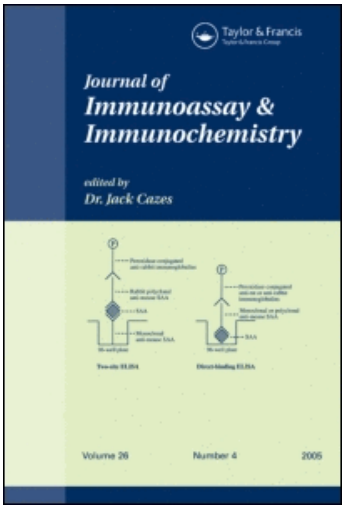

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Neutralizing and Binding Antibodies to Interferon Beta in Patients with Multiple Sclerosis: A Comparison of Assay Results from Three Italian Centres

Francesca Bellomi[a]; Placido Bramanti[b]; Maria Trojano[c]; Carolina Scagnolari[a]; Antonella Muto[a]; Edoardo Sessa[b]; Vito La Volpe[c]; Pierluigi Russo[d]; Guido Antonelli[a]

[a] Department of Experimental Medicine - Virology Section, University "La Sapienza", Rome, Italy [b] IRRCS Centro Neurolesi "Bonino-Pulejo", Messina, Italy [c] Department of Neurological and Psychiatric Science, University of Bari, Italy [d] Department of Human Physiology and Pharmacology, University "Sapienza", Rome, Italy

To cite this Article Bellomi, Francesca , Bramanti, Placido , Trojano, Maria , Scagnolari, Carolina , Muto, Antonella , Sessa, Edoardo , Volpe, Vito La , Russo, Pierluigi and Antonelli, Guido(2009) 'Neutralizing and Binding Antibodies to Interferon Beta in Patients with Multiple Sclerosis: A Comparison of Assay Results from Three Italian Centres', Journal of Immunoassay and Immunochemistry, 30: 1, 40 — 50

To link to this Article: DOI: 10.1080/15321810802569857

URL: http://dx.doi.org/10.1080/15321810802569857

Journal of Immunoassay and Immunochemistry, 30: 40–50, 2009

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810802569857

Taylor & Francis
Taylor & Francis Group

Neutralizing and Binding Antibodies to Interferon Beta in Patients with Multiple Sclerosis: A Comparison of Assay Results from Three Italian Centres

Francesca Bellomi,[1] Placido Bramanti,[2] Maria Trojano,[3] Carolina Scagnolari,[1] Antonella Muto,[1] Edoardo Sessa,[2] Vito La Volpe,[3] Pierluigi Russo,[4] and Guido Antonelli[1]

[1]Department of Experimental Medicine – Virology Section, University "La Sapienza", Rome, Italy
[2]IRRCS Centro Neurolesi "Bonino-Pulejo", Messina, Italy
[3]Department of Neurological and Psychiatric Science, University of Bari, Italy
[4]Department of Human Physiology and Pharmacology, University "Sapienza", Rome, Italy

Abstract: Interferon (IFN) beta therapy for multiple sclerosis (MS) is associated with the development of binding antibodies (BAbs) and neutralizing antibodies (NAbs) in a percentage of patients. This study investigated the reproducibility of results of two different antibody detection techniques using serum from 100 patients with MS who were receiving IFN beta therapy.

Fifty samples were analysed using a commercially available kit-based BAb assay and a further 50 different samples were analysed using a widely used NAb cytopathic effect assay, at three different laboratories.

All three centres agreed on the BAb status of all serum samples. However, only 84% agreement was reached on serum NAb status, and there was significant inter-laboratory variation in NAb titre values. Further analysis of these data revealed a correlation between the mean NAb titre and the coefficient of variation of serum samples, indicating greater discordance with higher NAb titres.

Address correspondence to Guido Antonelli, University "La Sapienza", Viale di Porta Tiburtina, 28, 00185 Rome, Italy. E-mail: guido.antonelli@uniroma1.it

A significant interlaboratory variation in NAb titres does exist; thus, caution is required when comparing titres from different centres. It is clear that validated detection assays are needed to accurately quantify NAb titres.

Keywords: Antibodies to interferon, Interferon beta, Multiple sclerosis

INTRODUCTION

Interferon (IFN) beta belongs to a large group of related glycoproteins produced in response to virus infections and other biological agents that activate host defence and immunomodulatory mechanisms.[1–3]

IFN beta was the first intervention to be approved for the treatment of multiple sclerosis (MS) and many studies have demonstrated its effectiveness in patients with relapsing–remitting MS (RRMS). Treatment with IFN beta decreases the frequency and severity of clinical relapses, reduces accumulation of new and active lesions on brain magnetic resonance imaging activity and slows disability progression.[4–9]

Since 1993, three parenteral formulations of IFN beta have been approved for the treatment of MS in the USA and Europe: IFN beta-1a 30 μg, which is administered intramuscularly once weekly (Avonex®, Biogen IDEC); IFN beta-1a 22 or 44 μg, which is administered subcutaneously (sc) three times weekly (Rebif®, Merck Serono International S.A.); and IFN beta-1b 250 μg sc, which is administered once every other day (Betaferon®, Bayer Schering Pharma).[10,11] As with other therapeutically administered cytokines, administration of IFN beta, regardless of formulation, is associated with the development of binding antibodies (BAbs) and neutralizing antibodies (NAbs) in a percentage of patients.[12–15] BAbs and NAbs may disappear spontaneously (seroreversion) during or after cessation of therapy.[16] The immunogenicity of recombinant proteins depends on many factors, including their structure and glycosylation, the presence of contaminants or aggregates, and route of administration.[17]

BAbs can react either with epitopes on the outer IFN molecule or within the specific target cell receptor-binding site.[18] No clear biological functions have been attributed to BAbs,[18] but their detection is recommended by some as a screening tool prior to performing complex NAb assays.[19] NAbs are a subset of BAbs that bind to the active sites of IFN beta molecules to prevent interaction with its receptor.[20] In vitro data suggest that the presence of NAbs restricts the bioavailability of IFN beta,[21] but reduction in clinical efficacy for patients with MS is controversial due to conflicting results from clinical trials.[22] Indeed, evidence shows that individual patients treated with IFN beta may continue to have stable MS, regardless of their NAb status.[23] Nevertheless, NAb

testing has been proposed as a monitoring tool during IFN beta therapy,[12,24] and the potential for NAb development has been used to compare the relative merits of different formulations of IFN beta.[25,26]

Such management strategies and comparisons must be based on accurate and highly reproducible antibody detection methods. Thus, we formally analysed the inter-laboratory reproducibility of antibody titres obtained with two different detection techniques using serum samples from patients with MS who were receiving IFN beta therapy. We tested a commercially available BAb assay and a widely used NAb bioassay at three laboratories that are routinely involved in anti-IFN beta antibody assessments.

EXPERIMENTAL

Patients and Study Design

One hundred serum samples from patients with confirmed RRMS who were receiving IFN beta therapy were tested. Patients were enrolled regardless of their demographic or disease characteristics, or the IFN beta formulation that they were receiving.

Fifty serum samples were used to test for the presence of BAbs and a further, different 50 samples were used to test for NAbs. Each serum sample was divided into six, labelled aliquots. Three laboratories, designated A, B and C, received two aliquots of each serum sample. All samples were tested and retested (with two different aliquots) for the presence of BAbs and NAbs.

The same protocols were followed at all three laboratories. All centres used the same commercially available test kit for the detection of BAbs and the same cytopathic effect (CPE) bioassay for the detection of NAbs. At each centre, all samples were prepared by the same person using the same equipment, but technicians were blinded to details of the samples. Each centre was highly experienced and routinely involved in anti-IFN beta antibody analyses.

Detection of Antibodies to IFN Beta

Binding Antibodies

As described previously by Bellomi et al.,[16] antibodies binding to IFN beta-1a and IFN beta-1b were detected using a non-competitive enzyme immunoassay kit specific for IFN beta (Bühlmann Laboratories AG, Allschwil, Switzerland), according to the manufacturer's recommendations.

Briefly, serum obtained from patients was diluted 1:50 in incubation buffer and incubated in 96-well microtitre plates that had been coated previously with a mixture of natural human IFN beta, recombinant (r) IFN beta-1a and rIFN beta-1b. This assay allows the detection of BAbs directed against any type of IFN beta.

The presence of bound antibody was detected using a horseradish peroxidase-conjugated antiserum to human immunoglobulin G, followed, after a washing step, by the addition of a buffered tetramethylbenzidine substrate solution. Two positive (one high and one low titre) control samples and one negative control were used in accordance with the manufacturer's instructions. Results were obtained in optical density (OD) units by spectrophotometric analysis. A value of 50 Bühlmann titre units (BTU) or above was considered positive for BAbs, as suggested by the manufacturer.

Neutralizing Antibodies

A CPE bioassay was selected according to current recommendations by the World Health Organization (WHO) and the International Society for Interferon and Cytokine Research (ISICR), as described previously by Bellomi et al.[16,27] The presence of NAbs to IFN beta was tested against 10 IU rIFN beta-1a.[28,29]

Two-fold serial dilutions (starting from 1:10) of sample or control sample in 60 μL volumes were incubated at 37°C containing 20 IU/mL IFN beta-1a. After 1 hour, 100 μL of the individual mixtures were added to duplicate monolayers of human lung carcinoma (A549) cells in 96-well microtitre plates. After 18–24 hours of culture and extensive washing, the cells were challenged with encephalomyocarditis murine virus and incubated at 37°C for 24 hours.

Control samples included cells, virus, serum and a titration of the IFN beta-1a used in the assays. No internal controls were used to analyse NAb titres. To reflect routine inter-laboratory practice, the same types of cells and virus were used in all centres, but virus stock and cell passage numbers differed.

Antiviral activity and its neutralization were assessed on the basis of the virus-induced CPE and, to quantitate this, cells were stained with crystal violet in 20% ethanol. The dye taken up by the cells was eluted with 33% acetic acid, and its absorbance was measured in a microdensitometer at 540 nm (OD_{540}). The extent of virus-induced CPE, its inhibition by IFN beta, and the reversal of this by NAbs were shown by the amount of dye eluted from each well.

Serum samples were routinely assayed for, and found free from, endogenous or residual IFN activity. Titres were calculated using the Kawade method and expressed as $t_{1/10}$, namely the dilution of serum

that reduces 10 laboratory units (LU)/mL of IFN to 1 LU/mL.[30,31] A titre of greater than 10 $t_{1/10}$ was considered positive for NAbs.

Statistical Analysis

The individual laboratory variability in titre results was calculated using the coefficient of variation (CV) of all test and retest results from each patient considered positive or discordant for NAbs. The CV was calculated as the SD divided by the mean titre value.

The CV for each serum sample was calculated using the SD and mean values of the six titres obtained from three laboratories. The correlation between each patient's log-transformed mean NAb titre and CV was analysed using Pearson's (r) coefficient. Statistical analyses were performed using STATISTICA version 6.0 (StatSoft s.r.l., Italy) and significance was assumed when p was lower than 0.05.

RESULTS

All laboratories completed both BAb and NAb assays successfully and submitted data for statistical analysis.

Binding Antibodies

All three centres agreed on the BAb status of all 50 serum samples: 38% (19/50) were positive for BAbs, while 62% (31/50) gave results below the technical cut-off of 50 BTU in all laboratories. Although values of the control titres varied between laboratories, all were within the range of assay validity.

Neutralizing Antibodies

Figure 1 shows that all three laboratories agreed on the NAb status of 84% (42/50) of serum samples examined. For 40% (20/50) of samples, all three laboratories agreed on NAb-positive status. Laboratories also concurred that 44% (22/50) of samples were negative for NAbs. However, there was discordance in NAb status between laboratories for 16% (8/50) of samples.

The overall mean CV of NAb titre values per serum sample was 38% (95% confidence interval [CI]: 26–50). When serum samples classified as NAb negative by all three laboratories were excluded, the mean CV of titre results increased to 68% (95% CI: 55–81).

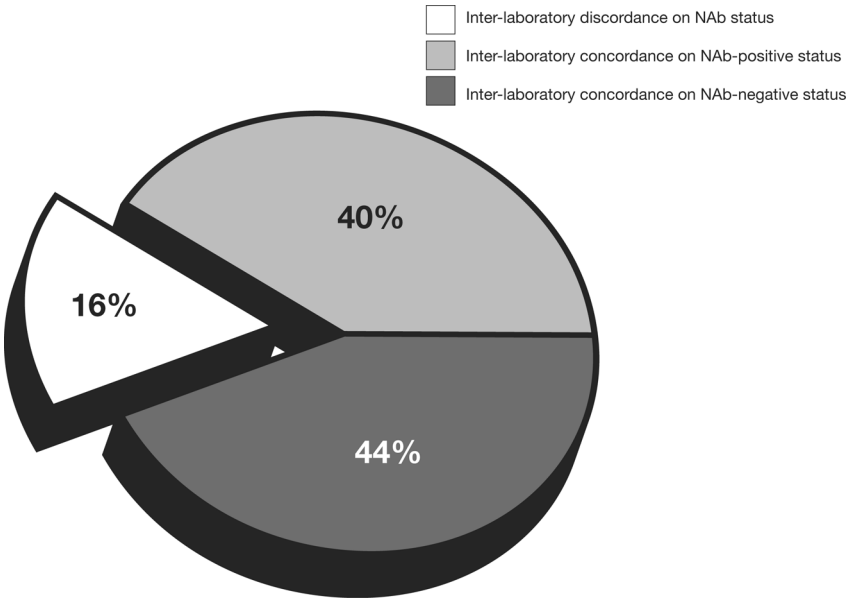

Figure 1. Interlaboratory data.

Further analysis of this subgroup revealed a significant correlation between the mean NAb titre and CV of each serum sample (Pearson's $r = 0.67$; $p < 0.001$; Figure 2). Data show that samples classified as positive, which had low positive (10–100 $t_{1/10}$) NAb titres showed less inter-laboratory variability than those with high (>100 $t_{1/10}$) NAb titres. Thus, inter-laboratory discordance is greatest at high NAb concentrations.

DISCUSSION

Although guidelines have proposed the use of NAb measurement in treatment decision-making,[12] the impact of NAbs on the clinical efficacy of IFN beta in patients with MS remains uncertain due to conflicting evidence from different studies.[8,12,18,22] This is made even more uncertain and confused by the lack of validated and standardized methods for measuring the development of antibodies, which are needed to enable reliable comparison of data obtained by different research groups.

In fact, although many methods are described for the detection of NAbs, agreement has not yet been reached on a definitive, standardized technique.[18] The two methods used most frequently for NAb measurement are the CPE assay,[16] currently recommended by the WHO and the ISICR, which measures the ability of antibodies in serum to

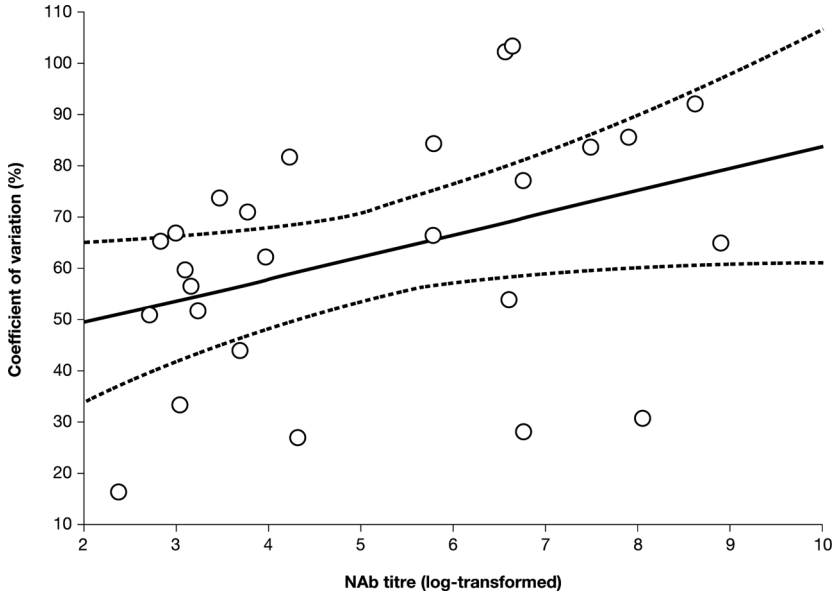

Figure 2. Nab titre vs. coefficient of variation.

neutralize IFN beta-mediated inhibition of virus-induced cell lysis, and the MxA gene-induction antibody assay, which evaluates the neutralizing capability of antibodies to prevent IFN-mediated induction of MxA protein.[32]

Interpretation of NAb results, from studies of patients with MS, is complicated by non-standardized methodology for measuring NAbs, differences in dose and route of administration of IFN beta formulations, expression of neutralizing capability of antibodies, data interpretation and definition of positive NAb titre.[17,18] Also, assay sensitivity, specificity and reliability may all profoundly affect detection of serum antibodies. Therefore, reliable and reproducible NAb detection methods that accurately represent the reduction in biological activity of therapeutically administered IFN beta are essential to realize the proposed clinical applications.

Our study demonstrated the existence of inter-laboratory variability in NAb titres and status, even when using the same biological assay for analysis of duplicate clinical specimens. Interpretation of our findings is somewhat limited because we did not test the reproducibility of results at each site. Nevertheless, our data are as expected and are consistent with findings of another study,[33] which demonstrated variability in results of NAb detection assays between different centres.

We believe that the observed discordance is related to the high number of variable components that may affect the accuracy and

reproducibility of all biological assays. Factors may include the conditions of sample distribution, assay timing, temperature regulation and operator skill. Biological factors such as virulence and the presence of metabolites in clinical material may affect the reproducibility of assays and the metabolic state of cell lines may also alter sensitivity to the cytopathic effect of the virus.

There is general agreement that serum NAb titres exceeding 1000 $t_{1/10}$ reduce the biological effects of therapeutically administered IFN beta but the impact of low titres is less certain. Indeed, the lowest NAb titre that is able to exert a significant biological effect is unknown and therefore the threshold value for positive NAb status is open to question. Our study has indicated considerable inter-laboratory variability at high titres, suggesting that interpretation of results may be complicated by a titre effect.

The NAb CPE bioassay could be improved by centralizing the titration in a limited number of laboratories that would use a calibrated and standardized assay. Reproducibility may also be enhanced by precise synchronization of cell division, close monitoring of experimental conditions and confirmation of adsorption of virus titre to cells. Strict external quality-control policies must also be implemented and laboratories should be strongly encouraged to participate in standardization schemes.

Consideration should also be given to the continuing development and use of accurate commercial kit-based detection methods. The highly reproducible results obtained from the commercially available BAb detection assay confirm that the use of kit-based methods produces less variable results than titre measurements from NAb bioassay. However, as different serum samples were tested for BAbs and NAbs in our study, no attempts have been made to compare the antibody titres detected using these different techniques.

CONCLUSIONS

Although we believe that development of NAbs has a clinical relevance and that monitoring NAb titres may be useful in many circumstances, we also believe that the fundamental clinical relevance of NAbs can only be accurately characterized using standardized assays performed by experienced operators. Caution should be taken when comparing NAb titre results obtained from different sites. Commercial, kit-based NAb assays, which are similar to the BAb assay used in this study, are now available and may provide a step towards the solution. Nonetheless, the clinical management of patients who have NAb-positive titres still needs careful consideration.

REFERENCES

1. Foster, G.R. Interferons in host defense. Semin. Liver. Dis. **1997**, *17*, 287–295.
2. Fernandez, O. Interferons in relapsing-remitting multiple sclerosis: are there benefits from long-term use? C.N.S. Drugs **2004**, *18*, 1057–1070.
3. Goodin, D.S. Treatment of multiple sclerosis with human beta interferon. Int. M.S. J. **2005**, *12*, 96–108.
4. PRISMS (Prevention or Relapses and Disability by Interferon-beta-1a Subcutaneously in Multiple Sclerosis) Study Group; the University of British Columbia MS/MRI Analysis Group: PRISMS-4. Long-term efficacy of interferon-beta-1a in relapsing MS. Neurology **2001**, *56*, 1628–1636.
5. Jacobs, L.D.; Cookfair, D.L.; Rudick, R.A.; Herndon, R.M.; Richert, J.R.; Salazar, A.M.; Fischer, J.S.; Goodkin, D.E.; Granger, C.V.; Simon, J.H.; Alam, J.J.; Bartoszak, D.M.; Bourdette, D.N.; Braiman, J.; Brownscheidle, C.M.; Coats, M.E.; Cohan, S.L.; Dougherty, D.S.; Kinkel, R.P.; Mass, M.K.; Munschauer, F.E. 3rd; Priore, R.L.; Pullicino, P.M.; Scherokman, B.J.; Whitham, R.H.; The Multiple Sclerosis Collaborative Research Group. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. Ann. Neurol. **1996**, *39*, 285–294.
6. Simon, J.H.; Jacobs, L.D.; Campion, M.; Wende, K.; Simonian, N.; Cookfair, D.L.; Rudick, R.A.; Herndon, R.M.; Richert, J.R.; Salazar, A.M.; Alam, J.J.; Fischer, J.S.; Goodkin, D.E.; Granger, C.V.; Lajaunie, M.; Martens-Davidson, A.L.; Meyer, M.; Sheeder, J.; Choi, K.; Scherzinger, A.L.; Bartoszak, D.M.; Bourdette, D.N.; Braiman, J.; Brownscheidle, C.M.; Whitham, R.H.; The Multiple Sclerosis Collaborative Research Group. Magnetic resonance studies of intramuscular interferon beta-1a for relapsing multiple sclerosis. Ann. Neurol. **1998**, *43*, 79–87.
7. The Once Weekly Interferon for MS Study Group. Evidence of interferon beta-1a dose response in relapsing-remitting MS: the OWIMS Study. Neurology **1999**, *53*, 679–686.
8. Arnason, B.G. Long-term experience with interferon beta-1b (Betaferon) in multiple sclerosis. J. Neurol. **2005**, *252* (Suppl 3), iii28–iii33.
9. Murdoch, D.; Lyseng-Williamson, K.A. Subcutaneous recombinant interferon-beta-1a (Rebif): a review of its use in relapsing-remitting multiple sclerosis. Drugs **2005**, *65*, 1295–1312.
10. Wingerchuk, D.M. Multiple sclerosis disease-modifying therapies: adverse effect surveillance and management. Expert Rev. Neurother. **2006**, *6*, 333–346.
11. Markowitz, C. Development of interferon-beta as a therapy for multiple sclerosis. Expert Opin. Emerg. Drugs **2004**, *9*, 363–374.
12. Goodin, D.S.; Frohman, E.M.; Garmany, G.P. Jr.; Halper, J.; Likosky, W.H.; Lublin, F.D.; Silberberg, D.H.; Stuart, W.H.; van den Noort, S. Disease modifying therapies in multiple sclerosis: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology and the MS Council for Clinical Practice Guidelines. Neurology **2002**, *58*, 169–178.

13. Durelli, L.; Ricci, A. Anti-interferon antibodies in multiple sclerosis. molecular basis and their impact on clinical efficacy. Front. Biosci. **2004**, *9*, 2192–2204.
14. Antonelli, G. Development of neutralizing and binding antibodies to interferon (IFN) in patients undergoing IFN therapy. Antiviral Res. **1994**, *24*, 235–244.
15. Schellekens, H.; Casadevall, N. Immunogenicity of recombinant human proteins: causes and consequences. J. Neurol. **2004**, *251* (Suppl. 2), II4–II9.
16. Bellomi, F.; Scagnolari, C.; Tomassini, V.; Gasperini, C.; Paolillo, A.; Pozzilli, C.; Antonelli, G. Fate of neutralizing and binding antibodies to IFN beta in MS patients treated with IFN beta for 6 years. J. Neurol. Sci. **2003**, *215*, 3–8.
17. Schellekens, H. The immunogenicity of biopharmaceuticals. Neurology **2003**, *61* (Suppl. 5), S11–S12.
18. Antonelli, G.; Bagnato, F.; Dianzani, F. Considerations on the development of serum antibodies to interferon-beta. New Microbiol. **2005**, *28*, 183–192.
19. Sorensen, P.S.; Deisenhammer, F.; Duda, P.; Hohlfeld, R.; Myhr, K.M.; Palace, J.; Polman, C.; Pozzilli, C.; Ross, C. Guidelines on use of anti-IFN-beta antibody measurements in multiple sclerosis: report of an EFNS Task Force on IFN-beta antibodies in multiple sclerosis. Eur. J. Neurol. **2005**, *12*, 817–827.
20. Runkel, L.; De Dios, C.; Karpusas, M.; Baker, D.; Li, Z.; Zafari, M.; Betzenhauser, M.; Muldowney, C.; Miller, S.; Redlich, P.N.; Grossberg, S.E.; Whitty, A.; Hochman, P.S. Mapping of IFN-beta epitopes important for receptor binding and biologic activation: comparison of results achieved using antibody-based methods and alanine substitution mutagenesis. J. Interferon Cytokine Res. **2001**, *21*, 931–941.
21. Deisenhammer, F.; Reindl, M.; Harvey, J.; Gasse, T.; Dilitz, E.; Berger, T. Bioavailability of interferon beta 1b in MS patients with and without neutralizing antibodies. Neurology **1999**, *52*, 1239–1243.
22. Francis, G.S.; Rice, G.P.; Alsop, J.C. Interferon beta-1a in MS: results following development of neutralizing antibodies in PRISMS. Neurology **2005**, *65*, 48–55.
23. Durelli, L.; Verdun, E.; Barbero, P.; Bergui, M.; Versino, E.; Ghezzi, A.; Montanari, E.; Zaffaroni, M. Every-other-day interferon beta-1b versus once-weekly interferon beta-1a for multiple sclerosis: results of a 2-year prospective randomised multicentre study (INCOMIN). Lancet **2002**, *359*, 1453–1460.
24. Giovannoni, G.; Goodman, A. Neutralizing anti-IFN-beta antibodies: how much more evidence do we need to use them in practice? Neurology **2005**, *65*, 6–8.
25. Perini, P.; Facchinetti, A.; Bulian, P.; Massaro, A.R.; Pascalis, D.D.; Bertolotto, A.; Biasi, G.; Gallo, P. Interferon-beta (IFN-beta) antibodies in interferon-beta1a- and interferon-beta1b-treated multiple sclerosis patients. Prevalence, kinetics, cross-reactivity, and factors enhancing interferon-beta immunogenicity in vivo. Eur. Cytokine Netw. **2001**, *12*, 56–61.

26. Bertolotto, A.; Deisenhammer, F.; Gallo, P.; Solberg Sorensen, P. Immunogenicity of interferon beta: differences among products. J. Neurol. **2004**, *251* (Suppl. 2), II15–II24.
27. WHO Expert Committee on Biological Standardization. Thirty-fifth report. World Health Organ. Tech. Rep. Ser. **1985**, *725*, 1–140.
28. Grossberg, S.E.; Kawade, Y. The expression of potency of neutralizing antibodies for interferons and other cytokines. Biotherapy **1997**, *10*, 93–98.
29. Grossberg, S.E.; Kawade, Y.; Kohase, M.; Yokoyama, H.; Finter, N. The neutralization of interferons by antibody. I. Quantitative and theoretical analyses of the neutralization reaction in different bioassay systems. J. Interferon Cytokine Res. **2001**, *21*, 729–742.
30. Kawade, Y. Quantitation of neutralization of interferon by antibody. Meth. Enzymol. **1986**, *119*, 558–573.
31. Grossberg, S.E.; Kawade, Y.; Kohase, M.; Klein, J.P. The neutralization of interferons by antibody. II. Neutralizing antibody unitage and its relationship to bioassay sensitivity: the tenfold reduction unit. J. Interferon Cytokine Res. **2001**, *21*, 743–755.
32. Pungor, E., Jr.; Files, J.G.; Gabe, J.D.; Do, L.T.; Foley, W.P.; Gray, J.L.; Nelson, J.W.; Nestaas, E.; Taylor, J.L.; Grossberg, S.E. A novel bioassay for the determination of neutralizing antibodies to IFN-beta1b. J. Interferon Cytokine Res. **1998**, *18*, 1025–1030.
33. Deisenhammer, F.; Schellekens, H.; Bertolotto, A. Measurement of neutralizing antibodies to interferon beta in patients with multiple sclerosis. J. Neurol. **2004**, *251* (Suppl. 2), II31–II39.

Received March 8, 2008
Accepted May 10, 2008
Manuscript 3300