glial cells within the central nervous system. Although systemic interferon beta does not access the central nervous system, titers of serum NAbs may be sufficient that some will access the central nervous system.

Objective: To address whether serum samples that contain high titers of NAbs could inhibit glial cell production of chemokines and cytokines that are regulated by endogenous IFN-β.

Design: We used an in vitro assay involving toll-like receptor 3 ligand (polyinosinic-polycytidylic acid) signaling to assess the effect of serum samples containing high titers of NAbs (1800-20,000 U) on production of the chemokine CXCL10 and the cytokine interleukin 6 by human astrocytes.

Results: Serum samples positive for NAbs significantly inhibited polyinosinic-polycytidylic acid–induced CXCL10 and IL-6 production by astrocytes.

Conclusion: High-titer NAbs to interferon beta may block endogenous IFN-β function and alter the chemokine/cytokine microenvironment within the central nervous system, thereby modulating the profile and course of the local inflammatory response.

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RECOMBINANT INTERFERON beta is a commonly used therapy for patients with multiple sclerosis (MS) (hereafter referred to as MS patients), an inflammatory disease of the central nervous system (CNS). A proportion of treated MS patients develop neutralizing antibodies (NAbs) against systemically administered interferon beta, which limits the drug’s efficacy. Interferon-β (INF-β) is a naturally occurring cytokine, initially shown to be produced by cells of the innate immune system and to be a mediator of rapid protective responses to viral infections. Interferon-β is now recognized to modulate interactions between the innate and adaptive immune systems with an effect on the generation and execution of a target-directed immune response. In the CNS, microglia and astrocytes are demonstrated sources of IFN-β.1 Experimental disruption of the IFNβ gene results in enhanced severity of experimental autoimmune encephalomyelitis, an effect attributed to a lack of endogenous IFN-β in the CNS leading to augmented microglia activation.2 Although systemic interferon beta does not access the CNS, titers of serum interferon (IFN) NAbs may be sufficiently high that some would be expected to access the CNS as a result of the limited diffusion of immunoglobulin across even the intact blood-brain barrier. Studies comparing serum and cerebrospinal fluid IgG levels suggest a relative concentration gradient of about 1:300.3 The effect of NAbs on endogenously produced IFN-β, specifically that produced within the CNS, is not defined and is the subject of the present study.

Endogenous IFN-β production can be regulated by signaling through toll-like receptors (TLRs), a family of pattern-recognition receptors that respond to ligands produced by pathogens.4 We have previously shown that astrocytes express relatively high levels of TLR3, which recognizes double-stranded RNA, a component of viruses.4 Stimulation of this receptor activates the MyD88-dependent and My88-independent intracellular signaling pathways, although cross talk may exist between them.5 The MyD88-independent pathway involves the production of endogenous IFN-β, which ligates its own type 1 IFN receptor.7 This sig-

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Cells were plated at a density of $3 \times 10^6$ to $5 \times 10^6$ cells/mL on poly-L-lysine–coated flask in Dulbecco modified Eagle medium containing 10% fetal calf serum (FCS), penicillin/streptomycin, glutamine, and glucose. Cell cultures were used at passage 3, at which point they were more than 90% pure, as previously determined by expression of glial fibrillary acidic protein. Astrocytes were then plated on 24-microwell plates, at $3 \times 10^5$ cells per well and grown to approximately 60% confluency. For our standard assay of functional antibody effects, the medium was then replaced with Dulbecco modified Eagle medium containing 1% FCS or 1% human test donor serum. The synthetic TLR3 ligand PIC (1–2 µg/mL) or recombinant interferon beta (2000 U/mL) was then added to assess the effects of the test serum samples on endogenous vs. exogenous IFN-β signaling, respectively. Cultures were incubated at 37°C for 24 hours. Supernatants were collected and quantified by means of enzyme-linked immunosorbent assay (BD Biosciences Pharmingen, Mississauga, Ontario) for the chemokine CXCL10 and the cytokine IL-6. Additional experiments were performed using serial dilutions of human serum samples; such cultures were supplemented with 1% FCS because serum-free cultures did not support optimal TLR3-induced cytokine responses. Preliminary studies indicated that higher concentrations of serum (≥2%) derived from healthy volunteers inhibited TLR3-mediated responses.

To assess whether antibody-containing serum samples had an effect on non–TLR-dependent signaling, astrocyte cultures were treated in parallel experiments with 100–U/mL interferon gamma or 10-ng/mL IL-1β and subsequently assayed for IL-6 production.

As additional controls for specificity of the inhibitory effects of NAB-containing serum samples, purified IgG fractions prepared from NAB-positive and NAB-negative MS patient serum samples using a protein G column specific for IgG were added at 1% concentration to TLR3 ligand-stimulated astrocyte cultures.

### EXPERIMENTAL PROCEDURE

Dissociated cultures of astrocytes were prepared as previously described from human fetal CNS tissue acquired from the Human Fetal Tissue Repository at Albert Einstein College of Medicine, Bronx, NY, following ethics committee–approved guidelines. Serum samples were collected from MS patients treated with interferon beta who underwent screening for anti–IFN-β NAbs using methods described by Grossberg et al. Serum samples were identified as containing high-titer NAbs (1800–20 000 U; n = 4) or nonsignificant titers (<20 U; n = 3). The clinical, treatment, and interferon beta antibody data on individual patients included in the study are given in the table. Serum samples were decomplemented by incubation at 56°C for 30 minutes and then stored at −80°C until ready for use. All studies received ethics committee approval.

### STATISTICAL ANALYSIS

We performed a paired, 2-tailed $t$ test at the 95% confidence level using statistical graphing software (Prism; GraphPad Software Inc, San Diego, Calif.). All data are recorded as mean ± SEM unless otherwise stated.

### METHODS

#### PATIENTS

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<th>Duration of Disease, y</th>
<th>EDSS Score</th>
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<th>NAb Titer, U</th>
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<th>No. of Assays†</th>
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<td>MS-A/M/26 RR 6 2.5 -1a</td>
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Table. Demographic, Clinical, and Laboratory Features of 7 MS Patients and 1 Control Subject

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Abbreviations: EDSS, Expanded Disability Status Scale; HC, healthy control; IM, intramuscular; MS, multiple sclerosis; NAb, neutralizing antibody; RR, relapsing remitting; SC, subcutaneous; ellipses, not applicable.
Aliquots of serum from the 4 high-titer NAb-seropositive and 3 NAb-seronegative MS patients treated with interferon beta and from a healthy control donor were tested on multiple occasions (Table) at a 1% final concentration on different astrocyte cultures. The mean level of PIC-induced CXCL10 in the high-titer NAb group was reduced to 27 713 ± 2747 pg/mL (n = 14 individual experiments), compared with 66 059 ± 3936 pg/mL (n = 12 individual experiments) in the NAb-seronegative group (P < .001). The values for the NAb-seronegative group were similar to values obtained using 1% FCS (69 560 ± 5930 pg/mL of CXCL10; n = 8 separate experiments; P = .6). Figure 1 provides mean results from individual donors. As shown in Figure 1, the mean percentage of suppression was significantly greater in the NAb-seropositive vs the NAb-seronegative groups (60% ± 5% vs 4% ± 6%; P < .001). Results from individual donors are provided in the Table.

The production of IL-6 was also significantly reduced in cultures containing NAb-positive serum (279 ± 33 pg/mL; n = 9 experiments; n = 3 donors) compared with cultures containing NAb-negative serum (1509 ± 261 pg/mL; n = 8 experiments; n = 2 donors; P < .001) and FCS-only control cultures (1703 ± 263 pg/mL; n = 6 experiments). The mean percentage of inhibition is shown in Figure 1. To ensure that this result was due to the presence of NAb rather than a nonspecific component inhibiting any cell activation, other stimuli that activate cells independently of the IFN-β pathway18 were used. Astrocytes treated with IL-1β and interferon gamma produced large quantities of IL-6 (mean, > 16 000 pg/mL) and were not affected by serum samples from NAb-seropositive (n = 2) or NAb-seronegative (n = 1) donors.

Mean levels of CXCL10 and IL-6 were low and not significantly different between cultures containing NAb-positive and NAb-negative serum samples in the absence of a stimulus (mean, <1000 pg/mL for CXCL10 and <30 pg/mL for IL-6).

Figure 1 further shows that NAb-positive serum samples could also inhibit IFN-β–induced CXCL10 production by astrocytes. As shown, high concentrations of interferon beta (2000 U/mL) were required to induce even a modest CXCL10 response (mean concentration, 704 ± 279 pg/mL). Interferon beta did not induce IL-6 in the 1% serum-containing cultures.

As shown in Figure 2, serial dilutions of the NAb-positive serum samples result in loss of the inhibitory effect, indicating a correlation between interferon beta NAb titers and the blocking effect on chemokine/cytokine responses. Figure 3 illustrates that the inhibitory effect of NAb-positive serum samples was retained when a purified IgG fraction was substituted for whole serum. Serum samples from patients MS-B (NAb seropositive) and MS-G (NAb seronegative) contained equivalent IgG concentrations.

In this study, we have demonstrated that high titers of anti–interferon beta antibodies found in serum samples from MS patients have the ability to inhibit endogenous IFN-β–mediated signaling induced after the ligation of TLR3. Although our study was performed in vitro, the serum concentrations in our study that were found to be
effective at blocking astrocyte chemokine and cytokine production were in the range of those predicted to accumulate in the CNS compartment on the basis of passive diffusion alone. It is possible that more antibodies could access the CNS at sites of blood-brain barrier breakdown, as found in active lesion sites in MS.

The role of individual cytokines and chemokines in the overall inflammatory response within the CNS that contributes to the MS disease process continues to be defined. As mentioned, the increased severity of experimental autoimmune encephalomyelitis in IFN-β-null mice has been attributed to a lack of endogenous IFN-β in the CNS. The effects of systemic administration of high concentrations of interferon beta NAb in this model have not been examined. Studies in which CXCL10 was universally deleted or in which CXCL10 antibody was administered systemically have given discrepant results in the different animal models of MS. When CXCL10 expression was selectively reduced within the CNS compartment by the administration of intrathecal antisense oligonucleotides, the clinical severity of experimental autoimmune encephalomyelitis decreased.

Interleukin 6 is implicated as playing a role as a proinflammatory molecule in MS lesions and in the repair process after relapses, being correlated with oligodendrocyte preservation.

Our study highlights the need to consider that anti-interferon beta antibodies will affect endogenous IFN-β and thus potentially modulate immune regulatory responses in the CNS and the periphery. Changes in the CNS chemokine/cytokine milieu could potentially amplify or inhibit the inflammatory response in this compartment; any clinical consequences of such changes would likely depend on the nature of the immunologic challenge.

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Author Contributions: The authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Shapiro, Jack, Arbour, and Antel. Acquisition of data: Shapiro, Lapiere, and Antel. Analysis and interpretation of data: Shapiro, Jack, Arbour, Bar-Or, and Antel. Drafting of the manuscript: Shapiro, Bar-Or, and Antel. Critical revision of the manuscript for important intellectual content: Jack, Lapiere, Arbour, and Antel. Statistical analysis: Shapiro, Arbour, and Bar-Or. Obtained funding: Antel. Administrative, technical, and material support: Bar-Or and Antel. Study supervision: Jack, Lapiere, Arbour, and Antel.

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References