The Neutralization of Interferons by Antibody III. The Constant Antibody Bioassay, A Highly Sensitive Quantitative Detector of Low Antibody Levels

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The neutralizing antibodies (NAbs) that develop in patients during interferon (IFN) therapy can reduce its beneficial effects. The universally employed method of NAb measurement currently is the *constant IFN* method, in which antigen at a single given concentration is mixed with serial dilutions of serum, the lowest final dilution of which (usually 1:20) is constrained by the potential adverse effect of human serum on human cells in culture. The *constant antibody (Ab)* method described herein uses serum at a certain set dilution (usually 1:20) mixed with a series of IFN concentrations. Theoretical neutralization curves based on the previously presented model of the Ab-IFN reaction are depicted herein in terms of experimentally observable quantities. As predicted by the theoretical studies, the *constant Ab* method was demonstrated experimentally to extend the lower limits of detection of Ab by a factor of 10–20. The excellent agreement observed between the theoretical prediction and experimental findings reinforces the validity of using as NAb unitage the titer based on 10-fold reduction of IFN activity, reportable as Tenfold Reduction Units (TRU)/mL, as previously recommended. Testing by the *constant Ab* method of sera previously considered negative (<20 TRU/mL by the *constant IFN* method) from patients treated with Rebif or Betaseron showed that ~50% had detectable NAb; such sera from Avonex-treated patients had titers of <1 TRU/mL. The *constant Ab* method can be used as a quantitative, sensitive IFN NAb screening bioassay of any nature, and should be able to detect low levels of NAb early in the course of IFN therapy. The method may be useful to test monoclonal antibodies for otherwise undetectable NAb. In principle, the *constant Ab* method should be applicable to the measurement of NAb against any cytokine or other protein-effector molecule.

Introduction

INTERFERONS (IFNs) HAVE BEEN used clinically for the treatment of a variety of diseases, including multiple sclerosis, hepatitis B and C, condylomata, and cancers of different types, such as renal carcinoma, non-Hodgkin's lymphoma, melanoma, as well as chronic myelogenous, hairy cell, and B-cell leukemias (reviewed in Müller 2006). During such therapy neutralizing antibodies (NAb) can appear and interfere with the desired therapeutic effects (reviewed in Grossberg and Kawade 2006; Hartung and others 2007). While there is general agreement that determination of antibody (Ab) status is important, especially during therapy of multiple sclerosis patients with IFN- β , there is controversy about the methodology of IFN biological assays and how best to calculate and report NAb results (Sorensen and

others 2005a; Goodin and others 2007; Hartung and others 2007).

Virtually all IFN bioassays, whether based on IFN induction either of antiviral resistance or of a cellular gene product, utilize as titration endpoint the median point between the appropriate maximal and minimal effect control values. This 50% endpoint, which falls in the rectilinear portion of the typically sigmoidal dose-response curve, also operationally defines one Laboratory Unit (LU) of antigen, expressed as a concentration, that is, per unit volume, usually 1 mL (Grossberg and Kawade 1997). Assay sensitivity, an important element, can be defined in two ways. The relative sensitivity of a bioassay for an IFN product is established by comparing the potency, expressed in LU/mL as measured in that particular assay, of the homologous World

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Health Organization (WHO) IFN International Standard to its assigned potency unitage in International Units (IU) (Grossberg and Kawade 1997; Grossberg and others 2001a). The sensitivity of an assay for NABs, on the other hand, relates to the ability of the bioassay to detect antibody, the subject addressed in this paper.

The early work by Kawade and colleagues (Kawade 1980; Kawade and Watanabe 1984; Kawade and Watanabe 1985; Kawade 1986), based on thermodynamic considerations and experimental observations of IFN-NAB interactions, led to the operational approach to standardizing NAB measurement, approved and repeatedly affirmed by WHO, whereby 10 LU/mL is reduced to 1 LU/mL (Berg and others 1983; Billiau and others 1985; Andzhaparidze and others 1988; Calam and others 1995). To account for the available data and theoretical constructs, two hypotheses were posed: (i) Ab acts to neutralize a certain amount of biologically active IFN molecules, the Fixed Amount hypothesis, or (ii) NAB reduces IFN activity in a set ratio of added-to-residual, biologically active IFN molecules, the Constant Proportion hypothesis (Grossberg and others 2001a). The insight that the Constant Proportion hypothesis was the correctly applicable circumstance was substantiated by analyses of the data from several laboratories involved in a WHO international collaborative study on two human serum anti-IFN WHO Reference Reagents (Grossberg and others 2001a, 2001b; NIH Reference Reagent Notes #44 and #45 1994). These data and more detailed theoretical analyses resulted in a recommendation that permits the calculation of results from an appropriately designed bioassay to be expressed as: $t = f(n - 1)/(10 - 1)^*$, where t is the titer, f is the dilution of Ab at the endpoint, and n is the amount of antigen measured in LU/mL mixed with Ab, where one LU/mL is the endpoint (Grossberg and others 2001a, 2001b). Use of this formula defines the standardized unit of Ab neutralization, the Tenfold Reduction Unit, expressed as TRU/mL (Grossberg and others 2001a, 2001b). This expression of unitage can be utilized with any type of quantitative neutralization bioassay properly designed and sufficiently sensitive to the antigen.

Neutralization can be quantitated by reducing a determinable amount of total antigen, I_t , to a defined, measurable, residual amount of free antigen, I_f , in order to express a NAB potency value or titer, t . The amount as weight or mass of antigen is not measured directly in bioassays but is gauged instead in terms of quantifiable biological response. An experimentally observable measure, e.g., absorbance of a dye, can be used to establish a unitage of potency by defining 1 LU/mL at the 50% endpoint. To determine the total amount of IFN, I_t , in the mixture with Ab, an IFN dose-response control curve must be run simultaneously with the neutralization dose-response curves. The typical sigmoidal IFN potency titration curve in relation to dilutions of IFN product defines 1 LU/mL, the same biological response metric applied to the simultaneously determined neutralization curves to measure I_f , the residual (free) IFN (Grossberg and others 2001a, 2001b).

Two approaches can be taken to the design of the neutralization bioassay: (i) the *constant IFN* method, in which a chosen concentration of IFN is mixed with varying dilutions of Ab, and (ii) the *constant Ab* method, in which a set dilution

of serum is mixed with varying concentrations of IFN. The *constant IFN* method is almost universally employed currently. A practical constraint is the fact that high concentrations of serum may be either toxic or stimulatory for cells in culture, at least at lesser dilutions than the most usually employed 1:20 final serum dilution applied to the cells. In this paper we describe the use of the *constant Ab* method, which possesses great sensitivity to detect IFN NABs, permitting detection of as little as 1–2 TRU/mL at 1:20 final dilutions of serum. The theoretical neutralization curves presented are drawn in terms of experimentally observable quantities so that direct comparison with the experimental NAB titration curves is possible. The *constant Ab* approach should be applicable to the measurement of NABs against any cytokine or other protein-effector molecules.

Materials and Methods

IFNs

Human IFN- β 1a (Rebif) produced by Serono, Rockland, MA, Avonex (IFN- β 1a) by Biogen Idec, Cambridge, MA, and IFN- β 1b (Betaseron) by Berlex Schering (Emeryville, CA) were obtained as commercial products for injection. The WHO Second International Standard for Human IFN- β , Gb23-902-531, a natural fibroblast (IFN- β 1a) preparation (NIH Reference Reagent Note #35 1987), and Gxb02-901-535, the WHO First Human Standard IFN- β 1b recombinant preparation, having a serine-17 substitution for cysteine (NIH Reference Reagent Note #37 1987), were obtained from the National Institutes of Health, Bethesda, Maryland, USA, and from the Biodefense & Emerging Infections Research Resources Repository, Manassas, Virginia, USA. The WHO Third International Standard for Human IFN- β 1a, 00/572, was obtained from the National Institutes of Biological Standards and Control, South Mimms, Hertfordshire, UK.

Antibody preparations

The WHO International Reference Reagent human serum anti-HuIFN- β Ab G038-501-572 (NIH Reference Reagent Note #45 1994) has a WHO-assigned titer of 1,500 (Calam and others 1995). Clinical serum samples were from patients treated with Rebif (IFN- β 1a), Avonex (IFN- β 1a), or Betaseron (IFN- β 1b); prior to assay, these sera were heated at 56°C for 30 min.

IFN bioassay

The IFN bioassay employed was the objective, cytopathic effect (CPE), naphthol-blue-black dye-uptake procedure, utilizing the human bronchioloalveolar carcinoma A549 cells and encephalomyocarditis (EMC) rodent virus (Grossberg and others 1986). The IFN sensitivity of this bioassay is such that it measures $0.41 \times$ the 15,000 IU assigned to Gb23-902-531 and $0.77 \times$ the 6,000 IU assigned to Gxb02-901-535, the WHO International Standards for IFN- β (IU/LU = 2.4 and 1.3 respectively) (Grossberg and others 2001b). A549 cells were propagated in Dulbecco's minimal essential medium with 10% newborn calf serum, and dispensed into 96-well plates at 5×10^5 cells/mL or 5×10^4 cells/well/0.1 mL; following overnight growth to confluence, monolayers were maintained on medium with 2% serum. After incubation with

*Equations and variables are shown in bold for clarity.

IFN overnight at 36° C, cells were washed and challenged with EMC virus in a concentration intended to cause 80–90% CPE the following day. The extent of CPE was determined by absorbance at 620 nm (A_{620}) of extracted, cell-associated, naphthol blue-black dye measured in a spectrophotometric 96-well plate reader, Lab Systems MultiScan MS. The absorbance values were directly processed by customized computer programs to determine the sigmoidal dose-response curves and 50% endpoints, as well as calculate assay results and perform statistical analyses. Multiple wells of controls on each plate included uninfected (untreated) cells and EMC virus-infected cells.

Neutralization bioassay

In the *constant IFN* method, duplicate Ab samples serially diluted 2-fold were mixed with an equal volume of IFN- β product at a fixed concentration ~20 LU/mL (final concentration 10 LU/mL). In the *constant Ab* method, duplicate Ab samples at a fixed dilution (1:10 or higher) were added to an equal volume of serial 2-fold dilutions of IFN with an appropriate target dose (e.g., ~20 LU/mL), in a pattern identical to the simultaneous IFN titration in duplicate on each plate. Antigen-Ab mixtures prepared for either method were incubated for two hours at 36° C and added to the A549 cells, along with a simultaneous IFN titration on each plate. Control wells included cells treated with Ab alone, in addition to those for IFN assay. Challenge with EMC virus is carried out as described above for IFN bioassay. The *constant IFN* method seeks to determine the Ab dilution (f) that reduces the added IFN (n LU/mL) to 1 LU/mL, whereas the *constant Ab* method seeks to determine the IFN concentration (n LU/mL) that is reduced to 1 LU/mL by Ab at the given dilution used. The value of the neutralization titer t is calculated by the formula $t = f(n - 1)/9$ and reported as TRU/mL as recommended (Grossberg and others 2001a, 2001b; Grossberg and Kawade 2006). The calculations were carried out by customized computer programs.

Computational operations

Since existing ready-made software tools were not found to be sufficiently configurable or mathematically robust to perform the required computational tasks, a suite of computer applications were written in Mathematica (Wolfram Research, Inc.; Champaign, IL) to measure objectively and efficiently the results of cytokine assays having sigmoidal dose-response curves. The applications developed read as input operator-keyed values and spectrophotometer data files. A Marquardt-Levenberg nonlinear regression, informed by programmatically selected starting values, data-point weights, and a four-parameter logistic model, is used to fit the curves. This algorithm uses an iterative process to find the values for the parameters of the given sigmoidal model for which the sum of the squares of the vertical distances of the data points from the determined curve is a minimum. The equation of the model is $p_1 + (p_2 - p_1) / (1 + e^{-(x - p_3) / p_4})$ where p_1 through p_4 are treated as parameters. Assay results in quantitative characteristics (e.g., titer and slope) are calculated from the curve's equation. Statistical and quality control assessments are also performed.

Results

Theoretical neutralization curves depicted with experimentally determined values

Theoretical neutralization curves for both the *constant IFN* and *constant Ab* methods were shown in our previous papers (Grossberg and others 2001a; Kawade and others 2003). However, in order to be able to compare the experimentally observed curves directly with the theoretical ones, the quantity I_f , the residual IFN remaining free in the mixture with Ab, in those papers must be converted to the experimentally observed value, measurable as absorbance in the case of the dye-uptake assay. For this purpose, a typical sigmoidal dose-response IFN titration curve shown in Figure 1 can be used to relate the absorbance values on the ordinate to the dilutions of IFN product and thereby define 1 LU/mL. Only the case of the Constant Proportion mode of Ab action is considered here, because the theoretical curves for the Fixed Amount mode were shown to deviate far from the experimental findings from several laboratories involved in a WHO international collaborative study of human antibodies to IFN- α and - β (Grossberg and others 2001a, 2001b; Kawade and others 2003).

Constant IFN method. Theoretical curves, calculated as explained in detail in the Appendix, of the *constant IFN* method were constructed (see Fig. 2) for nine NABs with t ranging from 2 to 320 with residual, or free, IFN activity, I_f , and absorbance (A_{620}), both plotted on the ordinate, against the Ab dilution, f , on the abscissa. The range of Ab dilutions,

FIG. 1. A typical IFN- β simultaneous control curve obtained by the A549 cell/EMC virus, naphthol blue-black, dye-uptake method. The horizontal dashed line at the 50% endpoint, the median of the dynamic range between cell control (CC) and virus control (VC) defines 1 LU/mL and at its intersection with the dose-response curve establishes the potency (i.e., titer) of the antigen, in this case 1000 (LU/mL). The vertical dashed lines within the rectilinear portion of the dose-response curve relate the IFN dose as LU/mL to the IFN dilution of antigen, for which the upper and lower abscissas are drawn in opposite directions.

f , in the region smaller than 10 are not considered, because high serum concentrations can cause undesirable effects on cells that can adversely affect the assay. The lowest final serum dilution routinely used to test patient sera is 1:20, and the range of f values covered is generally from 20 to 640 in 2-fold dilutional steps.

Figure 2A illustrates the theoretical curves for nine NABs with t ranging from 2 to 320, when the IFN dose of 10 LU/mL is used. It can be seen that Abs with t less than or equal to 10 do not show properly measurable neutralization for Ab dilution of 20 and higher. Sera with $t = 20$ have a neutralization curve that reduces to 1 LU/mL at the highest Ab concentration ($f = 20$), but covers only half the dynamic range of the assay. Assays are more reliable for Abs with t values of 40 or higher, for which the neutralization curves cover all or a large part of the dynamic range.

In theory, the sensitivity of the *constant IFN* method can be increased by reducing the IFN dose, I_v , to 5 LU/mL as illustrated in Figure 2B. Antibodies with $t = 20$ or higher are seen to show satisfactory theoretical neutralization curves, demonstrating an approximately 2-fold increase in NAB sensitivity compared with the previous case of $I_v = 10$. When the I_v antigen dose is further decreased to 2 LU/mL, (Fig. 2C), the NAB sensitivity can be further increased. However, such a low level of IFN as antigen is not recommended because in practice the dynamic range is reduced to such an extent that assay results become unreliable (as shown in the experimental curves below).

Constant Ab method. The theoretical neutralization curve for the *constant Ab* method is plotted on the ordinate with both I_f and absorbance, and on the abscissa, added IFN, I_v , both in LU/mL and as a dilution. Figure 3 illustrates the curves of eight Ab samples with titer ranging from 2 to 320. The Ab dilution f is held to the value of 20, the generally acceptable threshold dilution level, explained above. The control IFN titration curve without Ab is plotted in the same frame on the far right.

There are two important points to note: (i) the neutralization curves cover the whole dynamic A_{620} range, provided that an appropriate series of IFN concentration values (I_v) is utilized, and (ii) the curves are always parallel to the control IFN curve.

Most importantly, Figure 3 demonstrates how Abs with relatively low titers, such as $t = 2$ and 5, yield curves that cover the whole dynamic range and are clearly distinguishable from the control IFN curve. Assuming conservatively that the lowest detectable limit to detect neutralization is a 2-fold reduction of IFN titer, it can be concluded that the NAB sensitivity of the *constant Ab* method is ~10-fold higher than the *constant IFN* method, inasmuch as with the *constant Ab* method low serum titers such as $t = 2$ can be reliably titrated, whereas with the *constant IFN* method, reliable assays are generally possible only with antibodies having titers greater than 20. Further, the parallelism of the *constant Ab* neutralization curves to the control IFN curve provides a gauge of the reliability of the results obtained.

For the case of Ab dilutions other than $f = 20$, theoretical curves are easily derived. For any value of f , the curves are the same as those in Figure 3 except that the value of t for each curve can be changed to $f/20$ times the number indicated in the figure. Thus, if an Ab dilution of 1:10 were used, the curve labeled $t = 2$ would be relabeled $t = 2 \times (10/20) = 1$.

Experimental comparisons of the constant Ab and constant IFN methods

In order to test experimentally the predicted sensitivity of the *constant Ab* method in comparison with the *constant IFN* method, five model serum samples with a desired range of $t = 2$ to 40 TRU/mL were created from a serum having a titer in excess of 100,000 TRU/mL from a Rebif (IFN- β 1a)-treated patient. The serum was initially diluted in medium containing 2% bovine serum to an estimated concentration of 400 TRU/mL, which was titrated to have an actual mean titer of 375 TRU/mL by the original *constant IFN* method, and diluted further to prepare samples with an expected range of 1.9 to 37.5 TRU/mL. This experimental design establishes a dilutional grid such that the serum sample, of a potency initially measured by the *constant IFN* method, is then measured repeatedly as points on the grid by the *constant Ab* method. The most dilute sample in the grid is a gauge of sensitivity and a possible detection limit; the spacing between the dilutions is a gauge of both error and variation, i.e., accuracy and precision.

Figure 4 shows the neutralization curves and summaries of the calculated expected and actual titers of these five model serum samples, designated respectively A through E, with the titrations on one plate for A, B, and C represented in panel 4A, and on another plate, those for C, D, and E in panel 4B. The neutralization curves (absorbance vs. IFN dose) are generally parallel to the simultaneous control IFN curves without Ab, as predicted by the theory, and the values of the titers obtained using an Ab dilution of 1:20 are in good agreement with the expected values calculated from the preliminary titrations of the serum pool. The expected values shown in the figure summary are based on the measured titer of the pool, as indicated above, from which the model sera were produced as dilutions of that pool. Inasmuch as a serum neutralizes 10 LU/mL of IFN to 1 LU/mL at a 1:20 dilution, its titer is stated to be 20 TRU/mL, the lowest titer observable with the *constant IFN* method. Therefore, antibodies A, B, C, and D, with the expected calculated titers ranging from 1.9 to 18.75, would have usually been reported as <20 TRU/mL, following titration with the *constant IFN* method with 10 LU/mL of IFN as antigen (the procedure most commonly used currently by investigators in the field); whereas the *constant Ab* method could measure titers well below that level, for example, 1.7 TRU/mL in sample A. Sample Ab C was included for comparability in the two sets of titrations to give a titer of 7.8 TRU/mL in one titration and 7.4 TRU/mL in the other.

Table 1 summarizes repeated titrations by the *constant IFN* and *constant Ab* methods, as well as statistical evaluations that demonstrate the precision of the *constant Ab* method, even at 1–2 TRU/mL. The *constant IFN* method utilized antigen at 10 LU/mL and 2-fold dilution series of Ab starting at 1:20. As expected, the neutralization of sample E was readily determined, whereas samples A, B, and C were clearly below the level of detection; the titer of serum D could be calculated at the limits of the *constant IFN* curves, but should be considered unreliable, a matter considered experimentally separately below. Repeated measurements even at very low titers were very reproducible by the *constant Ab* method. With this method NABs with titers higher than 20 will register as >20 TRU/mL, using as a routine serial dilutions of IFN beginning at 10 LU/mL; to determine their titers

FIG. 2. Theoretical neutralization curves for the *constant IFN* method. The relationship between A_{620} and antibody dilution f is depicted for nine antibody samples with the indicated values of titer t ranging from 2 to 320. Note that the control IFN- β curve shown in Figure 1 is opposite in direction to the antibody neutralization curves. With regard to the range of f to be used in assays of antisera, the lowest value (the highest antibody concentration) is set at 20 (indicated by the vertical dotted line) and extended usually to 640 (six steps of a 2-fold dilution series). The panels show the cases when the test IFN level, I_t , was equal to (A) 10 LU/mL, (B) 5 LU/mL, or (C) 2 LU/mL.

FIG. 3. Theoretical neutralization curves for the *constant Ab* method in the case of a fixed antibody sample dilution of 1:20 ($f = 20$). The curves are of eight antibodies with a titer value, t , ranging from 2 to 320. Note that I_t (LU/mL) is shown on the upper abscissa, necessarily opposite in direction from the increasing IFN dilutions on the lower abscissa. The simultaneous control IFN titration curve (no Ab) is on the far right of the graph. Note that the neutralization curves are all parallel to the control IFN curve. In actual titrations, the variable that is directly observed is not I_t in LU/mL but IFN dilution, as indicated on the lower abscissa. The IFN dilution scale observed is translated into the I_t (LU/mL) scale by means of the control IFN curve that indicates the value of the IFN dilution that represents $I_t = 1$ LU/mL; in this figure a dilution value of 1:1,000 is assigned arbitrarily for illustrative purposes to give 1 LU/mL.

the assays require either higher constant dilutions of Ab or higher concentrations of IFN with 1:20 serum dilutions (also see below). These results clearly show that the higher sensitivity of the *constant Ab* method compared to the *constant IFN* method is readily attainable in practice.

Effect of low antigen doses on the constant IFN method

To test the previously stated theoretical concept that reducing the doses of IFN antigen might increase the sensitivity of the *constant IFN* method, additional antigen doses of 6, 4 and 2 LU/mL were used to assay the model sera A to E, utilizing 2-fold dilution series beginning with a 1:20 dilution as before. The neutralization curves are shown in Figure 5, together with the simultaneous IFN control curves run on each plate, on which the model sera were titrated at the indicated IFN antigen doses (I_i); the titers obtained are summarized in Table 2. The neutralization curves obtained are in good agreement with the theoretical ones in Figure 2. It is clear that no endpoint could be reliably obtained for the low-potency model sera A and B at the lowest antigen doses, where even the measurement of the amount of antigen in the control curves was problematic. The curve for serum sample C, with the expected titer of 10 TRU/mL, reached the midpoint at 1 LU/mL at the antigen doses of 6 and 4 LU/mL and less, but the results

cannot be considered as reliable. With model sera D and E, estimated to contain Ab in the range of 15 to 40 TRU/mL, it was possible to obtain titers at the lower antigen doses of 6 and 4 LU/mL, but problematic in measuring the amount of control IFN at 2 LU/mL. Thus, it appears that the *constant IFN* method cannot provide titers reliably with antigen doses less than about 6 LU/mL with the antiviral bioassay system utilized.

Measurement of NAbS by the constant Ab method in patients' sera with undetectable (<20) titers by the constant IFN method

To determine whether the *constant Ab* method could measure NAbS in patients' sera previously determined as either negative (<20 TRU/mL) or with low titers (23–31 TRU/mL) by the *constant IFN* technique, such sera were taken at random that had been stored frozen at -80°C and tested by the *constant Ab* assay, with the IFN product as antigen homologous to that the patient had been receiving as therapy. The results shown in Table 3 reveal striking differences between the two methods in the NAb positivity of sera of multiple sclerosis patients treated with Rebif and Betaseron. Of sera with titers previously shown to be <20 from Rebif (IFN- β 1a)-treated patients, 12 of 22, or 54%, had detectable titers between 1 and 16.5 TRU/mL by the *constant Ab* technique; and of 12 sera with titers previously shown to be <20 of Betaseron (IFN-

FIG. 4. The experimental neutralization curves for the five model serum samples A to E by the *constant Ab* method with summaries of their calculated expected titers and their actual observed titers. Note that the simultaneous IFN control titration curve and the NAb curves are parallel. Panel (A) provides the titration data for serum samples A, B, and C, and demonstrates that the *constant Ab* method can measure NAb titers approaching 1 TRU/mL. Panel (B) provides the titration data for serum samples C, D, and E. Sample C was included in both titrations for comparability.

β 1b)-treated patients, six of 12, or 50%, had titers between 1.5 and 14.5 TRU/mL by the *constant Ab* method. Contrary to expectation, of the 10 sera of Avonex (IFN- β 1a)-treated patients, none had detectable NAb (<1 TRU/mL) by the *constant Ab* method. These results clearly illustrate the 10- to 20-fold greater sensitivity of the *constant Ab* method to detect

NAb in patients' sera compared to the *constant IFN* technique in which the titers were undetectable (<20 TRU/mL).

Also shown in Table 3 are the results of six sera (R23–R26, B13 and B14) with NAb titers greater than 20 TRU/mL. Whereas such titers (23–32 TRU/mL) were readily measured by the *constant IFN* method with 10 LU/mL, assignment of titers >20

TABLE 1. COMPARISON OF THE SENSITIVITY OF THE *CONSTANT IFN* AND *CONSTANT AB* METHODS TO DETERMINE THE IFN NEUTRALIZING ANTIBODY TITERS (TRU/mL) OF EXPERIMENTAL MODEL SERUM SAMPLES

Model serum	Desired value ^a	Expected value ^a	Constant IFN mean titer	Constant Ab titer			
				n	Mean	SEM ^b	95% CI ^c
A	2	1.9	<20	4	1.88	0.05	1.66–2.11
B	5	4.65	<20	4	4.16	0.25	3.10–5.23
C	10	9.3	<20	12	8.37	0.42	7.28–9.46
D	20	18.75	<20	8	15.87	0.99	13.05–18.68
E	40	37.5	30.9 $\pm$ 2.0 ^b	6	35.09	2.33	28.16–42.01

^aA patient's serum with antibodies to Rebif (IFN- β 1a) was initially diluted to a desired potency value of 400 TRU/mL, which was verified repeatedly by the *constant IFN* method to obtain a mean value of 375 TRU/mL and diluted further to create a grid of the model serum samples with expected (calculated) NAb values based on dilution. ^bStandard error of the mean. ^cConfidence interval (CI) at 95% level.

FIG. 5. The experimental neutralization curves for model sera A to E obtained by the *constant IFN* method using antigen doses 10, 6, 4, and 2 LU/mL with sera having nominal titers of 40 or less TRU/mL. The *constant IFN* method does not reliably measure neutralizing antibody when antigen doses are below 6 LU/mL.

TRU/mL by routine titrations of the *constant Ab* method, in which serial 2-fold dilutions of 10 LU/mL were mixed with 1:20 serum dilutions, gave less readily calculable results.

The use of the *constant Ab* method in the case of higher titer Abs, such as the WHO anti-IFN- β human serum International Reference Reagent G038-501-572 (NIH Reference Reagent Note #45 1994), having a WHO-assigned titer of 1,500 (Calam and others 1995), is instructive. It was

necessary to dilute the serum to 1:2,000 or 1:3,000 to obtain a titer of 1,042 or 1,007 TRU/mL, respectively, *versus* 2-fold dilutions of IFN beginning with 10 LU/mL of Rebif (IFN- β 1a) compared with a mean titer of 1,255 TRU/mL by the *constant IFN* method. Thus, so long as the IFN dose used routinely is fixed at about 10 LU/mL, the *constant IFN* method is more advantageous for the measurement of high-titered NABs.

TABLE 2. NEUTRALIZATION TITERS OF MODEL SERA A THROUGH E OBTAINED BY THE *CONSTANT IFN* ASSAY USING DIFFERENT IFN DOSES, 10 LU/mL AND LESS, ILLUSTRATED IN FIGURE 5

Model serum	Expected value (TRU/mL)	Neutralization titer <i>t</i> (TRU/mL) in relation to indicated IFN dose <i>I_i</i>			
		10 LU/mL	6 LU/mL	4 LU/mL	2 LU/mL
A	1.9	<20	<11	<6	<2.2 ^b
B	4.65	<20	<11	<6	<2.2 ^b
C	9.3	<20	[12] ^a	[8] ^a	[3.8] ^{a,b}
D	18.75	23	31	19	[4.2] ^b
E	37.5	35	44	30	[12] ^b

The values shown in italics for samples A and B at 6, 4, and 2 LU/mL are based on calculations from the formula $t = f(n - 1)/9$ where <20 is taken as the serum dilution, e.g., with *I_i* at 2 LU/mL, $t = <20 (2 - 1)/9 = <2.2$.

^aThe value in brackets is considered unreliable because the neutralization curve does not adequately cover the dynamic range.

^bThese values are considered unreliable because the low antigen dose resulted in curves that do not adequately cover the dynamic range.

Discussion

The experimental observations described herein are in excellent agreement with the theoretical calculations based on the simplest model of Kawade and others (Kawade and Watanabe 1984; Grossberg and others 2001a, 2001b; Grossberg and Kawade 2006), adding substantively to the evidence already accumulated for the validity of the theory, its applicability to the determination of 10-fold reduction unitage, and its utility in expressing NAb titers. These data demonstrate unequivocally the much greater sensitivity of the *constant Ab* method than the *constant IFN* approach commonly employed for the quantitative measurement of human serum IFN- β antibodies, a conclusion that should apply also to the neutralization by antibodies to other cytokines. The *constant Ab* method can reproducibly detect as little as 1–2 TRU/mL, a more than 10-fold greater sensitivity than the *constant IFN* method as predicted by the theory. Although the sensitivity of the *constant IFN* method can theoretically be increased by reducing the amount of antigen, significant antigen reductions, as shown in Figure 5, make the dose–response and NAb measurements unreliable. A major virtue of the *constant Ab* technique is the quality assurance provided by the parallelism of the IFN control curves with the Ab neutralization curves, in contrast to the Ab curves with the *constant IFN* method in which the IFN control curves are opposite in direction to the NAb curves. A practical constraint on the *constant IFN* method is the adverse effect of human serum for cells in culture at relatively high serum concentrations, that is, dilutions 1:10 and less. No such constraint applies to hybridoma cell supernatants, but the great sensitivity of the *constant Ab* method recommends its use in screening monoclonal antibodies for NABs.

Evidence of the negative impact of NABs on clinical efficacy of IFN- β has been noted with Rebif (PRISMS, 2001) and Betaseron (The IFN β Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group 1996; Malucchi and others 2004; Boz and others 2007; Hartung and others 2007), although the issue remains controversial (Goodin and others 2007). Some decreases in NAb titers have occurred over time, although Sorensen and

others (2005b) showed that the majority of patients who were NAB-positive in year three remained NAB-positive for years more. However, NAB-positive patients can revert to negative Ab status over a 2–5-year follow-up, more often in patients with relatively low titers of NABs, but also in some with higher titers. Patients who remained NAB-negative during the first 18–24 months of IFN- β therapy would only rarely develop NABs (Rice and others 1999; Rice 2001; Perini and others 2004; Sorensen and others 2005b). It should be appreciated that such studies usually consider NAB positivity as titers >20. A more sensitive technique such as the *constant Ab* method might provide a different perspective on the above clinical correlations, especially in view of our observations (Table 3) that 50% of sera we tested from patients treated with Rebif and Betaseron previously considered as negative (<20 TRU/mL) had measurable NABs by the *constant Ab* technique. The unanticipated finding of no detectable NABs in 10 sera of Avonex-treated patients should also be extended.

Persistent serum NAB levels reduce the induction of MxA protein and mRNA in peripheral blood mononuclear cells from MS patients injected about 12 hours before with IFN- β , indicating its reduced bioavailability (Deisenhammer and others 1999; Vallittu and others 2002; Bertolotto and others 2003). Even at a neutralizing titer of 20, the bioavailability of IFN was compromised (Bertolotto and others 2003). It will be of interest to determine by the *constant Ab* technique at what NAB Ab level less than 20 TRU/mL correlates further with reduced IFN bioavailability. The need for a highly sensitive NAB assay that allows determination of low titers seems evident. One should note that a titer of 20 TRU/mL means reduction of IFN activity by 200-fold; even a titer of 1 TRU/mL indicates a 10-fold reduction.

It might be said that a possible drawback to the *constant Ab* method is that it requires a larger amount of the test serum sample than the *constant IFN* method. In our *constant IFN* assay consisting of six duplicate, 2-fold dilutional steps of serum (plus two serum control wells), the total quantity of serum required for the 1:10 initial dilution step needed for a titration is 80 μ L. Our *constant Ab* method that uses an initial 1:10 serum dilution to mix with six IFN dilutional steps plus

TABLE 3. ANTI-IFN- β NEUTRALIZING ANTIBODY TITERS (TRU/mL) MEASURED IN 50 SERA OF IFN- β -TREATED PATIENTS BY THE *CONSTANT IFN* AND *CONSTANT Ab* BIOASSAY METHODS

Serum no.	IFN- β antigen ^a	<i>Constant IFN</i> (TRU/mL)	<i>Constant Ab</i> (TRU/mL)
R1	Rebif (β 1a)	<20	<1
R2	Rebif (β 1a)	<20	<1
R3	Rebif (β 1a)	<20	<1
R4	Rebif (β 1a)	<20	<1
R5	Rebif (β 1a)	<20	<1
R6	Rebif (β 1a)	<20	<1
R7	Rebif (β 1a)	<20	<1
R8	Rebif (β 1a)	<20	<1
R9	Rebif (β 1a)	<20	<1
R10	Rebif (β 1a)	<20	<1
R11	Rebif (β 1a)	<20	1.1
R12	Rebif (β 1a)	<20	1.25
R13	Rebif (β 1a)	<20	1.3
R14	Rebif (β 1a)	<20	2.5
R15	Rebif (β 1a)	<20	2.8
R16	Rebif (β 1a)	<20	3.6
R17	Rebif (β 1a)	<20	3.9
R18	Rebif (β 1a)	<20	5.1
R19	Rebif (β 1a)	<20	5.6
R20	Rebif (β 1a)	<20	5.7
R21	Rebif (β 1a)	<20	5.8
R22	Rebif (β 1a)	<20	16.5
R23	Rebif (β 1a)	23	>20
R24	Rebif (β 1a)	28	>20
R25	Rebif (β 1a)	30	>20
R26	Rebif (β 1a)	31	>20
A1	Avonex (β 1a)	<20	<1
A2	Avonex (β 1a)	<20	<1
A3	Avonex (β 1a)	<20	<1
A4	Avonex (β 1a)	<20	<1
A5	Avonex (β 1a)	<20	<1
A6	Avonex (β 1a)	<20	<1
A7	Avonex (β 1a)	<20	<1
A8	Avonex (β 1a)	<20	<1
A9	Avonex (β 1a)	<20	<1
A10	Avonex (β 1a)	<20	<1
B1	Betaseron (β 1b)	<20	<1
B2	Betaseron (β 1b)	<20	<1
B3	Betaseron (β 1b)	<20	<1
B4	Betaseron (β 1b)	<20	<1
B5	Betaseron (β 1b)	<20	<1
B6	Betaseron (β 1b)	<20	<1
B7	Betaseron (β 1b)	<20	1.5
B8	Betaseron (β 1b)	<20	2.2
B9	Betaseron (β 1b)	<20	4.4
B10	Betaseron (β 1b)	<20	5.25
B11	Betaseron (β 1b)	<20	6.4
B12	Betaseron (β 1b)	<20	14.5
B13	Betaseron (β 1b)	24	>20
B14	Betaseron (β 1b)	26	>20

^aThe antigen used in assay is homologous to the specific IFN- β product with which the patient was treated.

duplicate serum controls requires a total quantity of 150 μ l of serum, about twice as much as the *constant IFN* method, but still considered to be a small sample volume.

Antibodies detected by *in vitro* immunoassays such as ELISAs of either the direct or sandwich type have been referred to as binding antibodies (BAbs), although it is obvious that NABs also bind to IFN molecules. BAbs have been described as developing sooner than NABs after therapy begins in MS patients who are treated with the three commercially available IFN- β products, as early as 2–3 months after therapy has started, achieving maximum levels in 9–21 months (Perini and others 2004). BAbs have been observed in (i) greater than 80% of patients treated with subcutaneous Betaseron/Betaferon (IFN- β 1b), (ii) more than 40% of patients treated with subcutaneous Rebif (IFN- β 1a), and (iii) about 15% of those given intramuscular Avonex (IFN- β 1a). These titers tend to diminish with time, but can remain detectable, certainly in the case of both Betaseron/Betaferon and Rebif, for at least 48 months. The question whether these binding antibodies, or some great proportion of them, are in fact NABs can now be answered with the *constant Ab* method that can measure NAb titers as low as 1–2 TRU/mL. Since approximately two-thirds of sera submitted for IFN- β NAb testing have been considered negative by the *constant IFN* method at the <20 TRU/mL cutoff, the *constant Ab* method can be used as a screening procedure with assays of any nature, be they based on induction of either antiviral effects, or MxA protein (Pungor and others 1998) and mRNA (Bertolotto and others 2007), or luciferase and other gene-reporter products (Lallemand and others 2008).

The sensitive *constant Ab* method should be equally applicable to other cytokines and other biologically active soluble protein-effector molecules having a single, nonrepetitive epitope, provided that the nature of the neutralization reaction is in the Constant Proportion mode, which appears to be the case with most neutralization reactions (Grossberg and others 2001a, 2001b).

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Appendix

Calculation of theoretical neutralization curves with experimentally determined quantities

The theoretical formulas that describe the quantitative relationships between the concentrations of IFN and antibody were derived by thermodynamic considerations of the previously described simplest model of the IFN-Ab reaction (Kawade and Watanabe 1984), which assumes that an IFN molecule binds 1:1 to an Ab binding site and loses its biological activity completely (Grossberg and others 2001a). These theoretical results were amply supported by experimental data collected from several laboratories (Grossberg and others 2001a and 2001b).

In the case of the *constant IFN* method (see Fig. 2), the theoretical neutralization curve relates the residual IFN activity

I_f (LU/mL) that remains in the mixture with Ab shown on the ordinate and the Ab concentration, that can be represented by Ab dilution f , on the abscissa. In the case of the *constant Ab* method (see Fig. 3), the curve has I_f on the ordinate and the total IFN activity I_t (LU/mL) on the abscissa. When the Ab acts in the Constant Proportion mode, as was found to be the case with practically all Ab samples examined, the relationship between I_f and f in the *constant IFN* method and that between I_f and I_t in the *constant Ab* method is informed by the formula:

$$t = f(I_t/I_f - 1)/9 \quad (1)$$

where t is the titer of the Ab sample defined as the Ab dilution f that reduces the IFN activity 10-fold, e.g., from $I_t = 10$ LU/mL to $I_f = 1$ LU/mL, the endpoint of IFN titration (Grossberg and others 2001a and 2001b; Kawade and others 2003). The neutralization curve of a NAb is calculated with this formula by assigning a value to the titer t . The range we used of the values of t was from 2 up to 320, as our attention was directed especially to the behavior of low-titered Abs.

Constant IFN method

When an IFN dose I_t of 10 LU/mL is used, the theoretical curve of a NAb with an assigned value of t is calculated by rearranging Eq. (1):

$$f = 9 t/(10/I_f - 1) \quad (2)$$

The values of f are calculated by substituting various values for I_f . For instance, for an Ab with $t = 10$, the values of f obtained for $I_f = 4, 2, 1, 0.5$ and 0.25 LU/mL are 60, 22.5, 10, 4.7, and 2.3, respectively. These values of I_f are correlated with the A_{620} scale by means of the IFN titration curve shown in Figure 1, and both I_f and A_{620} are plotted on the ordinate against the f values obtained on the abscissa, as shown in Figure 2A. Since the lowest final serum dilution routinely used to test patient sera is 1:20, the range of Ab dilution, f , in the region smaller than 10 are not considered.

The case of an IFN dose I_t other than 10 LU/mL can similarly be dealt with (Fig. 2B and C).

Constant Ab method

The theoretical neutralization curves for the *constant Ab* method are calculated using the formula derived from Eq. (1):

$$I_t = I_f(1 + 9tf) \quad (3)$$

Let us consider the case of Ab dilution f held to the value of 20, since the highest antibody concentration generally considered to be utilizable for patients' sera is 1:20. For an antibody with an assigned value of t , the value of I_t can be calculated for each of the values of I_f at 4, 2, 1, 0.5, and 0.25 LU/mL. For example, for an antibody with $t = 10$, I_t is equal to $I_f(1 + 9 \times 10/20) = 5.5 I_f$; the value of I_t then for $I_f = 4$ is 22, that for $I_f = 2$ is 11, etc. The I_f to I_t relationship thus obtained is illustrated in Figure 3. As explained in the main text, the theoretical curves for the case of an Ab dilution f other than 20 can be readily obtained.

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