Research paper

Neutralizing antibodies against IFN beta in patients with multiple sclerosis: A comparative study of two cytopathic effect tests (CPE) for their detection

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Neutralizing antibodies (NABs) against IFN beta should be measured in specialized laboratories, using a test of inhibition of the cytopathic effect (bioassay or CPE test), based on the capacity of IFNß to block the infection of live monolayer-cultured cells by a virus, depending on the presence or absence of NABs. The European Federation of Neurological Societies (EFNS) considers this assay to be the gold standard. However, the various different ways to perform this assay complicate comparison of the results between laboratories. The World Health Organization (WHO) has published several recommendations to perform this assay using the A549 cell line and the murine encephalomyocarditis virus (EMCV). In order to validate the results previously obtained in our laboratory with HEP2/VSV, we undertook a comparative analysis of the two bioassays, HEP2/VSV and A549/EMCV, to assess whether the use of different cell lines and viruses influences sensitivity. We also calibrated the A549/EMCV assay with a reference IFNß. Our results confirm that the bioassay with HEP2/VSV is as sensitive as the assay with A549/EMCV and that a significant association and correlation exist in the results between both assays. Thus, past results with HEP2/VSV in our laboratory could be comparable with those obtained with A549/EMCV in both our laboratory and others.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (SNC) involving numerous pathophysiological mechanisms, including inflammation, demyelinization, axonal damage and damage repair. MS is characterized by the presence of focal lesions in the white and grey matter of the SNC, called plaques, in which there is loss of myelin and a certain degree of axonal degeneration (Trapp et al., 1998).

Interferon beta (IFNß) is a first line drug for the treatment of patients with clinically defined MS. Following the administration of this immunomodulating drug, patients experience an improvement, with a reduction in the frequency and severity of the relapses, and the number and volume of MR lesions, and progress on the physical disability scale (Panitch et al., 2002). Nevertheless, an important number of patients fail to respond adequately to this treatment or else respond suboptimally, thereby reducing the efficacy of the drug.

A few patients treated with IFNß develop antibodies against this molecule, as the IFNs are glycoproteins with an antigenic capacity, despite being identical molecules to the endogenous molecule (Rudick et al., 1998a,b; Sorensen et al., 2005). An association has been found between the lack of response to IFNß and the presence of neutralizing antibodies (NABs) when these are permanently present at high titres (Sorensen et al., 2006; Hesse and Sørensen, 2007). One study by our group found that the time to appearance of the first relapse after treatment was greater in NAB-negative patients (Fernandez-Fernandez et al., 2006).

Two types of antibodies exist, depending on the antigenic determinants they recognize. The most common type is

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binding antibodies (BABs), which are specific to IFN epitopes not found at the area of interaction with its receptor (Pachner et al., 2003a,b, 2005). The second type, NABs, is specific for antigenic determinants found at the binding site of IFNß and its receptor, blocking this interaction. NABs are therefore associated with loss of bioactivity of the molecule, reducing its therapeutic effects (Bertolotto et al., 2002; Sorensen et al., 2003).

For the detection of antibodies in serum, including both BABs and NABs, the European Federation of Neurological Societies (EFNS) recommends the use of immunoprecipitation assays or capture ELISA, which are rapid and easy to perform, and advises against the use of Western Blot or direct ELISA methods due to their high percentage of false positive or false negative results (Sørensen et al., 2005).

The use of capture ELISA enables detection of the presence of antibodies against IFNβ, but it is not possible to determine whether they are NABs or not. It is therefore necessary to carry out a functional study. The EFNS recommends that NABs should be measured at 12 and 24 months after the onset of treatment, in specialized laboratories, using a test of inhibition of the cytopathic effect (Bioassay) or the MxA inhibition assay (Sørensen et al., 2005). The bioassay is based on the capacity of IFNß to block the infection of live monolayer-cultured cells by a virus, depending on the presence or absence of NABs. This is done by confronting serial dilutions of serum from the patient against the same concentration of IFNß that are added to the cellular monolayer and, after virus infection, the percentage of live cells is measured.

Many variables may affect the method and, therefore, comparison of the results. These include the dilutions of the initial amount of serum and IFNß, the virus titre, incubation times and the interpretation of the results. In addition to these variables, the bioassay has also been performed in different laboratories using different cell lines and different viruses. The World Health Organization (WHO) recommends performing this method with the A549 cell line (lung cancer cells), as this line does not change its sensitivity to interferons during the various steps, is easy to manage and grows quickly. Likewise, the WHO also recommends the use of the murine encephalomyocarditis virus (EMCV), due to the fact that it is stable, easily cultured, has a wide range of hosts and is relatively non-pathogenic to man (WHO. Expert Committee on Biological Standardization).

For years, different laboratories, including ours, have performed the bioassay using the HEP2 cell line and the vesicular stomatitis virus (VSV). This cell line is also stable, sensitive to IFNß and easy to manage in culture but in order to obtain results that can be compared between different laboratories we changed the cell line and the virus in the bioassay, in accordance with the WHO recommendation. The aim of this study, therefore, was to determine whether the results obtained with the HEP2 cell line and the VSV, since starting measurement of NABs to IFNβ, correlated well with those obtained with effect from 2007 with A549/EMC. Accordingly, we undertook a comparative analysis of the two bioassays, in order to assess whether the use of different cell lines influences sensitivity. In addition, we calibrated and validated the A549/EMCV assay in accordance with the WHO recommendations.

2. Materials and methods

2.1. Patients

The study included 69 patients with MS. Peripheral blood was drawn from all of them 24 h after the last injection of IFNß. All the patients had been receiving IFNß treatment for at least one year, 19 (27.53%) with IFNß-1b (Betaferon®) and 50 with IFNß-1a: 25 (36.23%) with Avonex® and 25 (36.23%) with Rebif®.

2.2. Reagents

The cells and the virus for the bioassays were obtained from the American collection (ATTC: American Type Culture Collection): HEP2 (No: CCL-23)/VSV (No: VR1421) and A549 (No: CCl-185)/EMCV (No: VR-129B) as well as the A929 line (No: CCL-1) for replication of the EMCV virus.

Prior to doing the bioassay, the patient sera were decomplemented at 56 °C for 30 min. To determine the amount of virus to add in each bioassay a prior titration was performed, such that 50% cell death was obtained between IFNß dilutions 16 and 32.

2.3. HEP2/VSV assay

The capacity of the serum samples to neutralize the IFNß antiviral capacity was determined. For this, 18,000 cells of HEP2 per well were sown on flat-bottom 96-well plates in 100 µl of RPMI medium supplemented with 2% foetal bovine serum. These were incubated at 37 °C and 5% CO_2 for 24 h. Serial dilutions of patient serum (1/5-1/640) were incubated for 1 h against a constant concentration of IFNß and later added to the HEP cell culture, sown the day before. Because the different commercial preparations have different biological activities, we worked with 10 final laboratory units (LU)/ml for IFNß-1a and 50 LU/ml for (IFNß-1b). After 24 h of incubation, 100 µl of previously titrated VSV were added (except to the cell control). This was again incubated for 24 h at 37 °C and 5% CO_2. Each well included a viral control, to which no IFNß or serum was added, to control the cytopathic efficacy of the virus. Likewise, a cell control was included, which did not contain virus, IFNß, or serum, to verify the presence of good cell viability, and an IFNß standard (serial dilutions: 1/1 to 1/128). To observe inhibition of the cytopathic effect, the cells were stained for 10 min with crystal violet vital staining diluted 1/5 and the absorbance read at 630 lambdas using a spectrophotometer. The calculation of the antibody titres is explained below.

2.4. A549/EMCV assay

For this part of the bioassay, 30,000 cells per well of the A549 line were sown on 96 well plates in 100 µl of DMEM medium supplemented with 2% foetal bovine serum. These were incubated at 37 °C and 5% CO_2 for 24 h. Serial dilutions of the patient sera (1/10-1/640) were incubated for 1 h against the same concentration of IFNß (final 10 LU of each of the commercially available IFNß) (McKay et al., 2006) and the same dilutions without IFNß were placed in another column to measure the presence of endogenous IFN in the patient

serum. These dilutions were added to the A549 cell culture, sown the day before. Each plate included a viral control which did not contain IFNß, a cell control which did not contain virus, and a standard of IFNß (serial dilutions: 1/1 to 1/128). After 24 h incubation, 100 µl of the EMCV virus diluted in DMEM medium without foetal bovine serum were added (except for the cell control, to which only DMEM medium without serum was added). After another 24 h of incubation at 37 °C and 5% CO_2, the cells were stained for 10 min with crystal violet. The plates were washed to eliminate excess staining and, once dry, the absorbance was read at 630 lambdas in a spectrophotometer. The antibody titres were calculated using the Kawade formula: $t = f(n-1)/9$ where f is the reciprocal of the antibody and n the IFNß concentration in LU/ml measured on the day of the assay (Kawade et al., 2003). The WHO and other authors (Grossberg and Kawade, 1997; Grossberg et al., 2001) recommend expressing the NAB titre as the dilution of serum that reduces the activity of IFNß-1a or IFNß-1b from 10 LU/ml to 1 LU/ml [TRU/ml (ten-fold reduction unit/ml)].

2.5. Statistical analysis

Comparison of the positive and negative results obtained with both methods was done with a chi-square test and the Kappa test was used to determine the agreement between the two types of bioassay. Numerical variables were compared with a correlation analysis.

3. Results

3.1. Precision of the HEP2/VSV and A549/EMCV assays

The precision of each assay was evaluated using the repeatability or intra-assay variation, testing the same serum 10 times in the same assay. The reproducibility or inter-assay variation was determined by measuring the absorbance of the same serum in 10 assays done on different days. The results obtained for each assay, with IFNß-1a and IFNß-1b, are shown in Table 1.

3.2. Comparison of the results obtained with the two types of bioassay

Although no consensus exists concerning the criteria for positivity, a patient is accepted to be positive when the titre is ≥20 TRU/ml, as this level of antibodies has been associated with a reduction in the biological activity of IFNß (Rudick et al., 1998a,b; Bertolotto and Gilli, 2003; Phillips et al., 2004). Of the 69 patients studied, 27 (39.1%) were positive with the A549/EMCV assay and 30 (43.5%) with the HEP-2/VSV assay. Chi-square analysis of the results obtained with both assays showed that the methods were not independent ($P < 0.001$). The Kappa test showed a significant association between the two methods ($P < 0.001$) (Table 2).

Of the patients who were positive in the assay with the A549/EMCV line, 3 had low levels of NABs (< 180 TRU/ml) and 24 medium-high levels (≥180 TRU/ml). The measurement performed with the HEP-2/VSV assay showed 6 patients with low levels and 24 with medium-high titres.

Comparison of the NAB levels obtained using the Pearson test showed a significant correlation between the two methods ($P < 0.001$) and $R = 0.904$ (Fig. 1).

3.3. Calibration of the A549/EMCV assay for the measurement of NABs

For the calibration of the A549/EMCV assay, the NIAID (National Institute of Allergy and Infectious Diseases) was requested to donate IFNß (no. Gb23-902-531) so that we could calibrate the standards of IFNß-1a and IFNß-1b. The dose-response curves were made in five tests done on different days. One laboratory unit (LU/ml) is the concentration of IFNß that protects 50% of the A549 cells from the cytopathic effect of the virus (CE50). For each IFN the value of CE50 was determined and, applying the Kawade formula, the equivalence was measured between LU/ml and IU/ml. Thus, 5 LU/ml of IFNß-1b was found to be equivalent to 1 IU/ml and 2 LU/ml of IFNß-1a equivalent to 1 IU/ml (Fig. 2).

4. Discussion

Several different authors have shown that NABs reduce the therapeutic efficacy of IFNß. Measurement of these antibodies in patients is therefore recommended to be done 12 to 24 months after starting treatment with IFNß (Sørensen et al., 2005). Bioassay, based on the antiviral capacity of IFNß, is the method of choice for the measurement of NABs. However, comparison of the results obtained at different laboratories is currently difficult, for several reasons, such as the cell lines and virus used, incubation times, the bioactivity of IFNβ and the interpretation of the results. Because of a lack of any standardized assay for NAB detection, the WHO expert committee on biological standardization published several recommendations on the measurement of NABs, using the A549 line and the EMCV (WHO Expert Committee on Biological Standardisation).

For many years in our laboratory, we performed the bioassay using the HEP2 cell line and the VSV. In order to perform the method according to the WHO recommendations, we started using the A549 cell line and the EMCV, with the calculation of the neutralizing titre obtained using the Kawade method (Kawade et al., 2003).

Table 1
Precision and reproducibility of the assays.

		Inter-assay variation	Intra-assay variation
HEP2/VSV	IFNß-1a	10.3%	4.2%
	IFNß-1b	9.4%	3.5%
A549/EMCV	IFNß-1a	9.1%	3.7%
	IFNß-1b	12.93%	3.2%

Table 2
Positive and negative results obtained with the two types of bioassay.

		HEP2/VSV assay		Total
		Negative	Positive	
A549/EMCV assay	Negative	39	3	42
	Positive	0	27	27
	Total	39	30	69

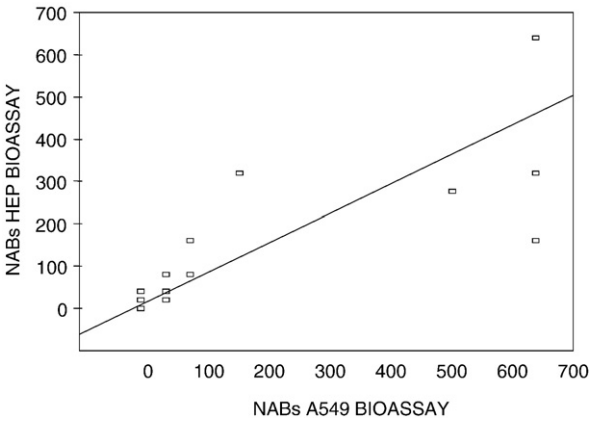

Fig. 1. Comparison of the NAB levels obtained with the two types of bioassay (Pearson test).

The first part of the study involved a comparative analysis between the two types of bioassay. For the purpose of routine clinical use in MS patients treated with IFNß, we determined the precision and sensitivity of the two assays. For the intra-assay variation we measured the same serum 10 times, obtaining variations of 3-4% for IFNß-1a and IFNß-1b. The inter-assay variations were made on 10 different days, with results of 10-12% for IFNß-1a and IFNß-1b. These variations are acceptable for a biological test, especially considering the great variability of NAB assays reported (Sørensen et al., 2005).

The statistical tests showed that the two methods were not independent and that a significant association between the two assays exists. Comparison of the positive or negative results showed just three discordant samples, which were positive with HEP2/VSV and negative with A549/EMCV. Analysis of the titres of these samples shows that the lack of concordance between the two methods occurs in samples with low antibody titres, as all three positive samples for the HEP2/VSV assay had titres of 20 TRU/ml. Thus, the discordance between the two methods is probably due to the fact that the samples contained titres at the limit of positivity and the presence of antibodies should be reassessed 6 months later. This situation has also been seen in other laboratories

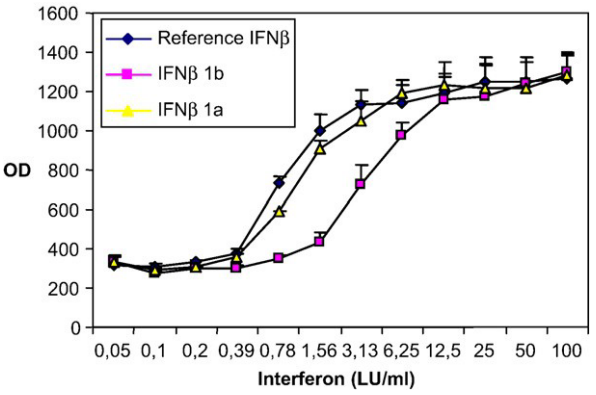

Fig. 2. Dose-response curves of IFNß-1a, IFNß-1b, and reference IFN to determine the dose of IFN that protects 50% of the cells against the cytopathic effect of the virus. OD: optical density; LU: laboratory unit.

(Massart et al., 2007). Nevertheless, the EFNS recommends withdrawal of treatment with IFN if the patient has permanently high antibody titres (Sørensen et al., 2005), so the difference detected in our samples with low NAB titres would not interfere in the management of the patients treated with IFNß. In the samples with high antibody titres, no differences were found in any of the samples analyzed between the two methods.

Because of the WHO recommendations, our laboratory, which previously used the HEP2/VSV assay, changed to the A549/EMCV assay. We therefore calibrated the A549/EMCV assay with reference IFNß from the NIAID, as recommended by the WHO. We made some dose-response curves with the reference IFNβ and with IFNß-1a and IFNß-1b and established the equivalence between LU and IU. According to our data, 5 LU/ml of IFNß-1b are equivalent to 1 IU/ml and 2 LU/ml of IFN beta 1a are equivalent to 1 IU/ml. These results show that a greater amount of IFNß-1b than IFNß-1a is necessary to achieve the same effect, as it has less activity (Runkel et al., 1998; Antonetti et al., 2002).

The results obtained in this study enable us to conclude that the bioassay with the HEP2 or the A549 cell lines introduces no variation in the sensitivity of the method for the measurement of neutralizing antibodies in patients' serum, neither with the use of the vesicular stomatitis virus nor the encephalomyocarditis virus. Our results confirm that the bioassay with HEP2/VSV is as sensitive as the assay with A549/EMCV and that a significant correlation exists between both assays; accordingly, both can be used for the follow-up of patients treated with IFNß. However, we understand the need for a standardized assay for NAB detection to allow comparison of the results from different laboratories and, in accordance with the WHO recommendations, we will in future perform our bioassays only with the A549 cell line and the EMCV.

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