Use of a Standardized MxA Protein Measurement-Based Assay for Validation of Assays for the Assessment of Neutralizing Antibodies Against Interferon- β

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Effective monitoring of the development of neutralizing antibodies (NAbs) against IFN- β in multiple sclerosis (MS) patients on IFN- β therapy is important for clinical decision making and disease management. To date, antiviral assays have been the favored approach for NAb determination, but variations in assay conditions between laboratories and the increasing use of novel assays have contributed to the reporting of inconsistent antibody data between laboratories and between products. This study, undertaken at the request of the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA), is a joint effort by manufacturers of IFN- β products (approved in Europe) towards harmonization of a NAb assay that facilitates generation of comparable NAb data, which, in conjunction with clinical outcomes, should prove useful for clinicians treating MS patients with IFN- β products. This article describes the standardized cellular myxovirus resistance protein A (MxA) protein measurement-based assay for detection of IFN- β NAbs and its use for the validation of assays used for the quantitative determination of such antibodies. Although titers varied between laboratories and the products used, utilization of IFN- β 1a rather than IFN- β 1b as the challenge antigen produced more consistent results in the NAb assay. Adoption of the standardized assay improves comparability between laboratories circumventing problems that arise when different, nonstandardized assays are employed for immunogenicity assessment. Based on the data, the EMA recommended for standardization purposes, the use of IFN- β 1a in NAb assays, independent of the therapeutic product used for therapy and validation of new NAb procedures against the standardized assay described.

Introduction

ADVANCES IN MULTIPLE SCLEROSIS (MS) research have led to an improved understanding of the disease and availability of a number of disease-modifying therapies. Among these is Interferon- β (IFN- β), an immunomodulatory protein that in clinical trials in relapsing remitting MS (RRMS) patients has been shown to reduce relapses and diminish development of new inflammatory lesions as evidenced by magnetic resonance imaging (Jacobs and others 1996; PRISMS Study Group 1998; Goodin 2005; Clerico and others 2007). In RRMS, therefore, IFN- β products are widely

used as first-line treatment. Defining a significant benefit in some MS patients, however, is very difficult, as MS is a complex, multi-faceted disease and patients continue to relapse, develop active lesions, or show increased disability despite treatment. The timely recognition of factors that affect the efficacy in individual patients is important, as such recognition could enable intervention and switching to other licensed therapeutics for effective management of disease.

Currently, 3 recombinant IFN- β products—subcutaneous IFN- β 1b (Betaferon, Bayer, United Kingdom; also marketed as Extavia, Novartis, United Kingdom), intramuscular IFN- β 1a (Avonex, Biogen Idec, United Kingdom), and

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subcutaneous IFN- β 1a (Rebif, Merck Serono, United Kingdom)—which vary in structure, formulation, dose, and route of administration are indicated in the EU for patients with relapsing MS or at high risk of developing MS after a single demyelinating event. None of the products are approved for patients with primary progressive MS (PPMS). While the IFN- β 1b product is an *Escherichia coli* expressed, non-glycosylated IFN- β , Met-1, Ser17 mutein (in which the cysteine residue at position 17 is replaced by a serine), both IFN- β 1a products are glycosylated preparations (with an amino-acid sequence identical to the natural human protein) that are produced using Chinese hamster ovary (CHO) cells (Karpusas and others 1998; Runkel and others 1998). Despite their differences, all products show clinical efficacy in RRMS, as they reduce the rate of relapses by 17% to 30% and new lesion activity by 65% to 88% and also slow the progression of physical disability and brain atrophy [Avonex FDA label; (Li and Paty 1999; Durelli and others 2002; Rudick and Goelz 2011)]. In trials in secondary progressive (SP)MS and PPMS, however, results have been inconsistent, largely due to differences in the characteristics of the patients treated (Rudick and Goelz 2011).

The effectiveness of IFN- β in RRMS varies with a robust response and disease stabilization in some patients; while in others, the disease activity continues. A significant number of patients discontinue treatment either due to side effects or due to a perceived lack of efficacy with some patients developing antibodies against IFN- β (Rio and others 2005).

The incidence and characteristics of the induced antibodies (eg, non-neutralizing or neutralizing) is variable and dependent on the administered IFN- β product as well as on a host of other factors, including the dose, frequency, route of administration, patient status, and the method used for antibody detection.

It is now increasingly recognized that antibodies can have an impact on clinical outcome, although there is also evidence showing no discernible effect on clinical outcome despite an association of neutralizing antibody (Nab) positivity with increased brain lesions (Goodin and others 2012). Persistent IFN- β NAb (>2 consecutive positive samples) are associated with reduced pharmacodynamics and loss of bioactivity (Bertolotto and others 2003; Scagnolari and others 2007; Deisenhammer 2009). A reduction in the clinical efficacy of IFN- β as noted by an increased number of exacerbations and/or new lesions detected by MRI in Nab-positive (high titers) patients compared with those who were Nab negative is evident from some but not all clinical trials (Francis and others 2005; Kappos and others 2005; Goodin and others 2007; Pachner and others 2009). The unfavorable effect of the developed antibodies when noted appears to be independent of the type of IFN- β used (Koch-Henriksen and others 2009).

Since IFN- β products are intended for chronic use, the formation of NAb is an important consideration affecting patient therapy and has aroused much debate. Based on accumulated evidence, expert panels have concluded that persistently high titers of NAb (>200_{t_{1/10}}) are likely to be associated with frequent relapses and exacerbation in disease (Goodin and others 2007; Polman and others 2010). Indeed, a recent study showed that high titers of NAb, which persisted years after end of therapy, were associated with increased relapse rates and progressive disability. Switching patients from one IFN- β product to another does not sig-

nificantly change NAb titers, as induced antibodies cross-react with other IFN- β products (Gneiss and others 2009). Importantly, effective monitoring of the development of NAb using appropriate assay systems is crucial for clinical decision making and disease management (Polman and others 2010; Farrell and others 2011).

Establishing the exact magnitude of the clinical impact of NAb on the efficacy of IFN- β has been complicated due to the plethora of different assays used for determining NAb, variable experimental conditions, differences in methods for calculating and defining NAb titers, and discrepancies in sampling times (Bertolotto 2009). Assays based on the inhibition of IFN activity using either antiviral (AVA) bioassays based on the cytopathic effect reduction (CPE) approach or, more recently, inhibition of an IFN-inducible gene, myxovirus resistance protein A (MxA), or secreted MxA protein are largely favored for monitoring and quantifying the levels of NAb (Pungor and others 1998; Meager and Gaines-Das 2005; McKay and others 2006; Bertolotto and others 2007; Goodin and others 2007). A number of reporter gene assays (RGA) have also been described (Lallemand and others 2008; Lam and others 2008). However, even among the AVA (the assay described in the European Pharmacopoeia for determination of biological activity of IFNs), comparisons have been difficult due to divergence in assay protocols with regard to cell-virus combinations, the amounts of virus, and the dose and type of IFN- β used (Hartung and others 2007). This has resulted in a wide variation in the reporting of NAb frequencies and titers for the different products in various studies. For example, incidences of NAb in patients treated with Avonex, Rebif, and Betaseron have been reported as 2%–6%, 12%–28%, and 28%–47%, respectively (Bertolotto and others 2004; Deisenhammer 2009; Rudick and Goelz 2011) or as 13% (Avonex), 30%–39% (Rebif), and 43% (Betaseron) with each of the products separately (Sominanda and others 2007). However, the issue is further complicated, because in many of these assays, the correlation between titer level and assay signal to a clinically meaningful effect on the PK/PD or efficacy of the product has not been established and provides little useful information, if any, to the clinician in terms of disease management, emphasizing the need for well-standardized NAb assays (Goodin and others 2007).

This lack of standardization instigated the need for a common standardized NAb assay for facilitating a comparison for data generated across studies using different products so that a better understanding of the impact of NAb on clinical outcome can be achieved. Consequently, the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) requested the development of a standardized assay for measuring NAb. Following this recommendation, the marketing authorization holders agreed to validate a common bioassay (EMA, 2008). The selection of an assay measures cytoplasmic accumulation of the IFN-inducible MxA protein, a 75 kD cytoplasmic protein that is one of the markers of the biological activity of IFN- β and is known to be applicable to the measurement of neutralizing IFN- β NAb (Towbin and others 1992; Files and others 1998; Pungor and others 1998). Moreover, it offered distinct advantages compared with the CPE assay, as it enabled easier standardization with possibility of automation, faster data turn-around time and eliminated the safety concerns associated with virus handling.

Box 1. MxA Blind-Panel Study.

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Study Participants:

Biogen Idec, USA—Goelz S, Jones W, Goyal J, Jethwa V
Berlex (now Bayer), USA—Files James G and others
Serono (now Merck Serono), Italy—Antonetti F and others

NIBSC for the planning, assembly and distribution of panels, data collation and statistical analysis.

This article describes the standardized cell-based assay for detection of IFN- β NABs based on results from a collaborative study in which a blind panel of serum samples ($n=62$) was assessed for neutralizing activity by the 3 marketing authorization holders ("participants"—See Box 1) for IFN- β products in the EU using a common IFN- β preparation as the challenge antigen in all NAb assays. Data from assays in which each participant employed their own therapeutic IFN- β as the antigen is also shown. In addition, data derived for a

set of samples ($n=11$) by each participant using the 3 commercial IFN- β products are also presented. The use of the MxA protein assay for validation of another representative NAb assay is also described.

Materials and Methods**Materials**

Human fibroblast-derived IFN- β (reference reagent Gb-23-902-531, available from NIH, Bethesda, USA) and all approved IFN- β products, Betaseron (IFN- β 1b), Rebif (IFN- β 1a), and Avonex (IFN- β 1a), obtained from their respective manufacturers, were used in the NAb assays. A human IFN- β NAb reference serum (G038-501-572; NIH) was used in validation studies. Serum test samples were obtained from NAb-positive MS patients undergoing therapy with 1 of 3 products—Betaseron, Avonex, or Rebif. Some samples were prepared as pools of similar titer sera (negative, low, medium, and high titer) from different patients who were being treated with the same therapeutic (Table 1). Some normal serum samples were isolated at the National Institute of Biological Standards and Control, NIBSC, United Kingdom. For the collaborative study, 62 serum samples from patients treated

TABLE 1. BLIND-PANEL SERUM SAMPLES TESTED IN THE MxA ASSAY

Sample	Origin (code)	Previous titer	Selected subset	Pos/Neg*	Sample	Origin (code)	Previous titer	Selected subset	Pos/Neg*
6	HC	—	S	—	15	Berlex (18)	93		+
9	M. Serono (2)	ND		—	12	Berlex (21)	63		+
16	HC	—		—	33	Berlex (26)	22		+
29	HC	—		—	8	Berlex (28)	49		+
38	HC	—		—	11	Biogen (5)	50–200		+
39	HC	—		—	13	Biogen (9)	50–200		+
43	M. Serono (1)	ND	S	—	46	Berlex (29)	39		+
44	HC	—		—	55	Berlex (17)	103		+
49	HC	—		—	45	M. Serono (4)	10–50	S	+
50	HC	—		—	59	Biogen (1)	> 200		+
60	HC	—		—	48	Berlex (23)	64		+
61	Other	49	1:100	+	27	Berlex (19, 22)	75, 72	S	+
4	Berlex (32)	<10		+	36	Berlex (12)	253		+
52	Other	+	1:50	+	53	Berlex (14)	180		+
18	M. Serono (1)	10–50		+	10	Berlex (16)	113		+
19	Other	+	1:25 S ²	+	26	Berlex (15)	141		+
56	Biogen (4)	10–50		+	35	Berlex (13)	391		+
31	Other	+	1:25 S ²	+	28	Berlex (11)	250		+
54	Biogen (8)	10–50		+	5	Berlex (10)	389		+
47	Biogen (7)	10–50		+	1	M. Serono (7)	> 200		+
17	Berlex (31)	29		+	25	NIBSC (99/606)	*****		+
24	Biogen (3)	50–200		+	30	Berlex (7)	783		+
34	Biogen (6)	10–50		+	58	M. Serono (8)	> 200	S	+
20	Other	453	1:10	+	57	Berlex (6)	1366		+
37	Berlex (30)	21		+	22	Berlex (33)	<10		+
14	M. Serono (5)	50–200	S	+	32	Berlex (5)	1574		+
7	Berlex (27)	32		+	40	Biogen (10)	> 200		+
42	Berlex (20)	53		+	23	Berlex (8,9)	463, 465	S	+
41	Berlex(24, 25)	40, 47	S	+	51	Berlex (3,4)	2185, 2038	S	+
62	M. Serono (6)	50–200		+	3	Berlex (1)	5409		+
2	Biogen (2)	> 200		+	21	Berlex (2)	3051		+

S denotes selected samples, Other indicates patient sera obtained from another source; this was diluted as a dilution series for the study; sample at a 1:25 dilution was included as coded duplicates of the same sample S²; HC, healthy control (untreated); ND, not detectable; ***** denotes not determined; M Serono, Merck Serono.

Sample codes, origin, previous titer (where available using in-house assays), and antibody status* as+ for positive and – for negative confirmed by all participants in MxA assay using IFN- β (NIH) as antigen in MxA assay is provided.

MxA, myxovirus resistance protein A.

with each of the 3 products (sera sent by the manufacturers and a clinician to NIBSC, United Kingdom) and normal healthy sera were aliquotted, prepared as a blind panel (coded) at NIBSC, and distributed to the study participants.

Participants

Three participants [Biogen Idec; Merck-Serono; Berlex (now Bayer)] contributed NAb assay data for the blind-panel study.

MxA protein NAb assay

The NAb assay is a modification of the MxA induction assay for type I IFNs involving IFN- β stimulation of the human A549 adenocarcinoma cell line previously described (Files and others 1998; Pungor and others 1998).

Briefly, serum samples were initially diluted 1:5 in assay medium containing 20 IU/mL of IFN- β and serially diluted 2-fold (to achieve up to a 1:1,280 dilution) in IFN- β containing medium previously added into a 96-well plate. After 1 h at room temperature, 50 μ L of medium from each well was transferred, in duplicate, to wells of another 96-well sterile microtiter plate containing 5,000 A549 cells (ATCC CCL-185) in 50 μ L medium, seeded for 20–24 h earlier, and incubated at 37°C. This provided a final concentration of 10 IU/mL IFN- β and serum dilutions of 1:10–1:2560. After further incubation for 24 h at 37°C, the media were aspirated gently, and the cells were lysed by addition of 100 μ L of lysis buffer (25 mM Tris-HCl-pH 7.2, 0.02% sodium azide, and 2% NP-40) for approximately 2 h at room temperature. The lysates were frozen and assayed for accumulated cellular MxA protein using an ELISA.

MxA protein was determined using a rat anti-MxA monoclonal antibody (2D12; Biogen Idec) to capture and a biotinylated mouse anti-MxA monoclonal antibody (4E5; Biogen Idec) to detect the protein. Briefly, 96-well plates were immobilized with 100 μ L of capture antibody (2 μ g/mL in 0.2 M sodium-carbonate-bicarbonate buffer, pH 9.4; Pierce Cat.no. 28382), stored overnight at 2°C–8°C, washed (PBS containing 0.05% tween-20), and blocked (PBS with 3% BSA) for 1–2 h at room temperature or overnight at 2°C–8°C. After a wash step, 50 μ L of samples (cell lysates, MxA-positive control cell lysate) and 50 μ L of the detector antibody (0.5 μ g/mL in PBS with 3% BSA) were added to the wells, and the plates were incubated overnight on a plate rotator at 2°C–8°C. Subsequent to washing, the wells were incubated with 100 μ L of streptavidin-HRP (1:10,000 dilution; Jackson ImmunoResearch Cat No., 016-030-084) for 30 min, washed, and 100 μ L of TMB substrate peroxidase solution (BioRx, TMBW-001-01) was added. Color development was monitored, the reaction was stopped (100 μ L of 1N H₂SO₄), and the optical densities (OD) were measured in a microplate reader (Molecular Devices) at 450 nm. The OD value is directly proportional to the concentration of MxA in each well.

A standard curve consisting of lysates from cells incubated with various concentrations of IFN- β was included on each plate. The EC₅₀ value from a 4-parameter curve fit of the IFN- β standard curve was defined as 1 Lab Unit (LU) value for the assay. The sample titer was calculated using the Kawade formula (Kawade 1986): $\text{Reciprocal Dilution of Sample at Cutoff} \times [(10/\text{C value}) - 1] / 9 = \text{titer}$; this defines the titer as the dilution of serum that reduces the amount of LU of IFN by 90% (1 LU = EC₅₀ ie, 50% MxA induction).

Blind-panel study design

Before assessing the blind-panel samples, the MxA NAb assay was validated by the 3 manufacturers and shown to have good reproducibility. All 3 participants were requested to assay all blind-panel samples ($n=62$), shown in Table 1, for neutralizing activity in the MxA NAb assay using a fibroblast-derived IFN- β and their own therapeutic IFN- β product as challenge antigens. A subset comprising 11 “selected samples” (codes 06, 14, 19, 23, 27, 31, 41, 43, 45, 51, 58), labeled S (Table 1), was included in the panel. This was tested by each participant using all 3 commercial IFN- β products. Two samples coded 19 and 31 were included in the subset as duplicates (ie, identical except for sample code), to provide a direct measure of intra-assay variation, with which the observed inter-assay variation can be compared. Participants were requested to provide calculations of titer and raw assay data for all study samples.

Participant data

Each of the 3 participants provided a single titer value (based on their calculations) for each sample and antigen, although repeat assays and dilution curves with different starting dilutions were performed for some samples. Raw assay data provided included duplicate responses in adjacent columns of the plate for each preparation/dose. One participant also provided data from their in-house assay (data not included).

Statistical analysis

Analyses of titers were carried out at NIBSC after transformation of titers to log titers. Variation of estimates is summarized as the geometric coefficient of variation (GCV). The information for some variances is limited, in which case the associated degrees of freedom (df) are also given. Correlations were calculated for the log titers of the non-negative selected samples.

Comparisons in independent laboratories

Two laboratories carried out a further evaluation of the MxA protein NAb assay. The assay was performed using the same specified conditions and reagents (as described earlier) where possible. In comparative experiments, concentrations of antibody pairs for MxA ELISAs were different; the original antibodies (Biogen Idec) were used as indicated earlier, while the antibody pair (from Novartis and procured later) was used at a concentration of 4 μ g/mL. The latter antibodies at 4 μ g/mL (for both) were also used in another laboratory for the cross-validation study in which the MxA assay, conducted using reagents available in house, was compared with a reporter gene NAb assay. The reporter gene NAb assay was carried out as previously described (Lallemant and others 2008) using assay-ready *iLite* cells obtained from Biomonitor Ltd.

Results

Before evaluation of blind-panel serum samples, the MxA NAb assay was assessed by the 3 companies for its suitability using a common validation protocol and assay design and found to demonstrate good reproducibility. The blind-panel

study was conducted with an understanding that the description of the method and reagents for executing the MxA NAb assay is publicly available.

Blind-panel study

The complete blind panel ($n=62$) was tested by each participant using fibroblast-derived IFN- β and the participant's antigen in the assay. Therefore, the complete panel was tested only by a single participant for each of the 3 commercial preparations. Analysis of the data from the participants showed that there was 100% consistency across the 3 laboratories, in terms of samples categorized "negative" for NAb. All laboratories identified the negative samples included in the panel (Table 1) as negative irrespective of whether the fibroblast-derived IFN- β (Table 2) or the manufacturer's unique antigen (Table 3) was used in the assay. On this basis, samples coded 06, 09, 16, 29, 38, 39, 43, 44, 49, 50, and 60 were assigned titers of <10 . Since all laboratories

classified these samples as negative ($n=11$), they have been omitted from a further analysis.

In terms of "non-negative" samples, all laboratories identified the remaining samples ($n=51$) in the panel as positive for NAb when using fibroblast-derived IFN- β or the IFN- β 1a antigen for challenge (Tables 2 and 3). For these preparations, therefore, a 100% concordance was observed between the different laboratories in terms of samples identified as "negative" and "positive" for NAb. However, when IFN- β 1b product (Betaseron) was used as challenge antigen, 2 (code 04, 56) of the 51 samples tested did not show quantifiable neutralization, resulting in 3% discordance between the different laboratories in terms of samples identified as "negative" and "positive" for NAb. The neutralization titers, derived by each of the participants, using either fibroblast-derived IFN- β or their own IFN- β product as the challenge antigen in the MxA assay, are shown in Tables 2 and 3, respectively. The 2 discordant samples, coded 04 and 56, were of low titer but showed some neutralizing activity

TABLE 2. NEUTRALIZATION TITERS OBTAINED BY THE 3 PARTICIPANTS USING FIBROBLAST-DERIVED IFN- β (2ND IS, NIH) AS CHALLENGE ANTIGEN IN THE MxA ASSAY

Sample	Biogen Idec/1	Merck Serono/2	Berlex/3	Sample	Biogen Idec/1	Merck Serono/2	Berlex/3
6	<10	<10	<10	15	326	350	279
9	<10	<10	<10	12	350	318	321
16	<10	<10	<10	33	370	357	486
29	<10	<10	<10	8	394	391	344
38	<10	<10	<10	11	412	394	423
39	<10	<10	<10	13	421	418	348
43	<10	<10	<10	46	426	379	329
44	<10	<10	<10	55	442	385	364
49	<10	<10	<10	45	553	467	425
50	<10	<10	<10	59	747	725	697
60	<10	<10	<10	48	784	849	814
61	24	29	27	27	798	595	582
4	37	30	46	36	824	678	898
52	48	42	50	53	951	780	586
18	79	64	57	10	1439	1198	1293
19	99	98	76	26	1711	968	982
56	102	73	77	35	2001	2080	1388
31	116	107	80	28	2118	1450	1196
54	145	100	118	5	2125	1580	1081
47	171	111	158	1	2401	2195	1625
17	179	108	89	25	2457	1200	1508
24	195	260	162	30	4098	2188	2151
34	212	162	247	58	4845	5545	3019
20	223	164	183	57	5009	3331	2722
37	228	161	173	22	5115	2420	2289
14	270	157	180	32	5166	3309	2956
7	271	174	161	40	10818	9329	10604
42	292	208	228	23	13973	12598	10082
41	294	235	224	51	24606	22487	13370
62	298	252	291	3	25348	25100	17333
2	301	265	291	21	30624	22269	23439

<10=neg.

10–100=low.

100–1000=mid.

>1000=high.

TABLE 3. NEUTRALIZATION TITERS OBTAINED BY THE 3 PARTICIPANTS USING THEIR OWN IFN- β PRODUCT AS CHALLENGE ANTIGEN IN THE MxA ASSAY

Sample	Avonex	Rebif	Betaferon	Sample	Avonex	Rebif	Betaferon
6	<10	<10	<10	15	534	284	110
9	<10	<10	<10	12	461	321	75
16	<10	<10	<10	33	623	342	86
29	<10	<10	<10	8	538	304	70
38	<10	<10	<10	11	473	324	91
39	<10	<10	<10	13	707	555	89
43	<10	<10	<10	46	815	400	63
44	<10	<10	<10	55	773	309	129
49	<10	<10	<10	45	828	337	79
50	<10	<10	<10	59	1189	622	84
60	<10	<10	<10	48	1009	693	170
61	46	19	13	27	924	609	129
4	83	31	<10*	36	2448	970	489
52	100	52	14	53	1318	827	301
18	110	38	18	10	866	734	199
19	208	104	25	26	1731	868	371
56	172	71	<10*	35	2731	1740	507
31	214	108	39	28	3174	1395	461
54	165	92	23	5	2452	1640	235
47	184	134	27	1	3802	2342	394
17	464	200	64	25	3505	1604	947
24	305	154	21	30	7504	3341	779
34	288	153	41	58	6754	4274	1029
20	392	328	89	57	7212	2476	1105
37	307	149	39	22	3473	1824	292
14	445	182	45	32	6447	2264	1035
7	312	144	27	40	14249	9434	1595
42	507	243	54	23	14426	15100	2698
41	476	282	47	51	33382	26800	5032
62	416	212	37	3	44893	44100	8071
2	525	324	37	21	46248	30016	6854

<10=neg.

10–100=low.

100–1000=mid.

>1000=high.

*Neutralization below limit of quantitation when Betaferon is used as challenge antigen (see results section).

when either of the IFN- β 1a products, Avonex or Rebif, were used. In particular, sample 56 showed moderate neutralization with both products. However, when tested using Betaferon, although the samples exhibited detectable neutralization, the titer values were below the lowest dilution and limit of quantitation (1:10)—these are shown by <10* in Table 3.

An examination of correlation coefficients for the 51 non-negative samples indicated a complex relationship. Correlation coefficients for values obtained with the common assay using the fibroblast-derived IFN- β in the different laboratories were ≥ 0.99 . Correlations among the values obtained using the fibroblast-derived IFN- β and the 2 IFN- β 1a products, Avonex and Rebif, were ≥ 0.98 . Correlations of values obtained using the IFN- β 1b product, Betaferon with those obtained using fibroblast-derived IFN- β , Rebif, or Avonex were ≥ 0.96 .

Selected samples

Among the subset of 11 “selected samples” (codes 06, 14, 19, 23, 27, 31, 41, 43, 45, 51, 58) tested by each participant using all 3 commercial IFN- β products, samples coded 06 and 43 were found to be negative and were, therefore, excluded from a further analysis. The reported titers for the 9 non-negative samples, including the 2 identical (which provide a direct measure of intra-assay variation, with which the observed inter-assay variation can be compared) samples coded 19 and 31, are shown in Table 4. For any preparation/laboratory, the correlation coefficient for these 9 samples was greater than 0.98.

The intra-assay variation was pooled over laboratories for each preparation (3 df for each preparation) and also within each laboratory over the 4 preparations (4 df for each laboratory). The associated GCVs for the commercial

TABLE 4. NEUTRALIZATION TITERS FOR THE SUBSET OF SELECTED (9 NON-NEGATIVE) SAMPLES USING 4 DIFFERENT IFN- β PREPARATIONS FOR NEUTRALIZATION IN THE MxA ASSAY

Sample	Biogen Idec			Fibroblast-derived	Berlex			Fibroblast-derived	Merck Serono			Fibroblast-derived
	Avonex	Betaseron	Rebif		Avonex	Betaseron	Rebif		Avonex	Betaseron	Rebif	
14	445	66	287	270	283	45	255	180	224	57	182	157
23	14426	6388	13392	13973	11778	2699	19770	10082	15337	4269	15100	2598
27	924	193	579	798	564	129	699	582	658	127	609	595
41	476	82	268	294	263	47	297	224	361	62	282	235
45	828	190	423	553	479	79	599	425	429	161	337	467
51	33382	14775	25621	24606	25842	5032	38838	13370	27613	8948	26800	22487
58	6754	1314	4040	4845	4234	1030	6223	3019	4344	1300	4274	5545
19*	208	61	154	99	144	25	157	76	102	48	104	98
31*	214	55	142	116	119	39	149	80	101	49	108	107

preparations, Avonex, Betaseron, and Rebif are 8%, 20%, and 4% respectively. For the fibroblast-derived preparation, the GCV was 8%. The GCV for participating laboratories was 18% (Berlex), 8% (Biogen), and 4% (Serono), respectively. Although these data are limited for drawing any reliable conclusions, it is consistent with dependence of the variance on both the laboratory and the preparation.

Comparisons of the between-laboratory variation with the within-laboratory variation for the coded duplicates using each preparation showed that the between-laboratory variation was significantly larger than the within-laboratory variation within assay variation (the reported titers do not provide replicate assays for these samples, and, hence, no estimate of the between assay within laboratory variation is available). The difference was significant for Avonex and Rebif ($P < 0.01$), and marginally significant for the fibroblast-derived ($P = \sim 0.05$) and Betaseron ($P = \sim 0.10$) preparations.

Inter-laboratory variation

The inter-laboratory variation has been determined for the common assay with each preparation, pooled across all samples. For the commercial preparations, 9 non-negative samples were analyzed in all laboratories, and for the fibroblast-derived IFN- β preparation, 51 non-negative samples were analyzed in all laboratories. The GCVs were 34%, 42%, 23%, and 25% for Avonex, Betaseron, Rebif, and fi-

broblast-derived IFN- β , respectively. These data suggest that the inter-laboratory variation for Betaseron may be larger than that for the other preparations, and that for Avonex, it may be slightly larger than that for Rebif and fibroblast-derived IFN- β .

The inter-laboratory variance of the reported titers for all samples using the common fibroblast-derived IFN- β preparation gave a GCV of 24% compared with GCV of 189% for the reported titers when each laboratory used the individual antigen. For the selected samples, the GCVs using any one of the preparations as the "common antigen" were found to be 34% (Avonex), 42% (Betaseron), and 23% (Rebif).

Analysis of variance of the log titers for each antigen shows that there are highly significant differences between laboratories, $P < 0.001$ for each preparation. This is consistent with the findings for the coded duplicate samples, where the limited number of observations limited the ability to detect clearly the significant differences between laboratories.

Ranks of titers

The close agreement among estimates is also shown by the consistency with which the subset of 11 selected samples is ranked (Table 5). The ranks of the coded duplicate samples are of interest. Considering only the 9 non-negative samples, the duplicates have ranks of either 1 or 2. For the 3 commercial preparations, either ranking appears to be equally likely; whereas for the fibroblast-derived IFN- β preparation,

TABLE 5. RANKING FOR THE SUBSET OF SELECTED (9 NON-NEGATIVE) SAMPLES USING 4 DIFFERENT IFN- β PREPARATIONS FOR NEUTRALIZATION IN THE MxA ASSAY

Sample	Biogen			Fibroblast-derived	Berlex			Fibroblast-derived	Merck Serono			Fibroblast-derived
	Avonex	Betaseron	Rebif		Avonex	Betaseron	Rebif		Avonex	Betaseron	Rebif	
14	3	3	4	3	4	3	3	3	3	3	3	3
23	8	8	8	8	8	8	8	8	8	8	8	8
27	6	6	6	6	6	6	6	6	6	5	6	6
41	4	4	3	4	3	4	4	4	4	4	4	4
45	5	5	5	5	5	5	5	5	5	6	5	5
51	9	9	9	9	9	9	9	9	9	9	9	9
58	7	7	7	7	7	7	7	7	7	7	7	7
19*	1	2	2	1	2	1	2	1	2	1	1	1
31*	2	1	1	2	1	2	1	2	1	2	2	2

*denotes coded duplicates.

FIG. 1. IFN- β NAb titers in antibody-positive patient sera using different antibody pairs in MxA ELISA for determination of MxA protein. IFN- β 1a was used as the challenge antigen in the MxA assay; using Pearson's correlation, $r=0.994$ ($n=28$ for antibody-positive samples; $n=31$ in total). MxA, myxovirus resistance protein A; Nab, neutralizing antibody.

sample 19 consistently has rank 1 and sample 31 has rank 2. For samples 14 and 41, the titers are also not significantly different, resulting in a consistent ranking of either 3 or 4. When all 51 samples are considered, the ranking order is 5, 5, and 6 for sample 19 or 7; 7, 8 for sample 31 when fibroblast-derived IFN- β is used by Berlex, Biogen, and Merck Serono, respectively (data not shown).

Further studies

MxA monoclonal antibodies produced by Biogen Idec were used in the blind-panel study. Antibodies derived from hybridomas provided by Novartis (Towbin and others 1992) were compared with the original antibody pair (Biogen Idec) for their suitability to detect MxA protein in the MxA NAb assay. Serum samples from MS patients were evaluated using the common assay protocol described, and the MxA protein was detected by ELISA using either the original (coating and detector) antibody pair or the newly obtained antibody pair (mAb 32 for capture; mAb 16 for detection) and the antibody titers were estimated. Similar results were obtained for all samples using either of the antibody pairs for the MxA ELISA. Antibody-negative samples were negative in both assay systems, and all antibody-positive samples were positive in both assay systems. While NAb titers derived from both assays are broadly consistent, it was noted that titer values for antibody-positive samples were generally higher when using the original antibody pair in comparison with the substituted antibody pair. Despite this, titers derived from each assay were highly correlated, $r=0.994$ ($n=28$) as indicated in Fig. 1.

Comparative validation study using the standardized assay by an independent laboratory

A part of the aims of the project to develop a standardized assay for measuring IFN- β NAb was that the description of the assay and critical reagents for carrying it out are publicly available. Although it was clear from the study described

earlier that all 3 manufacturers of IFN- β products could successfully carry out the assay, it had not been established that it could be transferred to another laboratory for cross-validation of an in-house method. It was, therefore, decided to provide a brief description of the assay method and the mAbs for carrying out the ELISA for measuring MxA protein to an independent laboratory to establish that the assay could be used for cross-validation purposes.

For this, data derived using the standardized assay for assaying a number of serum samples containing different amounts and types of IFN- β NAb were compared with data derived using an RGA (Lallemant and others 2008) for measuring IFN- β NAb for the same samples in the same independent laboratory.

Results from this study clearly showed that the standardized assay was fit for purpose, as results derived using either assay method were essentially comparable (Fig. 2). An assessment of NAb titers for the antibody-positive patient samples using IFN- β 1a and IFN- β 1b as the challenge antigen gave Pearson's correlation coefficient, $r=0.951$ and $r=0.906$ respectively. Therefore, a good correlation was noted between the MxA protein assay and the RGA used in the study.

Discussion

MS is a chronic inflammatory autoimmune disease causing demyelination and formation of lesions in the central nervous system, often with progression of disability over time. It affects ~2.5 million people worldwide (100–150 per 100,000 people in UK) and comprises several disease subtypes. In a majority of patients (85%), the onset of MS begins with a clinically isolated syndrome (CIS), which usually leads to the relapsing-remitting form, RRMS. RRMS is initially characterized by acute exacerbations (or relapses) of neurological dysfunction followed by periods of remissions and further progresses to the SPMS associated with functional impairment and increased morbidity and mortality. A minority of patients (~15%) begin with PPMS with disease

FIG. 2. IFN- β NAb titers for antibody-positive patient sera using the MxA and luciferase NAb assays. **(A)** Using IFN- β 1a as the challenge antigen ($n=52$ in total), the Pearson's correlation, $r=0.951$ for the antibody-positive samples ($n=32$). **(B)** Using IFN- β 1b as the challenge antigen ($n=65$ in total), the Pearson's correlation, $r=0.906$ for the antibody-positive samples ($n=45$).

progression from onset, or progressive relapsing MS characterized by disease progression from onset but with superimposed acute relapses, with or without recovery (Lublin and Reingold 1996).

Despite promising results for newer therapies such as monoclonal antibodies (eg, natalizumab) and oral agents (eg, fingolimod), the favorable safety profile of IFN- β indicates that its use in RRMS as one of the first-line treatment options is likely to continue (Killestein and others 2011). Some neurologists also advocate its use at the CIS stage, as this represents the first opportunity that may be beneficial in the long term (Farrell and Giovannoni 2010; Comi and others 2012; Giovannoni 2012). Evidence suggests that IFN- β acts at

various levels for example, by inhibiting MHC class II expression and the recognition between antigen-presenting cells and T cells, reducing T-cell activation and proliferation, inhibiting development and activation of TH17 cells, inducing regulatory T cells, and modulating cytokine production toward an anti-inflammatory profile (Dhib-Jalbut and Marks 2010).

IFN- β treatment is, however, associated with antibody formation, which reduces the beneficial aspects of therapy. Testing for antibodies is imperative for disease management of these patients. Consistent with the systematic evaluation of immunogenicity advocated by regulatory authorities (EMA 2007), the spectrum of available cell-based assays for

IFNs should provide the means to accurately measure NABs to IFNs. Until recently, the measurement of NABs with a validated CPE assay was considered the gold standard for assessment of NABs in patient sera. Published guidelines recommended an NAB test performed in specialized laboratories using a validated bioassay employing A549 cells challenged with EMC virus for measuring CPE activity using a fixed amount of IFN- β for stimulation and serial dilutions of sera. However, other cell-virus combinations and novel approaches are increasingly being used. The broad choice of available assays and inharmonious methods of analysis have led to disparities in reported NAB incidence, titers, and controversies regarding clinical relevance of Nabs, emphasizing the need for a standardized approach for NAB determination.

A reference assay that uses the same reagents, format, and conditions was, therefore, considered by the EMA as a means of producing harmonious results between different laboratories. Consequently, at the request of the Biologics Working Party (BWP) of the EMA's CHMP, NIBSC coordinated a collaborative study among the manufacturers of IFN- β products in which a blinded panel of serum samples with variable titers (previously determined to be negative, high, medium, and low) from RRMS patients was assessed for NABs using a common "reference assay" based on NAB inhibition of IFN- β -induced MxA protein in the A549 human lung carcinoma cell line (Files and others 1998; Pungor and others 1998). All 3 study participants used the same cell line, experimental conditions, and reagents for the assay to minimize the reagent/procedure-related variance. The constant IFN method was applied, and the assay was performed using first, human fibroblast-derived IFN- β and second, each participants' own commercial IFN- β product. The assay was designed such that the results could be reported as the reciprocal dilution of serum required for the reduction of 10 to 1 LU/mL, following the procedure of Kawade (Kawade 1986; Grossberg and others 2001a, 2001b).

An analysis of the collaborative study data involving a cohort of 62 samples showed there was 100% consistency across the 3 laboratories, in terms of samples that were "negative" for NABs irrespective of the antigen (fibroblast-derived IFN- β or the manufacturer's antigen) used in the assay.

For the other "non-negative" samples, all laboratories identified them as positive for NABs when using either fibroblast-derived IFN- β (NIH, 2nd IS) or IFN- β 1a antigen for challenge. For these preparations, therefore, a 100% concordance was observed between the different laboratories for samples identified as "negative" and "positive" for NABs. However, when an IFN- β 1b product (Betaseron) was used as challenge antigen, 2 samples (which were of low titer when using any of the IFN- β 1a products) did not show quantifiable neutralization, resulting in 3% discordance between the different laboratories in terms of samples identified as "negative" and "positive" for NABs. For all non-negative samples, titers obtained against IFN- β 1b were consistently lower than those obtained against the 2 IFN- β 1a products. In addition, reported titers in Table 1 using in-house (non-standard) assays were generally lower than those seen with MxA assay. Original cohorts were low titer=10–50, mid titer=50–200, high titer>200 and negative samples<10 but the titers from the MxA assay are higher due to the improved sensitivity of the assay. This means that there is a need to re-establish clinically relevant titers with the MxA assay.

Statistical analysis of the data showed that the intra-assay variation based on coded duplicates was low but the magnitude may be dependent on the laboratory and/or antigen. However, the inter-laboratory variation in reported titers was significantly larger than within-assay variation for the duplicate samples. The magnitude of the inter-laboratory variation was larger for IFN- β 1b than for the other antigens. However, when a common antigen was used in the different laboratories as opposed to a different antigen in each laboratory, the inter-laboratory variation was reduced. Correlation coefficients showed that the agreement between estimates for the samples is more consistent when a common antigen (rather than different antigens) is used in the different laboratories. Estimates were more consistent when fibroblast-derived IFN- β or either of the 2 IFN- β 1a antigens was used as opposed to IFN- β 1b. In accordance with this, the neutralization titers were highly concordant when fibroblast-derived IFN- β or the 2 IFN- β 1a products were used as the challenge antigens but often lower when tested against the IFN- β 1b antigen regardless of whether the serum sample was obtained from patients treated with IFN- β 1a or IFN- β 1b. The reason for the variation in titers between IFN- β 1a and IFN- β 1b is not fully understood, although several reasons have been postulated including differences in specific activity, protein folding, glycosylation, and degree of aggregation (Runkel and others 1998; Files and others 2007; van Beers and others 2010).

On the basis of these results, it was concluded that the standardized MxA NAB assay is a suitable assay for detection of NABs against IFN- β . However, based on the limited supply of the natural IFN- β (fibroblast-derived, 2nd IS) standard, its use as a challenge antigen for routine testing was not appropriate. Since statistical analysis undertaken showed high concordance in results when using the IFN- β 1a antigen (CHO derived), the BWP (EMA CHMP) recommended the use of IFN- β 1a antigen as the challenge antigen for the MxA assay as well as the CPE assay. In further studies, evaluation of 2 different MxA antibody pairs generated essentially similar results, showing that either antibody pair can be used for the common assay.

More recently, several novel assays, including RGA, have been described for measuring NABs (Lallemand and others 2008; Lam and others 2008). An RGA assay has also been incorporated in routine clinical practice, in conjunction with assessment of *in vivo* IFN- β bioactivity by measuring MxA gene expression (Farrell and Giovannoni 2010). In the RGA, an IFN-responsive cell line is transfected with a plasmid in which an IFN-inducible promoter controls the expression of an enzyme, for example, firefly luciferase or alkaline phosphatase, which is measured by simple addition of enzyme substrate, often within hours of IFN stimulation. Since the IFN-induced enzymatic activity is directly related to IFN concentration, the presence of NABs inhibits the amount of enzyme produced (Lallemand and others 2008; Lam and others 2008). Using sensitive quantitative reverse transcription-polymerase chain reaction technology to determine the levels of specific IFN-induced mRNA, for example, MxA mRNA or 6–16 mRNA provides assays that require a shorter period of stimulation with IFN- β and can be completed within a day (Bertolotto and others 2007; Aarskog and others 2009; Moore and others 2009) in common with the RGA (Lallemand and others 2008). The potential for high throughput applications is increased if branched DNA

technology is used, as gene expression is measured without the requirement for RNA extraction and cDNA synthesis (Moore and others 2009). However, if different methods are used, it is possible that noncomparable results will be produced. By validating such new procedures or indeed older procedures for example, CPE assays against the standardized assay described here, such inconsistencies should be avoided. The data in Fig. 2 of our study have clearly demonstrated that the NAb assay can be successfully transferred to an independent laboratory for cross-validation studies with an in-house assay. Furthermore, a high correlation was noted between the MxA protein assay and the RGA used in the study.

Use of the standardized NAb assay will enable compliance with the EMA statement "it should be stressed that in case the sponsors use (those) new technologies, they will have to demonstrate how the new assay compares to the agreed upon common assay, so as to guarantee standardisation in the expression of the results in antibody formation and incidence rate" (EMA, 2008).

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