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EFNS TASK FORCE/CME ARTICLE

Guidelines on use of anti-IFN-β antibody measurements in multiple sclerosis: report of an EFNS Task Force on IFN-β antibodies in multiple sclerosis

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Therapy-induced binding and neutralizing antibodies is a major problem in interferon (IFN)-β treatment of multiple sclerosis. The objective of this study was to provide guidelines outlining the methods and clinical use of the measurements of binding and neutralizing antibodies. Systematic search of the Medline database for available publications on binding and neutralizing antibodies was undertaken. Appropriate publications were reviewed by one or more of the task force members. Grading of evidence and recommendations was based on consensus by all task force members. Measurements of binding antibodies are recommended for IFN-β antibody screening before performing a neutralizing antibody (NAB) assay (Level A recommendation). Measurement of NABs should be performed in specialized laboratories with a validated cytopathic effect assay or MxA production assay using serial dilution of the test sera. The NAB titre should be calculated using the Kawade formula (Level A recommendation). Tests for the presence of NABs should be performed in all patients at 12 and 24 months of therapy (Level A recommendation). In patients who remain NAB-negative during this period measurements of NABs can be discontinued (Level B recommendation). In patient with NABs, measurements should be repeated, and therapy with IFN-β should be discontinued in patients with high titres of NABs sustained at repeated measurements with 3- to 6-month intervals (Level A recommendation).

Background and objectives

Interferon (IFN)-β is a first-line therapy for relapsing–remitting multiple sclerosis (MS). In recent years, several publications have concordantly reported that binding antibodies (BABs) and neutralizing antibodies (NABs) occur during treatment with recombinant IFN-β products. The frequencies and titres of anti-IFN-β antibodies vary considerably depending on the IFN-β preparation, the frequency and route of administration, and the type of assay being used. There is no generally accepted standardized assay for measuring BABs and NABs. Clinical studies in patients with MS have demonstrated that when NABs to IFN-β develop, the therapeutic benefits of IFN-β are reduced or abolished.

The objectives of our task force were to: (i) evaluate differences in immunogenicity of IFN-β products, (ii) evaluate the reliability and give recommendations on BABs and NABs assays, (iii) evaluate the impact of NABs on clinical efficacy and give recommendation on the clinical use of measurement of IFN-β antibodies and (iv) review the evidence on prevention of NAB development and the management of patients with NABs.

Search strategy and consensus

The task force systematically searched the Medline database for available information published in English up to September 2004. Key words included: interferon

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beta, multiple sclerosis, immunogenicity, antibodies, binding antibody assays, neutralizing antibody assays. Articles related to this topic from the authors' personal literature databases were also included. For each specific issue at least one member of the task force assessed all published papers and omitted those that did not fulfil given criteria, read and rated the remaining articles according to the guidance for preparation of neurological management guidelines by EFNS Scientific task forces – revised recommendations 2004 (Brainin *et al.*, 2004). Each paragraph of the guidelines was drafted by one member of the task force and circulated to the other members. After appropriate revision the guidelines were finalized and consensus was reached amongst all task force members at meeting.

Immunogenicity

It is entirely predictable that patients treated with long-term recombinant IFN-β produce antibodies against the product. This observation follows in the footsteps of other biological products troubled by the production of antibodies including IFN-α, erythropoietin, factor VIII and human insulin. Understandably, the closer a product is to the species' natural antigen the less likely it is to provoke antibodies.

Immunogenicity of IFN-β products

The three commercially available IFN-β products vary substantially in their immunogenicity. The first licensed product, IFN-β-1b, is produced in *Escherichia coli* and it differs from the natural human product by methionin-1 deletion, cystein-17 to serine mutation, and lack of glycosylation. There is about a 10-fold increase in weight of protein present in a single IFN-β-1b dose compared with the IFN-β-1a versions in order to reach a suitable specific activity level. This is likely to lead to increased aggregation (Runkel *et al.*, 1998) which may enhance its antigenicity. IFN-β-1a in contrast is identical in primary and secondary structure to the native form and is produced in mammalian cells – a system associated with less host cell contaminants. The proportion of patients reported to have neutralizing antibodies range from 2% to >40%, and Table 1 summarizes the data from the initial pivotal relapsing–remitting and secondary progressive placebo-controlled trials. The immunogenicity of Avonex (Biogen Idec, Cambridge, MA, USA) after the initial studies was profoundly reduced for reasons that either are not clear or confidential, but it is possible that the tendency for aggregation was reduced.

Dynamics of NABs

The majority of patients destined to become NAB-positive do so within 6–18 months of treatment. Patients on IFN-β-1b tend to become positive earlier than those on IFN-β-1a. However the percentage becoming positive on IFN-β-1a (Rebif; Serono, Geneva, Switzerland) has been reported to catch up in frequency (Ross *et al.*, 2000; Dubois *et al.*, 2003). It seems that tolerance may occur over the long term during continued IFN-β therapy (Rice *et al.*, 1999; Sorensen *et al.*, 2005). For NAB-positive patients, the probability of reverting to NAB-negative status was significantly higher in patients treated with IFN-β-1b than in patients treated with IFN-β-1a (Rebif), when followed over 36–48 months (Gneiss *et al.*, 2004; Sorensen *et al.*, 2005). Thus it seems that tolerance is an earlier feature with the IFN-β-1b formulation than with the IFN-β-1a formulation. Antibody titre appears to be predictive, with lower NAB titres more likely to revert to a NAB-negative state (Rice *et al.*, 1999; Gneiss *et al.*, 2004).

Influence of dosage and route of administration

It is difficult to separate the relative influence of the (i) dosage frequency, (ii) total weekly dosage and

Table 1 Frequency of NAB-positive patients in initial pivotal placebo-controlled trials

Study	IFN-β product and dosage	Frequency of NAB-positive patients (%)
The IFNB Multiple Sclerosis Study Group (1993)	IFN-β-1b (Betaferon) 250 μg b.i.d.	42
European Study Group on interferon beta-1b in secondary progressive MS (1998)	IFN-β-1b (Betaferon) 250 μg b.i.d.	28
Jacobs *et al.* (1996)	IFN-β-1a (Avonex) 30 μg weekly	22
PRISMS Study Group (1998)	IFN-β-1a (Rebif) 22 μg thrice weekly	24
PRISMS Study Group (1998)	IFN-β-1a (Rebif) 44 μg thrice weekly	13
SPECTRIMS Study Group (2001)	IFN-β-1a (Rebif) 22 μg thrice weekly	21
SPECTRIMS Study Group (2001)	IFN-β-1a (Rebif) 44 μg thrice weekly	15
Clanet *et al.* (2002)	IFN-beta 1a (Avonex) 60 μg weekly	3.3
The North American Study Group on Interferon beta-1b in Secondary Progressive MS (2004)	IFN-beta 1b 250 μg b.i.d.	23
The North American Study Group on Interferon beta-1b in Secondary Progressive MS (2004)	IFN-beta 1b 160 μg/m^2 b.i.d.	33

(iii) method of administration from the present available evidence.

Intramuscular (i.m.) administration of IFN-β-1b once weekly at 250 μg delayed the appearance and reduced the levels of BABs detected by ELISA when compared with the standard regime (Perini *et al.*, 2001). However, NABs were present in 41% of patients treated with repeated i.m. IFN-β-1b and 38% of those treated by the subcutaneous route (The IFNB Multiple Sclerosis Study Group, 1993; The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1996). IFN-β-1a (Rebif) 22 μg subcutaneously (s.c.) once weekly was significantly less immunogenic than three times weekly (Ross *et al.*, 2000; Sorensen *et al.*, 2003). However, IFN-β-1a (Rebif) 22 μg i.m. once or twice weekly was not obviously different to conventional treatment (Perini *et al.*, 2001; Bertolotto *et al.*, 2002). No effect on antibody frequency was seen with two different doses of IFN-β-1b (1.6 and 8 MIU). An increase in NAB frequency was seen with an increased dose of Avonex (Clanet *et al.*, 2002). However, the higher dose of IFN-β-1a (Rebif 44 μg) was associated with a lower proportion of patients developing NABs than on the lower dose (22 μg) in the pivotal relapsing–remitting and secondary progressive studies (see Table 1). The presence of drug in the serum tested could reduce the sensitivity of the assay leading to an apparent but false reduction in antibody positive rates (von Wussow *et al.*, 1989; Ross *et al.*, 2000). Oddly, this Rebif dose effect was not noted in those 2-year placebo patients who were subsequently randomized to either IFN-β-1a (Rebif) 22 or 44 μg and in the EVIDENCE study IFN-β-1a (Rebif) 44 μg three times weekly was associated with a high rate of NAB-positive patients ($>20\%$) (Panitch *et al.*, 2002). Thus doubt exists as to whether Rebif 44 μg really does stimulate less antibody production than 22 μg three times weekly.

Evidence regarding immunogenicity

Overall, the immunogenicity of the recombinant IFN-β appears to be most influenced by the formulation itself although increasing the frequency of injections also appears to be important. The influence of the intramuscular versus the subcutaneous route appears minimal. The effect of different doses is less clear. There is general agreement that the IFN-β-1a (Avonex) is the least immunogenic. There is class I evidence that the majority of patients with two consecutive NAB-positive tests remain NAB-positive for more than 2 years, although a substantial number of patients, who become NAB-positive, may revert to NAB-negative status during continuous IFN-β 1b therapy.

Measurements of binding and neutralizing antibodies

Binding antibodies

A PubMed search using 'binding antibodies assay interferon beta' found that 21 of the 55 articles were relevant for detection of BABs with IFN-β treatment.

Although BABs against IFN-β are induced in a majority of such treated patients, only a subset develop NABs causing loss of bioactivity. Because the method of NAB detection is cumbersome many laboratories use a simpler binding assay for screening purposes and only BAB-positive samples are further analysed by the NAB assay. The different assays can be divided into three basic methods: ELISA, Western blotting (WB), and radioimmunoprecipitation (RIPA) or affinity chromatography (ACA) assays (Table 2).

ELISA methods

The ELISA methods most commonly are direct binding (dELISA) i.e. direct coating of test wells with IFN-β) assays or capture (cELISA) (i.e. coating of test wells with a capture anti-IFN-β antibody) assays. ELISA titres generally correlated only weakly with NAB titres, but BAB-negative samples measured by ELISA reliably predict NAB-negativity. Only one study compared different BAB assays and demonstrated that cELISA is superior to dELISA with respect to specificity for NABs and the correlation between the BAB and NAB titre.

Western blot (WB)

This method gave similar results to the ELISA and had a low false-negative rate when screening for NAB-positivity. BAB titres cannot be calculated using WB.

Affinity chromatography (ACA) and radioimmunoprecipitation assay (RIPA)

The advantage of ACA and RIPA is that the antigen is in solution and, therefore, no epitopes are obscured by binding to a solid phase. Radioactive isotopes usage limits the use of these assays. Affinity chromatography was very sensitive with up to 97% of treated patients being BAB-positive depending on the IFN-β preparation and time on treatment. In the RIPA no NAB-positive sample was negative and there was a moderate correlation with the NAB titre. RIPA state correlated better with MRI lesion burden change than NAB titres (Table 2).

Conclusion and recommendations

There are no existing recommendations on BAB assays. There is class I evidence that IFN-β BAB assays have a very high sensitivity and specificity, and can be reliably

Table 2 Methods used for BAB detection: the ELISA method, the Western Blot (WB) method and the radio-immunoprecipitation (RIPA) or affinity chromatography assays

Method	Type[a] and concentration of IFN	Validation/cut-off	Reference
dELISA	IFN-β-1a or 1b/0.2 μg	Mean + 3× SD of normal	Perini *et al.* (2004)
dELISA	IFN-β-1b/concentration not given	NAB (MxA induction)	Kremenchutzky (2003)
dELISA, cELISA	IFN-β-1a and 1b/1.5 μg/ml	NAB assay	Pachner *et al.* (2004)
dELISA	IFN-β-1a/1b/human IFN-β	Mean + 3× SD of normal	Bellomi *et al.* (2003)
dELISA	IFN-β-1b/1000 IU/ml	NAB assay	Mayr *et al.* (2003)
dELISA	IFN-β/1 μg/ml	2× background of uncoated wells	Slavikova *et al.* (2003)
dELISA	IFN-β-1a/1 μg/ml IFN-β/1.2 μg/ml	Mean + 3× SD of baseline sera	Monzani *et al.* (2002)
dELISA	IFN-β-1a/1 μg/ml	3× OD of background	Vallittu *et al.* (2002)
dELISA	IFN-β-1a and 1b/1 μg/ml	Arbitrary (OD > 0.5)	Fernandez *et al.* (2001)
Affinity chromatography	Radio-labelled IFN-β-1a/3000 cpm	Mean + 3× SD of controls	Ross *et al.* (2000)
cELISA	IFN-β-1a and 1b/10^4 U/ml	Standard curve[b]	Kivisakk *et al.* (2000)
dELISA	IFN-β-1a and 1b/1–312 ng per well	Mean + 2× SD of controls	Antonelli *et al.* (1999)
WB, dELISA	IFN-β-1b/5000 IU per well (ELISA) IFN-β-1b/2.5 μg per gel	NAB assay/detection limit of WB	Deisenhammer *et al.* (1999)
dELISA	IFN-β-1b/2 μg/ml	Control placebo samples/39 binding units	Pungor *et al.* (1998)
dELISA	IFN-β-1a and 1b/1000 IU/ml	Mean of control + 2× SD	Khan and Dhib-Jalbut (1998)
dELISA	IFN-β-1b/1 μg/ml	Mean of control + 3× SD	Ferrarini *et al.* (1998)
RIPA	Radio-labelled IFN-β-1a and 1b/10 μg	NAB assay/mean of control + 3× SD	Lawrence *et al.* (2003)

dELISA, direct enzyme-linked immunosorbent assay; cELISA, capture ELISA.
[a]This column refers to the antigen used in the assay. IFN-β-1a is a recombinant human glycosylated IFN-β preparation whereas IFN-β-1b is not glycosylated.
[b]For the standard curve an internal positive control was used which in turn was compared with a WHO reference antibody (G038-501-572).

used for IFN-β antibody screening before performing a NAB assay (Level A recommendation). Different BAB assays should be evaluated and compared using a large number of serum samples in order to identify the method with the best sensitivity and specificity for NAB detection (Level B recommendation).

Neutralizing antibodies

PubMed was searched using the terms 'neutralizing antibodies interferon beta assay'. Thirty-four of 54 articles covered methods of NAB detection and were included. About 50% of patients who develop BABs also develop NABs. There is no standardized assay for NAB detection and, although the principle of NAB measurement is more or less unique, the materials used vary immensely between different laboratories.

Test systems

Almost all reported NAB assays used cultured cell lines responsive to IFN-β. Test samples are incubated with IFN-β prior to addition of the cells. If the test samples contain NABs, IFN-β is bound, receptor activation is blocked and antiviral proteins will not be induced.

In most cases, one of two different methods are used: either to measure the antiviral effect of IFN-β by challenging the cells with viruses, i.e. the cytopathic effect (CPE), or to measure IFN-β-induced gene products, namely the MxA protein (a specific marker of class I IFNs), i.e. the MxA induction assay. The assays vary with respect to several variables including the cell line, the virus, the IFN-β preparations and dosage, the incubation times, and the methods of MxA detection (Table 3).

A few alternative methods have been reported. Measurement of IFN-β bioactivity showed that NAB-positive patients had significantly lower levels of *in vivo* IFN-β inducible genes at the mRNA and protein level. Although the different markers were not compared with each other directly, MxA mRNA appears to be the most sensitive and specific marker of NABs. Low MxA levels indicated the presence of NABs.

Validation

The MxA induction assay is one of the most thoroughly validated NAB assays and has been used by several authors. It was validated using a CPE assay as gold standard and two different IFN-β preparations for cell stimulation were compared. Most laboratories use internal standards for quality controls. One of these standards is the reference IFN-β antibody (NIH code GO38-501 572) which has a defined neutralizing titre of 1:1700 against 10 Laboratory Units (LU) of human IFN-β. In CPE assays cell viability and viral CPE controls are widely used.

Table 3 Overview of assays for NAB detection showing cell lines, viruses, IFN-β preparations and doses, incubation times, and methods of MxA detection

Type of assay (read-out)	Cells/virus	IFN-β type/concentration	Titre calculation/cut-off for NAB-positivity	Validation/QC	Reference
MxA protein	Human whole blood	Betaferon/1000 IU/ml	MxA increase <22.5 ng/ml	NAB assay (Pungor *et al.*, 1998) Standard curve with rMxA	Kob *et al.* (2003)
MxA RNA	Human PBMC	Avonex, Betaferon, Rebif, therapeutic dose	MxA RNA <132 fg/pgGAPDH	CPE assay (Bertolotto *et al.*, 2000)	Bertolotto *et al.* (2003)
MxA protein (Meditest)	Human lung carcinoma cells (A549)	Betaferon 10 IU	>20 neutralizing units	CPE assay (Pungor *et al.*, 1998)	Polman *et al.* (2003a)
CPE	A549/EMCV	rIFN-β1a and 1b/10 LU	Kawade titre >80	Internal positive and negative controls	Monzani *et al.* (2002)
CPE/MxA protein by FACS	WISH/VSV PBMC	IFN-β-1a/10 experimental units	Titer >20 for bioassay MxA protein $<2\times$ mean of baseline	Not stated	Vallittu *et al.* (2002)
CPE	A549/EMCV	IFN-β type used by the patient/ 3, 10, 100 LU	%Reduction of IFN activity		Ross *et al.* (2000)
CPE	A549/EMCV	Avonex, Betaferon, Rebif/ 10 IU/ml	Kawade titre >20	Internal positive and negative controls	Bertolotto *et al.* (2000)
CPE	A549/EMCV	IFN-β-1a	Kawade/50% CPE		Zang *et al.* (2000)
CPE	WISH/VSV	IFN-β-1b/100 IU/ml	Kawade titre >20	Reference Ab G038-501-572	Kivisakk *et al.* (2000)
CPE	Sindbis virus	IFN-β-1a and 1b/20 U/ml	Kawade/10 LU	Not stated	Antonelli *et al.* (1999)
CPE	FL-cells/Sindbis virus	IFN-β/10 U/ml	6× serum dilution of EC_{50}	Not stated	Kageshita *et al.* (1999)
MxA protein	A549	IFN-β-1b (Betaser) 10 LU	Kawade >20	CPE assay using EMCV	Pungor *et al.* (1998)
CPE	Human fibroblasts/VSV	IFN-β1-a and 1-b/100 U/ml	50% of CPE	Not stated	Khan and Dhib-Jalbut (1998)
CPE	A549/EMCV	IFN-β-1a/10 IU	Kawade, different cut-off values compared with neopterin as bioactivity marker	Internal positive and negative controls	Rudick *et al.* (1998)
CPE	WISH/VSV	IFN-β-1a/10 EU/ml	Kawade/titer >4	Controlled for cell survival	Abdul-Ahad *et al.* (1997)
CPE Cell proliferation	FS-4 fibroblasts/EMCV melanoma cells	Fiblaferon (natural IFN-β) and IFN-β-1a	NU as difference between original and remaining IFN-β activity Relative reduction of proliferation	Not stated	Fierlbeck *et al.* (1994)
Cell proliferation	Daudi cells	IFN-β 10 IU	50% inhibition of proliferation	CPE	Prummer *et al.* (1996)
CPE	FS-4 fibroblasts/EMCV	Fiblaferon (natural IFN-β)/ 100 U/ml	Neutralizing unit = one unit of neutralized IFN-β	Not stated	Dummer *et al.* (1991)
CPE	A549/EMCV	Betaser	Kawade		Redlich and Grossberg (1989)
CPE	Human fibroblasts	Betaser	Serum dilution that reduces activity of 3 to 1 LU/ml/once >100 NU/ml or three consecutive times >20 NU	Reference antibody no G023-902-527	Larocca *et al.* (1989)

CPE, cytopathic effect; FACS, fluorescence activated cell sorter; LU, laboratory unit; NU, neutralizing unit; PBMC, peripheral blood mononuclear cells; EMCV, encephalomyocarditis virus; VSV, vesicular stomatitis virus.

Existing recommendations

The WHO expert committee on biological standardization published informal recommendations on measurement of antibodies to IFN in the technical report series no. 725 in 1985. The reference assay in this international study group used A549 cells and the encephalomyocarditis (EMC) virus but recommended development of simpler assays. For NAB assays the expert committee recommended that the following details should be reported: (i) final concentration of IFN and serum in the reaction mixture; (ii) final volume in the reaction mixture; and (iii) the lowest final dilution of serum tested. For the calculation of the neutralizing titre the Kawade method was recommended. This method calculates the serum dilution that reduces the IFN potency from 10 to 1 LU/ml (Grossberg *et al.*, 2001a,b).

Conclusion and recommendations

Measurements of binding and neutralizing antibodies against IFN-β should be performed in specialized laboratories (Level A recommendation). Measurement of NABs with a validated CPE assay is still the gold standard. It is recommended that A549 cells are used with a fixed amount of IFN-β (the preparation used by the patient) for stimulation and serial dilution of the test sera. The stimulated cells can either be challenged with EMC viruses or MxA production determined. Standard curves should be obtained using increasing amounts of IFN-β until saturation is reached. The NAB titre should be calculated using the Kawade formula (Level A recommendation).

Titres above 20–60 (depending on the IFN-β preparation used in the assay) are associated with a loss of IFN-β bioactivity (class I evidence). As the EMEA currently validates a NAB assay based upon the MxA production of A549 cells (MxA induction assay), it is recommended to use the EMEA protocol. (This recommendation is only based on class IV evidence, but consensus was reached to offer this advice as good practice.) Validation of simpler NAB assay methods is strongly recommended such as the *in vivo* biological response to IFN-β administration (Level A recommendation).

Clinical use of measurements of antibodies against IFN-β

PubMed was searched for 'IFN-β antibodies and multiple sclerosis'. Of 236 articles, 103 were original articles or review articles on antibodies against IFN-β or controlled clinical trials of IFN-β in which measurements of antibodies were performed. For assessment of the impact of NABs we selected randomized controlled trials of IFN-β in MS with blindly analysed NABs and controlled non-randomized studies with blind evaluation of NABs of at least 3-year duration (Table 4). NABs usually appear as low-affinity antibodies in small concentrations and later as higher-affinity antibodies in larger concentrations. Therefore, whereas the effect on antibodies on the biological response to IFN-β may be apparent after 9–12 months, the clinical consequences of neutralizing antibodies are usually not seen until

Table 4 Effect of NABs to IFN-β on clinical and MRI outcomes in MS therapeutic trials[a]

Study	IFN-β product	No. of patients receiving IFN-β	Duration	Relapse rate[b]	MRI activity[b]	Disease progression[b]	MRI severity[b]	Class (primary end-point)	Class (NAB evaluation)[c]
The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group (1996)	Betaferon	249	3 years	+ (*)	+ (ns)	+ (ns)	+ (ns)	I	I
Rudick *et al.* (1998)	Avonex	158	2 years	– (ns)	+ (ns)	+ (ns)	ND	I	II
PRISMS Study Group (2001)	Rebif	373	4 years	+ (**)	+ (***)			I	I
SPECTRIMS Study Group (2001)	Rebif	413	3 years	+ (ns)	ND	– (ns)	ND	I	I
Durelli *et al.* (2002)	Betaferon/Avonex	188	2 years	+ (ns)	ND	ND	ND	III	II
Panitch *et al.* (2002)	Rebif/Avonex	767	48 weeks	+ (ns)	+ (***)	ND	ND	I	III
Polman *et al.* (2003a)	Betaferon	360	3 years	+ (**)	ND	+ (ns)	+ (**)	I	I
Sorensen *et al.* (2003)	Betaferon/Avonex/Rebif	541	5 years	+ (**)	ND	+ (ns)	+ (ns)	III	I
Kappos *et al.* (2005)	Avonec	802	4 years	+ (*)	+ (*)	+ (*)	+ (ns)	I	I

[a]See text for selection of trials and for definition of the different clinical and MRI outcomes.

[b]+, Outcome worse in the NAB-positive group than in the NAB-negative group; –, outcome better in the NAB-positive group than in the NAB-negative group; ND, not done. Statistical significance is given in parentheses (ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

[c]I, trials of sufficient duration (≥3 years) and blind evaluation of NAB status; II, trials of less sufficient duration (2–3 years) and blind evaluation of NAB status; III, trials of inappropriate duration (<2 years) and/or no blind evaluation of NAB status.

12–18 months after the start of IFN-β therapy. Hence, only trials of sufficient duration (≥3 years) and blind evaluation of NAB status were graded as class I evidence for effects of NABs. Trials of less sufficient duration (2–3 years) and blind evaluation of NAB status were graded as class II evidence, and trials of inappropriate duration (< 2 years) and/or no blind evaluation of NAB status were classified as class III evidence regarding clinical effects of NABs.

It has been common to classify patients as NAB-positive after two consecutive serum samples containing NABs in a titre of 20 or more (once positive, always positive) (The IFNB Multiple Sclerosis Study Group, 1993; The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1996). The use of this approach will invariably result in an underestimation of the clinical consequences of NABs in studies of 2 years or shorter. Therefore, methods that account for switches between NAB-positive and NAB-negative periods (interval analysis) theoretically provide a more accurate assessment of the clinical impact of NABs on relapse rate and MRI activity (Sorensen *et al.*, 2003).

Effect of NABs on relapses

In the pivotal phase III trial of IFN-β-1b, NAB-positive patients had significantly higher annual relapse rates during years 2 and 3 (1.08) than NAB-negative patients (0.56) ($P < 0.01$) and equivalent to patients given placebo (1.06) (The IFNB Multiple Sclerosis Study Group, 1993). In the pivotal phase III IFN-β-1a (Rebif) trial, no significant difference in relapse rate was seen over the study duration of 2 years between NAB-positive and NAB-negative patients, using the 'anytime positive, always positive' method, (PRISMS Study Group, 1998). But in the 2-year extension phase, NABs caused a clear reduction in efficacy on relapses (PRISMS Study Group, 2001).

There was no correlation observed between NAB status using the 'anytime positive, always positive' method and relapse rate in patients treated for 2 years in the pivotal phase III trial of IFN-β-1a (Avonex) (Rudick *et al.*, 1998).

In the secondary progressive Betaferon (Schering, Berlin, Germany) study (Polman *et al.*, 2003a), the 'once positive, always positive' method showed that NAB-positive patients had a 45% increase in relapse rates ($P = 0.009$) when they switched to being NAB-positive compared with their prior NAB-negative state. However, relapse rates in NAB-positive patients showed only a trend ($P = 0.07$) of increase when the interval analysis method was applied. Higher titres seemed to reduce the treatment effect more. In the secondary progressive IFN-β-1a (Rebif) study, using the 'anytime positive, always positive' method, the relapse effect was reduced in NAB-positive patients 44 μg such that the difference between NAB-positive and placebo patients was no longer statistically significant (SPECTRIMS Study Group, 2001). The INCOMIN study [an open randomized study comparing IFN-β-1b (Betaferon) with IFN-β-1a (Avonex)] reported that the frequency of NABs in patients with relapses was a little higher, using the 'anytime positive, always positive' method, than in patients without relapses (Durelli *et al.*, 2002). The EVIDENCE study [open randomized comparison of IFN-β-1a (Rebif) with IFN-β-1a (Avonex)] continued only for 48 weeks making this study inadequate for assessing the clinical impact of NABs (Panitch *et al.*, 2002).

In a Danish nationwide prospective study, NABs were measured blinded for up to 60 months in 541 randomly selected patients (Sorensen *et al.*, 2003). The presence of NABs had significant effect on relapse rates using the 'anytime positive, always positive' method. In NAB-positive periods the annual relapse rate increased more than 50% compared with NAB-negative periods. Comparing NAB-positive with NAB-negative patients the median time to first relapse was significantly reduced by 244 days ($P = 0.009$), and the proportion of relapse-free patients was significantly lower ($P = 0.0064$).

Effect on MRI outcomes

The pivotal study of IFN-β-1b (Betaferon) showed significantly more enlarging lesions in NAB-positive patients compared with NAB-negative patients during years 2 ($P = 0.03$) and 3 ($P = 0.01$) (The IFNB Multiple Sclerosis Study Group, 1993; The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1996). In the PRISMS study, there was a trend over the first 2 years towards more MRI activity in NAB-positive patients (PRISMS Study Group, 1998). Over 4 years (PRISMS Study Group, 2001) NAB-positive patients compared with NAB-negative patients had a nearly fivefold increase in the median number of T2 active lesions ($P < 0.001$), and a 17.6% increase compared with an 8.5% decrease in MRI burden of disease ($P < 0.001$). In the pivotal study of IFN-β-1a (Avonex), a trend was seen towards more gadolinium-enhanced lesions in NAB-positive patients ($P = 0.062$) (Rudick *et al.*, 1998). Secondary progressive patients on IFN-β-1b (Betaferon) showed a higher percentage increase from baseline in T2 lesion volume in NAB-positive patients compared with NAB-negative patients ($P = 0.004$) (Polman *et al.*, 2003a). Despite the short duration of the EVIDENCE study it was apparent that

NAB-positive patients had more T2 active lesions than NAB-negative patients ($P = 0.0004$) (Panitch *et al.*, 2002).

Effect of NABs on disease progression

None of the randomized studies have been powered to detect a NAB effect on disease progression. In the pivotal IFN-β-1b study (Betaferon), however, a strong trend was seen towards an effect of NABs on the mean change in EDSS from baseline in the third year ($P = 0.083$) (The IFNB Multiple Sclerosis Study Group, 1993; The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group, 1996). NAB-positive patients on high-dose Rebif showed a near significant ($P = 0.051$) increase in the mean number of EDSS progressions compared with NAB-negative patients in the 4-year PRISMS trial (Rice GPA, personal communication, poster presentation, ECTRIMS, 2000). The Danish study also showed a strong trend towards a higher mean EDSS in NAB-positive patients compared with NAB-negative patients at month 42 and 48, and towards shorter time to disease progression in NAB-positive patients ($P = 0.10$) (Sorensen *et al.*, 2003). Neither the SPECTRIMS study [IFN-β-1a (Rebif)] (Li *et al.*, 2001), nor the study of IFN-β-1b (Betaferon) in secondary progressive patients found a significant difference between NAB-positive and NAB-negative patients (Polman *et al.*, 2003b). Recently it was shown in a long-term follow-up study of patients taking either Avonex 30 μg or 60 μg weekly that NAB positive patients showed more progression on EDSS than NAB negative patients (Kappos *et al.*, 2005).

Safety issues

The presence of NABs has not been reported to be associated with adverse events or toxicity.

Conclusions and recommendations regarding the clinical use of NAB measurements

It is recommended that patients treated with IFN-β are tested for the presence of NABs at 12 and 24 months of therapy (Level A recommendation). Measurements of NABs can be discontinued in those patients remaining NAB-negative during this period but should be resumed if disease activity increases (Level B recommendation). There is class I evidence that the presence of NABs significantly hampers the effect of IFN-β on the relapse rate and on both active lesions and burden of disease seen on MRI. In patients with NABs, NAB measurements should be repeated at intervals of 3–6 months and therapeutic options should be re-evaluated (Level A recommendation). Therapy with IFN-β should be discontinued in patients with high titres of NABs (e.g. titres >100 in patients using IFN-β-1b) sustained at repeated measurements with 3- to 6-month intervals (Level A recommendation).

Prevention and treatment of NABs

Steroids

Short pulses of steroids have been demonstrated to be safe, well tolerated and clinically effective for patients with MS. A clinical trial randomly assigned 161 patients to receive IFN-β-1b, either alone or in combination with 1 g of methylprednisolone (MP) administered monthly intravenously (i.v.) (Pozzilli *et al.*, 2002). Using an MxA assay, there was a significant reduction in NAB development in patients treated with MP, when defined as titres ≥1:20 on one occasion but not when defined as twice consecutively positive. There was no difference in the frequency of patients that developed NABs at high titres ($>$1:100). The development of NAB-positivity was significantly delayed in the MP group [Kaplan–Meyer analysis, log-rank test ($P < 0.05$ by month 6 of therapy)]. These results suggest that the chronic administration of steroids prevents or delays the formation of NABs, but does not reduce the titre in NAB-positive patients.

Other immunosuppressive agents

A number of clinical trials have been performed with either RR-MS or SP-MS patients to evaluate the use of IFN-β in association with an immunosuppressive agent (Patti *et al.*, 2001; Calabresi *et al.*, 2002; Fernandez *et al.*, 2002). However, the NAB data originating from these small studies is inconsistent and does not allow any definitive conclusion as to whether additional immunosuppression reduces NAB formation.

Switching IFN-β preparations or increasing the dose of IFN-β

One of the possible strategies to overcome the formation of NABs in MS could be the switching from one preparation of IFN-β to another, but unfortunately many studies have showed that NABs are cross-reactive between IFN-β-1a and IFN-β-1b (Khan and Dhib-Jalbut, 1998; Bertolotto *et al.*, 2000; Kivisakk *et al.*, 2000). Thus, switching to an alternative IFN-β preparation is not of clinical benefit for a NAB-positive MS patient.

It is well known that the amount of antigen to which an individual is exposed influences the magnitude of the immune response and that very large dose or repeated

administrations of small amounts of antigen are often inhibitory in the production of antibodies (Dresser and Mitchison, 1968). However, at the present time, there is no evidence that increasing of the dosage of IFN-β is of benefit to NAB-positive patients.

Other strategies

Plasmapheresis and immunoglobulins (IgG) might be considered as possible procedures to diminish NAB generation. Presently, the effects of the IgG on blocking antibody production are widely accepted in patients with autoimmune diseases. However, IgG and plasmapheresis do not affect memory plasma cells (Rudick and Goodkin, 1999). Therefore, the concomitant administration of IgG or plasmapheresis may be useful in eliminating circulating NABs, but it would not be expected to impede the production of NABs once it has been triggered.

Conclusions and future considerations on prevention NABs formation

Limited evidence is available on managements that reduce NAB formation to IFN-β in MS. Monthly 1 g i.v. MP administration has been revealed to be safe and able to minimize the formation of NABs over time (Level C recommendation). However, no effect has been observed in reducing the amplitude of NABs titres once NABs have been formed. Further studies are warranted to strengthen these results and to expand our knowledge in such an intriguing matter.

Principal recommendations regarding measurements of antibodies against IFN-β and the clinical use of NAB measurements

- BAB assays can be reliably used for IFN-β antibody screening before performing a NAB assay (Level A recommendation).
- Measurements of binding and neutralizing antibodies against IFN-β should be performed in specialized laboratories (Level A recommendation).
- Measurement of NABs should be performed with a validated CPE assay or MxA production assay using serial dilution of the test sera. The NAB titre should be calculated using the Kawade formula (Level A recommendation).
- Tests for the presence of NABs should be performed at 12 and 24 months of therapy (Level A recommendation).
- Measurements of NABs can be discontinued in those patients remaining NAB-negative during this period but should be resumed if disease activity increases (Level B recommendation).
- In patient with NABs, measurements should be repeated after 3–6 months (Level A recommendation).
- Therapy with IFN-β should be discontinued in patients with high titres of NABs sustained at repeated measurements with 3- to 6-month intervals (Level A recommendation).

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