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Assessment of the sensitivity of anti-interferon binding and neutralizing antibody assays in patients with relapsing -remitting multiple sclerosis under interferon- beta treatment- A comparative study.

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ABSTRACT: Interferon beta (IFNβ) is a first line disease-modifying treatment for the management of relapsing-remitting multiple sclerosis. IFNβ preparations may elicit an immune response reflected by the development of binding- (Babs) and neutralizing- antibodies (Nabs). The detrimental effect of Nabs is depicted by attenuation of treatment effect and as a result the deterioration of clinical and radiological parameters. The incidence and titers of Nabs vary by the preparation of IFNβ used, dose, frequency and route of administration, treatment duration and type of assay being used. In this study we aimed to assess the sensitivity of the binding and the neutralizing assays in patients with relapsing-remitting multiple sclerosis under treatment with interferon-beta. The assessment of the results suggests that Western blot assay is more sensitive than ELISA for the detection of binding antibodies. The evaluation of CPE and Real-time-PCR results indicates that they possess similar sensitivity. However, CPE assay remains the gold standard method for the detection of neutralizing antibodies, based on the World Health Organization. Our results indicate that interlaboratory studies are needed for a commonly accepted assay that might reflect a reliable and cost-effective procedure for the Babs and Nabs detection in MS patients under IFNβ treatment.

Key Words: Neutralizing antibodies, Binding antibodies, Interferon beta, Multiple sclerosis.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system affecting young adults. The therapeutic strategies include treatment of acute relapse, symptomatic- and the disease-modifying- treatment. Interferon beta (IFNβ) is a first line disease-modifying treatment available for the management of relapsing-remitting multiple sclerosis (RRMS) since 1993[1]. The beneficial effect of IFNβ in MS is exerted by the reduction of disease activity as measured by decreased relapse rate, delayed progression of disability and reduced MRI activity in relapsing-remitting MS[1,2,3]. Two types of recombinant IFNβ are commercially available: IFNβ-1b (subcutaneous IFNβ-1b, Betaferon®: Schering, Berlin, Germany and subcutaneous IFNβ-1b, Extavia®: Novartis, Basel, Switzerland) and IFNβ-1a (intramuscular IFNβ-1a, Avonex®: Biogen, Cambridge, USA and subcutaneous IFNβ-1a, Rebif: Serono®, Genevè, Switzerland). The formulations of interferon-beta-1a and interferon-beta-1b show immunogenic properties and treated patients may develop binding (Babs) and neutralizing (Nabs) antibodies to these products. Nabs are a subset of Babs that prevent IFNβ from binding successfully to its receptor and in consequence blocking all the biologic effects of IFNβ[4,5]. In patients exhibiting Nabs an attenuation of treatment effect[6] and a

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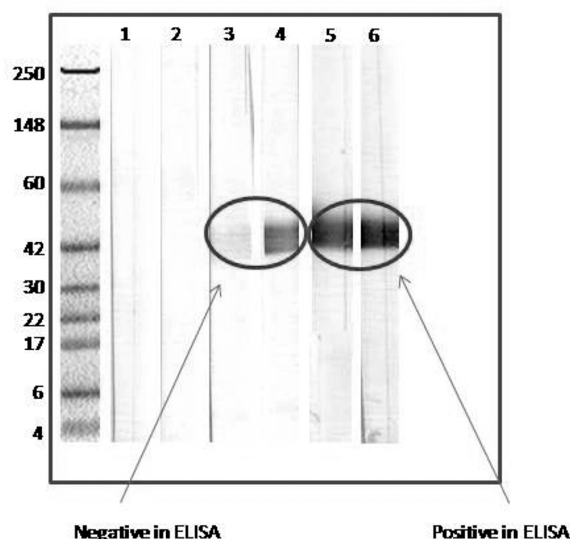

Figure 1. Western blot assay. The first strip is used as a molecular weight marker. The adjacent strips belong to patients demonstrating (strips 3, 4, 5 and 6) samples with a band at the molecular weight level of the mouse positive control band were it is considered positive. Strips 1 and 2 are negative.

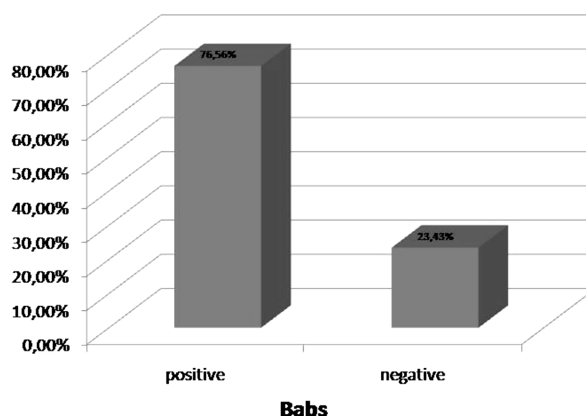

Figure 2. Diagrammatic presentation of Western blot assay results in blood samples which were characterized negative using the cELISA method. The first bar represents the positive and the second - one the negative samples.

decrease in the IFN inducible proteins (i.e. myxovirus protein A-MxA, neopterin, β2-mocroglobulin) have been reported[7,8]. Considering the disease activity, their presence is associated with deterioration of clinical and radiological parameters. The incidence and titers of Nabs vary by the preparation of IFNβ used, dose, frequency and route of administration, treatment duration and type of assay being used[1,2,4,9-12].

Two types of antibodies, ie, Babs and Nabs, indicate the antigenicity of IFNβ. Differences between detection assays, IFNβ subtype used in the assays, the timing of sampling, and differing product-specific cut-off levels for defining titers as negative, low, or high are all important considerations when measuring and interpreting Nab measurements.

In the present study we aimed to assess the sensitivity of two binding- and two neutralizing- available assays in patients with relapsing-remitting multiple sclerosis under treatment with interferon-beta.

METHODS

2.1. Study design and patients

A total of 124 patients were included in the study. We focused on a sub-group of 64 patients who exhibited neutralizing activity as indicated by the cytopathic effect assay without the presence of Babs based on the capture ELISA assay. The blood samples of these patients were further assessed with Western blot assay for the detection of binding antibodies, in order to compare the sensitivity of the two assays. In addition, we examined 30 blood samples positive for Babs as detected using cELISA, by Western blot assay for further confirmation of our results.

In the second part of the study, we performed real time PCR assay for MxA using 18s as a reference gene in 15 blood samples being positive and another 15 samples negative for Nabs detection as indicated by the CPE assay in order to validate the accuracy of both methods.

The patients included in this study (n = 124) experienced intermediate disease activity and poor response to their IFNβ treatment, based on the assessment of

their treating neurologist. The intermediate disease activity was defined as one relapse during therapy; no or limited MRI activity (contrast-enhancing lesions and/or new T2 lesions), while doing poorly was defined as multiple relapses or one relapse and extensive MRI activity (multiple contrast-enhancing lesions and new T2 lesions)[13].

Patients were diagnosed with relapsing-remitting multiple sclerosis based on the McDonald criteria and the revised McDonald criteria[14,15] and received disease-modifying treatment with any of the three formulations of interferon-β (INFβ-1b,Betaferon; INFβ-1a SC; INFβ-1a IM) for at least one year. Patients treated with subcutaneous IFNβ-1b (Extavia®) were not included in this study because this formulation was not available.

Patients underwent serologic examination (Babs), using capture ELISA (cELISA) and Western blot assay. Nabs presence was determined *via* the assessment of the biological responsiveness to IFNβ using *in vitro* neutralizing activity - cytopathic effect assay and the MxA gene expression analysis using quantitative real-time PCR.

Blood samples were drawn 12 hours after the last injection of correspondent IFNβ formulation. They were centrifuged and sera were stored in -20^0 C until their assessment.

2.2. Measurement of Nabs and Babs

2.2.1. Binding assays

2.2.1.1. Capture ELISA (cELISA)

Binding antibodies were detected using the cELISA method as previously described[16,17]. In brief, 96-well microtitre plates (Nunc, Denmark) were coated overnight with monoclonal anti-human IFNβ IgG BO2 (Yamasa Shoyu Co, LtD, Tokyo, Japan) 'capture' antibody, at a concentration of 0.1 μg/well in 0.1 M carbonate-bicarbonate buffer. After plate was washed with phosphate-buffered saline (PBS) 0.05% Tween-20 (PBS-T) and blocked with 0.5% non-fat dry milk solution for 1h. Then, wells were incubated for 1h either with PBS-T for the test wells, or with the appropriate type of IFNβ for each patient diluted in in PBS-T (competitor wells) at a concentration of 150 ng/mL or 1.5 μg/mL for IFNβ-1a and IFNβ-1b respectively. Sera samples and negative control were diluted 1:100 in PBS-T and incubated in duplicates in competitor wells and test wells for 2h. The plate was then washed three times and IFNβ binding antibodies were detected by 1.5h incubation with a rabbit anti-human IgG secondary antibody conjugated with horseradish peroxidase (DakoCytomation, Glostrup, Denmark), diluted 1:6400 in PBS-T. After washing the plate 3 times, TMB substrate (SIGMA, USA) was added resulting in color development. The reaction was stopped with 2N Sulfuric acid. Results were obtained in optical density (OD) units by spectrophotometric analysis at 405nm and were converted to binding units following comparison with a standard curve of serial dilutions of a positive reference sample known positive specimen.

2.2.1.2. Western blot assay (WB)

IFNβ was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 14% polyacrylamide). The proteins were then transferred to nitrocellulose membranes by electrotransfer. Then, free binding sites on the membranes were blocked in 2% non-fat dry milk in PBS for 1 hour. The membranes were then washed twice with PBS-0.1% Tween-20 (PBS-T) and cut into 2-mm strips.

A total of 1 mL test sample diluted 1:1000 in blocking buffer was incubated on a membrane strip overnight at room temperature. A mouse monoclonal anti-human IFNβ antibody (Axell, Japan) and an internal positive control sample served as positive control subjects. For the negative control, serum from an IFNβ-naïve MS patient was used. Next, the strips were washed three times for 10 minutes with PBS-T and then incubated for 1 hour at room temperature with phosphate-conjugated anti-mouse IgG antibodies (Axell, Japan) or phosphate-conjugated anti-human IgG antibodies (Axell, Japan) as appropriate. The strips were then washed twice with PBS-T and once with substrate buffer (100mM Tris HCl; pH 9.5; 100mM $MgCl_2$, 100mM NaCl). Finally, the strips were incubated with p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer-Mannheim, Mannheim, Germany). After development of the blue precipitate, strips were washed twice with double distilled water and then air dried. Samples with a band at the molecular weight level of the mouse positive control band were considered positive and the titer was determined by ELISA.

2.2.2. Neutralizing assays

2.2.2.1. Cytopathic Effect assay (CPE)

Neutralizing antibodies were detected using the antiviral cytopathic effect (CPE) assay since it is recommended by the World Health Organization (WHO)[18]. Briefly, each serum sample was serially diluted (from 1:5 to 1:40) in culture medium (D-MEM, 2% FBS, 2mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin solution, all from Invitrogen-Gibco, USA) and incubated for 1 hour with either the corresponding IFNβ preparations at a final concentration of 10IU/mL, or with culture medium as control. Every serum dilution was tested in duplicate. Thereafter, human lung carcinoma cell line A549 (ATCC) overnight plated into a microtiter 96-well plate (Corning, USA), were incubated overnight with serum-IFNβ mixture. The cytopathic effect was induced by the addition of encephalomyocarditis murine (EMC) virus, at known viral activity. After a 24-hour incubation, attached cells were stained with 0,2% cresyl violet solution (SIGMA, USA) and were diluted with the addition of 33% acetic acid solution. The absorbance was measured at 405nm. According to WHO recommendations, data from the neutralization assay are reported as the reciprocal of the highest dilution of serum including 50% neutralization (i.e. neutralizing 10IU/mL of IFNβ activity to an apparent 1IU/mL of activity). The neutralization titer of a serum sample was calculated according to Kawade's formula[19,20] and expressed in 10-fold reduction units per millilitre (TRU/mL)[21].

2.2.2.2. MxA gene expression analysis

In this method, Nabs titer is determined through the quantification of MxA mRNA transcription that is induced from the IFNβ activity in A549 cells.

Serum samples were diluted in culture medium (alpha-MEM, 2% FBS, 2mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin solution, all from Invitrogen-Gibco, USA) and the appropriate IFNβ type in a concentration of 10IU/mL. Then, samples were incubated for 6.5h with monolayer of A549 cells plated into 96-well plates (Corning, USA). After the incubation, total RNA was extracted using NucleoSpin RNA Extraction kit (Macherey-Nagel) following manufacturers' instructions. For the cDNA synthesis 1μg of RNA was reverse transcribed in a mixture containing 2 μg of random hexamers (Biorad) and the Omniscript reverse transcription kit (0.5mmol/L each dNTP and 0.2U/μL reverse transcriptase, Qiagen, Germany). Total reaction volume was 20μL and the thermocycler protocol used consisted of a5 min denaturation step at 84° C, a 5 min hexamer hybridization step at 64° C, a 60 min amplification step at 64° C and a final incubation for 10 min at 64° C. Three microlitres of cDNA were added in 27 μL of reaction mixture, containing TAQ Mann Universal Master Mix (Applied Biosystems, USA), 25 pmol of MxA gene probe (predesigned probes, Applied Biosystems, USA) and 25 pmol of 18S rRNA Endogenous control (VIC/MGB Probe, Applied Biosystems, USA). The latter was used as a housekeeping gene, for the normalization of the MxA gene expression. cDNA was amplified in a IQ5 Biorad system after an incubation at 95°C for 10min, followed by 40 cycles of denaturation at 95°C for 15sec and amplification at 60 ^{o}C for 60sec The relative quantification was estimated by the comparative cycle threshold as outlined by the manufacturer. The neutralization titer was determined by applying the Kawade's formula and titers higher than 20 TRU/mL were considered as positive.

RESULTS

A total of 124 patients were included in this study**.** 94 patients were tested with cELISA and Western blot assay for the detection of binding antibodies and 30 patients were tested with CPE assay and Real-Time–PCR for the detection of neutralizing antibodies.

In the subgroup of the 64 patients (negative for Babs in the cELISA assay), who were re-evaluated with the WB assay the results were as follows: 49/64 (76.56%) resulted positive and 15/64 (23.43%) resulted negative. (Figure 1, 2)

In succession, we verified that all 30 Bab-positive samples with cELISA resulted positive using the WB assay (100%). (Figure 2)

The CPE assay results revealed 15 positive and 15 negative for Nabs samples. Real-Time-PCR verified these results in the total of the samples tested (100.00%).

DISCUSSION

Several large double-blind placebo-controlled clinical trials suggest that the proportion of MS patients re-

ported to have Nabs range from 2% to 45%, whereas the respective percentage for the Babs was 81%[2,6,8,22-26]. The reason for the immunogenicity of INFβ is not known in full detail. There are several factors that determine whether administration of a recombinant human molecule like INFβ to a MS patient causes development of Nabs[27]. Some factors are patient-linked and others are harbored in the INFβ product, i.e., the structure of the protein, differences in the glycoproteins, dose, route and frequency of administration[27]. It is generally agreed that INFβ-1b is more immunogenic than INFβ-1a[28,29]. Furthermore, intramuscular administration induces antibodies less frequently than subcutaneous administration[28-30]. The common denominator in all cases is the abatement of therapeutic effect of INFβ[2,6,11]. Recently, new guidelines were postulated in order to incorporate information on Nabs status and/or MxA bioavailability in clinical practice and therapeutic decision making[13]. In this regard, it is prerequisite to establish and validate detection assays that would accurately depict the Babs and Nabs status of patients under treatment with INFβ. The existence of Inter-laboratory and inter-assay variability is a well-known negative aspect, even when using the same biological assay for analysis of duplicate clinical specimens[13,31,32]. Many factors contribute in impeding the reproducibility and accuracy of assays. These include the conditions of sample distribution, assay timing, temperature regulation, differing product-specific cut-off levels for defining titers as negative, low or high, interferon-β subtype used in the assays and operator skills[13,31]. Moreover, biological parameters may affect the results of the assays such as virulence and presence of metabolites in clinical material or the metabolic state of cell lines[31].

Neutralizing assays are expensive, have difficulties in methodology that limit standardization, and are time-consuming, and they cannot be used for screening[7,33]. Therefore binding assays are more appropriate because they provide a less expensive and a more rapid screening path. Many investigators utilize direct ELISA in which the cytokine is directly coated on the plates[7,34]. This assay presents disadvantages which could lead to false-negative and false-positive results[16]. In order to overcome the drawbacks capture ELISA was developed, using a monoclonal anti-IFNβ antibody to initially capture the antigen[16].

In the present study the assessment of the results suggests that Western blot assay is more sensitive than ELISA for the detection of binding antibodies. The superiority of Western blotting over ELISA was demonstrated in a previous study by Chan et al. regarding the detection of anti-ribosomal P antibodies in the diagnosis of cerebral lupus[35]. More recently, Di Marco et al addressed the problematic issue of detection of low-titer Nabs in patients with multiple sclerosis treated with interferon beta[36]. The method was found to be rapid, specific and sensitive and consistent with respect to well-established antiviral neutralization or commercial enzyme-linked immunosorbent assays.

The gold standard for detecting Nabs to IFNβ has been the antiviral CPE assay. It tests the ability of antibodies to interfere with receptor binding by measuring the antiviral effect of IFNβ. Due to the nature of this bioassay, it is more prone to variability between assays and between laboratories. The World Health Organization (WHO) has recommended the use of human lung carcinoma (A549) cell line and the encephalomyocarditis virus[18] in order to minimize the inter-laboratory variability. For quantitative purposes the Kawade method was chosen to calculate the titer[19]. The CPE assay possesses two advantages, it is very sensitive and it requires a complex cellular response to the IFNβ[32]. The latter is important because the inhibition of such a response is likely indicative of inhibition of multiple IFN pathways, including those important for the clinical effect in multiple sclerosis. The disadvantage of CPE assay is that it is not specific meaning that other compounds present in human serum, such interleukin-6 or IFN-γ, can interfere or mimic IFNβ antiviral activity and influence the results. Another drawback is the fact that it is cumbersome and can have high interassay variability[37]. The in vivo assay using myxovirus resistance protein A (MxA) mRNA measures the in vivo induction of the MxA gene that is specifically induced by IFNβ injected by the patient. In the present study the assay performed on the messenger RNA of this gene used the real-time-PCR to determine the in vivo biologic effect of administered IFNβ. Advantage of this assay is the fact that determines the in vivo biologic response of a specific patient to his or her IFNβ therapy. Additionally, it is relatively simple, there is large difference between pre- and post-dosing and the MxA gene is specific for

type I IFNs. A drawback is the time interval between IFNβ injection and blood collection[32].

The evaluation of CPE and Real-time-PCR results indicates that they possess similar sensitivity. The employment of the two assays combined has practical consequences in treating patients and supporting difficult or ambiguous decisions. RT-PCR and MxA bioactivity measurements provide complementary information about biological activity especially in patients with low Nab titers, intermediate disease activity or doing poorly while on treatment with IFNβ[13].

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Αξιολόγηση της ευαισθησίας των μεθόδων ανίχνευσης των συνδετικών και εξουδετερωτικών αντισωμάτων έναντι της ιντερφερόνης-β σε ασθενείς με υποτροπιάζουσα-διαλείπουσα Σκλήρυνση κατά Πλάκας υπό αγωγή με ιντερφερόνης-β - Συγκριτική μελέτη.

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ΠΕΡΙΛΗΨΗ: Η ιντερφερόνη-β (IFNβ) αποτελεί θεραπεία πρώτης γραμμής για την αντιμετώπιση της υποτροπιάζουσας-διαλείπουσας σκλήρυνσης κατά πλάκας. Τα σκευάσματα της IFNβ πυροδοτούν την ανάπτυξη συνδετικών (Babs) και εξουδετερωτικών αντισωμάτων (Nabs). Η καταστροφική επίδραση των Nabs αποτυπώνεται από την εξασθένιση του θεραπευτικού αποτελέσματος που συνεπάγεται την επιδείνωση κλινικών και ακτινολογικών παραμέτρων. Η συχνότητα παρουσίας και οι τίτλοι των Nabs ποικίλλουν ανάλογα με το σκεύασμα IFNβ που χρησιμοποιείται, τη δόση, την συχνότητα και την οδό χορήγησης, τη διάρκεια θεραπείας και την μέθοδο ανίχνευσης που χρησιμοποιείται. Σκοπός της παρούσας μελέτης είναι η εκτίμηση της ευαισθησίας των μεθόδων εντοπισμού των συνδετικών και εξουδετερωτικών αντισωμάτων σε ασθενείς με υποτροπιάζουσα-διαλείπουσα σκλήρυνση κατά πλάκας. Η ανάλυση των αποτελεσμάτων προτείνει ότι η μέθοδος ανοσοστυπώματος κατά Western είναι πιο ευαίσθητη από την ELISA. Επίσης, προκύπτει ότι η Real-time-PCR και η μέθοδος κυτταροπαθητικού αποτελέσματος κατέχουν ίδια αποτελεσματικότητα. Παρ' όλα αυτά, η μέθοδος κυτταροπαθητικού αποτελέσματος αποτελεί την μέθοδο επιλογής για την ανίχνευση των εξουδετερωτικών αντισωμάτων σύμφωνα με τον Παγκόσμιο Οργανισμό Υγείας. Τα αποτελέσματά μας συνηγορούν για τη διενέργεια δια-εργαστηριακών μελετών που θα καταλήξουν σε κοινά αποδεκτή μέθοδο η οποία θα είναι αξιόπιστη και οικονομική για τον εντοπισμό των Babs και Nabs σε ασθενείς με σκλήρυνση κατά πλάκας υπό θεραπεία με IFNβ.

Λέξεις Κλειδιά: Εξουδετερωτικά αντισώματα, Συνδετικά αντισώματα, Ιντερφερόνη-β, Σκλήρυνση κατά πλάκας.

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