

Importance of neutralizing antibody positivity in Turkish multiple sclerosis patients

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ABSTRACT

The frequency and the consequences of binding and neutralizing antibodies (BAbs and NABs) against Interferon beta (IFNbeta) in Turkish multiple sclerosis (MS) patients have not been determined yet, which could differ in such a country which is between Europa and Asia. The aim of the study is to assess the frequency of these antibodies, and to evaluate the impact of NABs, from the clinical and radiologic aspects in Turkish patients with MS. One hundred and two MS patients were included. BAbs were screened using capture enzyme-linked immunosorbent assay (cELISA), and NABs were detected via Myxovirus protein A (MxA) messenger RNA (mRNA) induction assay (real-time polymerase chain reaction-PCR) at the beginning and one year later. Relapse rate and expanded disability status scale (EDSS) were used to assess the clinical impact. Gadolinium enhanced lesions and T2 lesion volume were used as magnetic resonance imaging (MRI) parameters. Persistent NAB positivity defines to be positive both at first and then one year later. NABs were detected in 12.2% (6/49) of IFNbeta-1b treated patients, and in 7.5% (3/40) of IFNbeta-1a SC treated patients, but none of the IFNbeta-1a IM treated patients had detectable NABs. It was found that the mean relapse rate difference was significantly higher in persistent NAB negative patients ($p = 0.024$). Persistent NAB positivity had no effect on T2 lesion volume and contrast enhancing lesions. 60% of the persistent NAB positive patients had at least one relapse during one-year of follow-up. On the other hand, 32% of persistent NAB nega-

tive patients were detected to have at least one relapse. Data from this study suggest that patients may become unresponsive to IFNbeta therapy even when the frequency of NABs does not prove to be as high as those in the literature. Nevertheless, one should keep in mind that disease activity is not always equal to NAB positivity.

Keywords: Interferon Beta; Multiple Sclerosis; Neutralizing Antibody

1. INTRODUCTION

Interferon beta (IFNbeta) is one of the first line of immune treatment options for multiple sclerosis (MS) for over 20 years. However, repeated IFNbeta injections may induce IFNbeta antibody production in some patients. Such antibodies are called binding antibodies (BAbs), which do not affect the biological activity of the molecule, and neutralizing antibodies (NABs), which are associated with a decrease in the efficacy of the treatment [1,2]. In fact, NABs are a subset of BAbs which prevent the binding of the IFN β to its receptor on the surface of cells. When BAbs are detectable it is likely that NABs are also present [3].

There are a large number of papers addressing neutralizing antibodies against IFNbeta from Europa and North America. Recently, a group from Japan reported that the prevalence of NABs is similar to that in Caucasian populations and is associated with an increase in disease activity [4]. However, there is no information about Turkish MS patients' antibody status and their impacts. Neutralizing antibodies are not tested routinely in Turkey. When there is a need, the blood sample of the patient is sent to a center abroad. Mostly, in daily prac-

tice, in patients doing poorly clinically, a switch to a non-IFNbeta therapy is initiated independent of NAb.

To our knowledge, this is the first study that assesses the frequency of the neutralizing antibodies and their effect on disease activity in Turkish MS patients. The frequency and the impact of NABs could differ in our country which is located at the crossroads of *Europe and Asia*. From another point of view, it is important to re-evaluate the treatment of those whose disease activity is higher due to NAB.

In the present study, the frequency of BAbS and NABs, and impact of these antibodies, from the clinical and radiologic aspects in Turkish patients with MS were assessed.

3. METHODS

3.1. Participants

We included 274 consecutive MS patients between May 2008 and October 2009. All patients were routinely attending the Neurology Department of Dokuz Eylul University Hospital, Izmir. Patients were receiving one of three IFN-beta preparations: interferon b-1B (“Betaferon”, Bayer) or interferon b-1A (“Rebif 44 mg”, both Merck-Serono, or “Avonex”, Biogen). Inclusion criteria required: a) a neurologist-confirmed diagnosis of definite relapsing-remitting MS according to the McDonald criteria [5]; b) age of 18 - 55; c) to be already treated only with IFNbeta for at least 18 months; d) to be relapse-free for at least 30 days prior to testing. Exclusion criteria were: a) to receive immunosuppressive treatment within the preceding year; b) to receive *intravenous immunoglobulin* (IVIG) or plasmapheresis within the last six months; c) to have primary progressive MS; d) to have upper respiratory tract infection within three weeks; e) to receive corticosteroid treatment due to an attack within three months. The local University Hospital Medical Ethics Committee approved the research proposals for the study. Written informed consent was obtained from patients who participated in this study. A total of 102 patients were eligible for the study. Clinical characteristics of the 102 patients are shown in **Table 1**.

3.2. Binding Antibody Analysis

2.5 ml venous blood was drawn in serum tubes for BAb analysis, and samples were screened using capture enzyme-linked immunosorbent assay (cELISA) first at the time of study and later, the following year. Persistent BAb-positivity indicates patients who were BAb positive both at their first and final assessments.

3.3. Neutralizing Antibody Analysis

Blood samples were collected in PAXgene tubes (Pre Analytix GmbH, Hombrechticon, CH) 12 - 14 hours af-

ter an injection of IFN, and NABs were detected via Myxovirus protein A (MxA) messenger RNA (mRNA) induction assay (real-time polymerase chain reaction-PCR), first at the time of study and later, the following year.

RNA extraction and cDNA synthesis were done using commercially available kits (Preanalytix by Qiagen, and Superscript II Reverse Transcriptase, Invitrogen, Carlsbad, CA). PCR was performed on an ABI 7500 Fast Real Time PCR System (Applied Biosystems) using a commercially available TaqMan Universal PCR Master Mix and primer/probe kits. Gene expression in each sample of the target mRNA relative to GAPDH (ΔCt) was compared to a calibrator consisting of pooled cDNA from healthy controls. A normalization ratio (NR) was calculated using the formula $NR = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{pool})$. NR reflects fold induction of gene expression as compared to expression in the control pool. Samples were run in duplicate.

Persistent NAB-positivity indicates patients who were NAB positive both at their first and final assessments. Both binding and neutralizing antibody analyses were performed in the neuroimmunology laboratory of Faculty of Medicine of Dokuz Eylul University, Izmir.

3.4. Clinical and Radiologic Evaluation

A relapse was recorded only if the physician described new findings consistent with the patient’s reported symptoms, and had excluded the possibility of a pseudorelapse. Relapse rate and Expanded Disability Status Scale (EDSS) [6] were used to assess the clinical impact.

Table 1. Demographical and clinical features of the patients.

	Patients
Gender n (%):	
Female	68 (66.7)
Male	34 (33.3)
Mean age (\pm SD) (range)	34.2 \pm 7.9 (19 - 53)
Mean disease duration (year) (\pm SD) (range)	7.9 \pm 4.9 (2 - 23)
Disease course: RRMS (n)/SPMS (n)	98/4
Mean IFN β treatment duration (month) (\pm SD) (range)	43.5 \pm 24.7 (18 - 120)
Mean relapse rate before IFN β treatment (\pm SD) (range)	1.1 \pm 0.6 (0 - 3.5)
IFN β preparation	n (%)
IFN β -1b	49 (48)
IFN β -1a SC	40 (39.2)
IFN β -1a IM	13 (12.8)

SD: standard deviation; RRMS: relapsing remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; IFN β : interferon beta; SC: subcutaneous; IM: intramuscular.

EDSS scoring was performed at baseline and one year later. Also, number of relapses was recorded within one year of study.

Magnetic resonance imaging (MRI) scans were performed after blood sampling at the beginning and than one year later. Hyperintense lesions on T1-weighted post-gadolinium sequences were counted. T2 lesion total volume was calculated within the proton density/T2 weighted images semi-automatically using “Lesion Annotation and Volume Assessment (LAVA) software, Medical Image Mining Laboratories (New York)” in Windows XP operating system on personal computers. All radiologic images were assessed by a blind radiologist.

3.5. Statistical Analysis

SPSS 15.0 for Windows was used for the statistical analysis. Descriptive features for continuous variables were implied as mean and standard deviation (SD), and for discontinuous variables as number and percent. The comparison of continuous variables between two independent groups was done with Mann-Whitney U test, and when more than two groups with Kruskal-Wallis test if the normal distribution could not be fulfilled. Wilcoxon test was used for the time effect in between dependent groups due to lack of normal distribution of the difference between continuous variables. The significance of <0.05 had been accepted.

4. RESULTS

Demographic and clinical characteristics were similar in three different treatment groups (Table 2).

4.1. The First Evaluation

BABs were detected in 26.5% (27/102) of the patients. Of 49 patients treated with IFN β -1b, 40.8% were BAB positive at the beginning of our study, whereas of 40 patients treated with IFN β -1a SC, 15% were BAB positive; and of 13 patients treated with IFN β -1a IM, 7.7% were BAB positive. BAB positivity was higher in IFN β -1b treated group than IFN β -1a treated group, and in IFN β -1a preparation, the patients treated with SC form had higher BAB positivity than IM form ($p = 0.006$).

NABs were detected in 8.8% (9/102) of all the patients. NABs were present in 12.2% (6/49) of IFN β -1b treated patients, and in 7.5% (3/40) of IFN β -1a SC treated patients, but none of the IFN β -1a IM (0/13) treated patients had detectable NABs. The treatment duration was longer in NAB negative patients than in NAB positive patients, but it was not statistically significant (respectively 45.28 months; $p = 0.055$) (Table 3).

98.7% of BAB negative patients were NAB negative, and 29.6% of BAB positive patients were NAB positive.

Table 2. Demographic and clinical characteristics of the three different treatment groups.

	IFN β -1b	IFN β -1a SC	IFN β -1a IM	p*
	Mean \pm SD	Mean \pm SD	Mean \pm SD	
Mean Age	35.4 \pm 7.9	33.8 \pm 8.0	31.0 \pm 7.3	0.210
Mean Disease Duration (y)	8.7 \pm 5.6	7.3 \pm 4.6	6.9 \pm 2.2	0.532
Mean EDSS before IFN β	2.0 \pm 0.9	2.2 \pm 1.2	1.9 \pm 1.3	0.627
Mean EDSS in the First Evaluation	2.0 \pm 1.1	2.5 \pm 1.7	1.7 \pm 1.0	0.262
Mean Relapse Rate before IFN β	1.2 \pm 0.6	1.2 \pm 0.7	1.0 \pm 0.4	0.771
Mean Treatment Duration	41.2 \pm 26.0	42.2 \pm 22.6	56.2 \pm 24.4	0.054

y: year; IFN β : interferon beta; m: month; *Kruskal Wallis test.

There was only one NAB positive patient who was found to be BAB negative.

4.2. The Second Evaluation

Six of 102 patients dropped out one year after the first sampling time. 38.5% ($n = 37/96$) of patients were BAB positive. The increase in BAB positivity during one year follow-up was statistically significant only in IFN β -1b treated patients (respectively 41%, 56%; $p = 0.039$). Persistent BAB positivity was found 25% (24/96).

In the second evaluation (one year later), NAB was present in 7.3 (7/96) of all the patients. Similar to the first evaluation, none of the IFN β -1a IM treated patients had detectable NABs. NAB had disappeared in three of the IFN β -1b treated NAB positive patients and in one of the IFN β -1a SC treated NAB positive patient, whereas NAB had appeared in two of the IFN β -1b SC treated NAB negative patients. Persistent NAB positivity was found 5.2% (5/96).

4.3. Clinical Evaluation

Neither BAB positivity in the first evaluation nor persistent BAB positivity had any effect on relapse rate and progression of disability in terms of EDSS scoring.

The mean relapse rate difference was significantly higher in persistent NAB negative patients than in persistent NAB positive patients ($p = 0.024$) (Table 4). Mean EDSS change did not differ significantly between patients who were persistent NAB positive or NAB negative ($p = 0.22$). 60% of the persistent NAB positive patients were detected to have at least one relapse, whereas it was 32% of persistent NAB negative patients during one-year

Table 3. Features of patients regarding to NAb status.

	NAb		p
	negative	positive	
Mean Age (\pm SD)	34.4 \pm 7.9	32.9 \pm 6.1	0.816
Mean Disease Duration (y) (\pm SD)	8.3 \pm 5.1	4.6 \pm 2.0	0.050
Mean IFN β Duration (m) (\pm SD)	44.8 \pm 25.0	28.1 \pm 10.1	0.055
Mean EDSS in the First Evaluation (\pm SD)	2.1 \pm 1.4	1.9 \pm 0.8	0.835
Mean Relapse Rate before IFN β (\pm SD)	1.1 \pm 0.6	0.9 \pm 0.5	0.205

y: year; IFN β : interferon beta; m: month.

of follow-up ($p = 0.330$).

4.4. Radiologic Evaluation

It was shown that persistent NAb positivity had no effect on T2 lesion volume (**Table 5**) and contrast enhancing lesions in MRI (**Table 6**).

5. DISCUSSION

In the literature, it was reported that 28% - 47% of IFN β -1b treated patients, 12% - 28% of IFN β -1a SC treated patients, and 2% - 6% of IFN β -1a IM treated patients had developed NAb [7-13]. In our study, NAb were present in 12.2% of IFN β -1b treated patients, and in 7.5% of IFN β -1a SC treated patients, but none of the IFN β -1a IM treated patients had detectable NAb. The frequency of persistent NAb positive patients was also lower when compared to other similar studies [14-16], which might be due to genetic features of Turkish MS patients. Another possible explanation for the low proportion of persistent NAb positive patients could be about mean IFNbeta treatment duration. It was roughly 3.6 years in the present study. Several studies showed that 40% of patients treated with IFN β -1b reverted to NAb negative status within 4 years [17]. So that, some of our NAb positive patients could have reverted to NAb negative before the study. Three NAb positive patients receiving IFNbeta-1b and one NAb positive patient receiving IFNbeta-1a reverted to NAb negative status even in one year time in our study. Consistent with the results to other studies, IFN β -1b was found more immunogenic than IFN β -1a SC, and IFN β -1a SC was more immunogenic than IFN β -1a IM [9,18].

Patients whose IFNbeta treatment duration was at least 18 months were included in the present study. The reasons for that were, firstly, NAb appear between 3 to 18 months after the treatment [19], and secondly, determining persistence of NAb does not seem to be reliable

Table 4. The mean relapse rate difference between persistent NAb positive and negative patients.

	NAb	Before IFN β Therapy	After IFN β Therapy	p*
		Mean \pm SD	Mean \pm SD	
Relapse Rate	PP (n = 5)	1.00 \pm 0.35	0.60 \pm 0.55	0.34
	PN (n = 85)	1.57 \pm 0.80	0.28 \pm 0.40	<0.001
Difference in Relapse Rate	PP (n = 5)	-0.40 \pm 0.82		p**
	PN (n = 85)	-1.37 \pm 0.85		
		0.024		

IFN β : interferon beta; Nab: neutralizing antibodies; PP: persistent positive; PN: persistent negative; *Wilcoxon Test; **Mann Whitney U Test.

Table 5. Mean difference in T2 lesion total volume between persistent NAb positive and negative patients.

	NAb	First Evaluation	Second Evaluation	p*
		Mean \pm SD	Mean \pm SD	
T2 Lesion Volume	PP (n = 5)	3.67 \pm 0.51	2.83 \pm 0.66	0.18
	PN (n = 85)	8.59 \pm 11.04	9.41 \pm 11.91	0.02
T2 Lesion Volume Difference	PP (n = 5)	-0.83 \pm 0.15		p**
	PN (n = 85)	0.82 \pm 2.24		
		0.15		

NAb: neutralizing antibodies; PP: persistent positive; PN: persistent negative; SD: standard deviation; *Wilcoxon Test; **Mann Whitney U Test.

Table 6. Mean difference in CELs between persistent NAb positive and negative patients.

	NAb	First Evaluation	Second Evaluation	p*
		Mean \pm SD	Mean \pm SD	
CELs	PP (n = 5)	0.50 \pm 0.71	1.50 \pm 2.12	0.31
	PN (n = 85)	0.17 \pm 0.79	0.33 \pm 1.00	0.32
CELs Difference	PP (n = 5)	1.00 \pm 1.41		p**
	PN (n = 85)	0.16 \pm 1.22		
		0.17		

CELs: contrast enhancing lesions; Nab: neutralizing antibodies; PP: persistent positive; PN: persistent negative; SD: standard deviation; *Wilcoxon Test; **Mann Whitney U Test.

before month 12 - 18 on treatment [20,21] and finally, it was shown that NAb against IFNbeta were characterized by low affinity antibodies in the first 6 - 12 months that have a protective effect on IFNbeta and hence increase the effect of IFNbeta [22].

There are a number of methods that have been developed to detect NABs. Antiviral assays (AVA) in which IFN- β inhibits viral replication have been commonly used [17]. Another approach for measuring NABs is the myxovirus resistance protein A (MxA) induction assay, which measures the expression of the IFN-inducible GTPase MxA in cultured cells [23]. A reporter gene assay has been developed [24,25]. Recently, a new non-cell-based NAb assay has been described [26]. Besides, there is still ongoing researches for the development of a reliable, quick, standard and cost-effective NAb assay. In our study, NABs are not detected using functional assays of interferon-responsive cell lines in culture, because cell cultures are not feasible for our laboratory condition. Alternatively, NABs were detected via MxA mRNA induction assay. It was shown that treatment of patients with IFN β leads to maximal MxA increase at about 12 h post injection [27]. Following treatment of the patient with IFN β at 12 - 14 h, we collected blood samples into special tubes designed to lyse the cells and stabilize the mRNA. The mRNA is extracted, converted to cDNA by reverse transcription and analyzed by real-time PCR. If the patient has NABs to IFN β the amount of MxA produced is reduced or, in the case of particularly high NAB titres, abolished [27-29].

It was shown that BAb positivity precedes the development of NABs and BAb positivity appears to be a predictor of subsequent NAB development [30,31]. Regarding this, we found that 98.7% of BAb negative patients were NAB negative. As studies focused on the standardization and detecting techniques of NAB analyses [16,28, 32-35], importance of BAb analysis, which is cheaper and less time consuming, has been neglected. We think that if there are patients doing poorly clinically that are considered due to NABs, BAb analysis using ELISA could be used as a first step. When BAb is negative, it is most likely that the reason is not NAB. In relation to this, a recent paper also suggest to use ELISA measurements of BAb to identify patients with high titres of NABs, and in patients with low titres, they suggest to supplement ELISA with measurement of MX1 mRNA to establish whether the bioavailability of IFN- β is preserved [36].

During one-year of follow up, 60% of the persistent NAB positive patients had at least one relapse; this finding can be significant even for a clinically stable NAB positive patient and she/he should be followed up more closely. On the other hand, 32% of persistent NAB negative patients were detected to have relapses which may be due to mechanisms by non-NAB mediated molecules [31]. This finding could also reinforce that NABs account for only a minor part of breakthrough disease [37].

The only parameter that NAB was shown to be associated with a decline in therapeutic effects was relapse rate. We found that NAB positive patients had a higher relapse

rate. Our study failed to demonstrate significant impact of NABs on progression of disability and MRI measures of disease activity. This lack of effect on MRI parameters may be due to small number of persistent NAB positive patients.

The main limitation of this study relates to the number of patients in the study. It was mostly due to our study population included those who were treated only with IFN β for at least 18 months from one center. Therefore, a confirmation prospective multicenter study with a large number of MS patients would help to prove the real power of NABs. Moreover, our study is a short-term one. The evaluation of the impact of NABs on MS disease progression requires long-term studies in large cohorts of IFN β -treated MS patients.

6. CONCLUSION

From a clinical point of view, reliably identifying NAB positive and NAB negative patients is crucial for the development of a new treatment algorithm for the patient. The present study observation reinforces the importance of NAB on relapse rate and shows that patients may become unresponsive to IFN β therapy even when the frequency of NABs does not prove to be as high as those in the literature. Nevertheless, further studies with a larger number of Turkish MS patients should examine to confirm our clinical results.

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