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Title: Study of binding and neutralising antibodies to interferon-β in two groups of relapsing-remitting multiple sclerosis patients.

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Abstract

Interferon (IFN)-β is generally considered an effective treatment for multiple sclerosis (MS); however, some patients do not respond to this therapy, possibly due to the production of neutralising antibodies (NAB) which can prevent the biological effect of IFN-β. We compared the two types of IFN-β, the glycosylated IFN-β_{1a} and the non-glycosylated IFN-β1b, as their chemical differences may entail differing immunogenic capacities.

We studied 22 relapsing-remitting MS patients treated with IFN-β_{1a} and 31 treated with IFN-β_{1b} for 1 year, using the same assay and criteria, to compare the two types of IFN-β in their ability to induce binding and neutralising antibodies and examined the correlation of the findings with the clinical data. Binding antibodies to IFN-β_{1a} and IFN-β_{1b} were determined by enzyme-linked immunosorbent assay. A bioassay was used to detect and quantify the NABs to IFN-β, measuring the capacity of NABs to block the antiviral resistance induced by IFNs. Binding antibodies were found in 32% of those treated with IFN-β_{1a} and in 52% of those treated with IFN-β_{1b}; NABs were found in 14% and 24%, respectively. Both groups showed a significant decrease in relapse rate during the first year of treatment. These results demonstrate that the IFN-β_{1b} molecule is more immunogenic than the IFN-β_{1a} molecule. This may be due to the non-glycosylated, chemical structure of the former, which can produce aggregates and enhance antibody production. No association was found between the presence of NABs and the clinical status of the patients.

Key words: neutralising antibodies, interferon-β, multiple sclerosis.

Introduction

Multiple sclerosis (MS) is an inflammatory-demyelinating disease of the central nervous system caused presumably by an autoimmune process that induces demyelination, destruction of oligodendrocytes, and axonal injury [29]. The immunology of MS is associated with the production of several cytokines that can vary with the course of the disease [5, 15], having a Th1 pattern during relapses [6, 10], with an increase in the proinflammatory cytokines such as interleukin–2, interferon (IFN)-γ and tumor necrosis factor-α [10], and a down-regulation of these cytokines during the non-active phase of the disease. IFN-β possesses an immunomodulatory effect, which provides this cytokine with an important role in the treatment of this disease. On the other hand, the production of IFN-β resulting from a viral infection has been observed to be lower in MS patients than in controls [19, 30]. In these cases, treatment with exogenous IFN-β could also prove important.

The efficacy of treatment in MS by both IFN-β_{1a} and IFN-β_{1b} has been demonstrated in the reduced exacerbations and number of lesions observed on magnetic resonance imaging [12, 26] and in a slowed progression of the disease [8, 30]. However, there are some MS patients in whom this therapy is less effective, and who are considered non-responders. One possible reason for this lack of biological efficacy is the presence of specific antibodies to IFN-β administered in the therapy and which might block its activity in the cell [2].

Two recombinant IFN-β products are presently used in the treatment of MS patients: IFN-β_{1a} (glycosylated) and IFN-β_{1b} (non-glycosylated and with a Met–1 deletion and a Cys–17 to Ser mutation) [25]. These chemical structural variations can induce differences in both biological activity and immunological capacity. Although both of these types of IFN-β are very similar in chemical structure to endogenous IFN-

β, it has been reported that IFN-β treatment is able to induce binding antibodies and, more importantly, neutralising antibodies (NAB) [1, 19, 28]. These NABs are the antibodies that bind to the portion of the IFN-β molecule which interacts with its receptor in the target cells, preventing the biological activity of IFN-β [19, 22]. NABs have been demonstrated in 10% of MS patients treated with human natural IFN-β [17]. Studies on antibody production during treatment with recombinant IFN-β_{1a} [1, 3, 24] or IFN-β_{1b} [7, 14, 23, 28] show differences in percentage; however, because these studies have used varying methods, it is not possible to compare the immunogenic capacities of the two in treating MS. Moreover, it has not yet been possible to establish a relationship between the clinical state of the patient and the detection of NABs [5].

We examined the differences in immunogenic capacity of the two types of recombinant IFN-β used in the therapy of MS. Using the same assay and criteria, we compared the presence of binding antibodies and NABs induced in two groups of MS patients treated with IFN-β_{1a} or IFN-β_{1b}.We also studied the possible association of antibody presence with the clinical status of the patients.

Material and Methods

- **Patients**

The study included 53 patients with relapsing-remitting MS (28 women, 25 men; mean age 39.13 ± 9.2 years; mean disease duration 12.17 ± 6.1 years). Of these, 22 were treated with intramuscularly IFN-β_{1a} (Avonex, Biogen, Cambridge, Mass., USA) at 6 MIU/week (30 μg/week) and 31 subcutaneously with IFN-β_{1b} (Betaferon, Schering, Berlin, Germany) at 8 MIU/48 h (250 μg/48 h). After 1 year, serum samples were collected from each patient 24 h following the last IFN-β injection and stored at –20°C until use. The samples collected were run in duplicate in the same assays.

The patients' clinical data were monitored throughout the treatment, and we calculated the annual relapse rate, expressed as the total number of relapses in a year per group with respect to the total number of patients in the group. To define relapses, we followed the Poser et al. criteria [20], i. e. the occurrence of a symptom or symptoms of neurological dysfunction lasting more than 24 h, but we also required the existence of an objective confirmation. A control group of 211 healthy subjects was included in the study to establish the cutoff point for binding antibody levels.

- **Determination of binding antibodies to IFN-β**

Microtitre plates (Immulon 4 HBX, Dynex Technologies, Chantilly, Va.,USA) were coated overnight at 4°C with the two IFN-β types used in the treatment,with 100 μl/well at 1 μg/ml in phosphate-buffered solution (PBS). After washing with PBS with 0.05% Tween 20, the wells were blocked with 200 μl PBS–0.5% dry milk for 1 h at 37°C, and the plates were washed ten times. Two-fold serial dilutions (ranging from 1:160 to 1:2560) of rabbit anti-human IFN-β diluted in PBS–Tween 20 were prepared as

controls. Patient samples were used at 1:100 dilution in PBS–Tween 20. From the different dilutions, 50 µl was added and incubated for 1 h at 37°C. After washing, 50 µl/well alkaline phosphatase anti-rabbit IgG (1:1000; Sigma, St. Louis, Mo., USA), and alkaline phosphatase anti-human polyvalent Ig (1:1000; Sigma) were added and incubated for 1 h at 37°C. Plates were washed and developed with 50 µl/well phosphatase substrate (Sigma) at 2 mg/ml in glycine buffer and incubated for 1 h at 37°C. The optical densities were read at 405 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader (Labsystem Multiskan, Helsinki, Finland). This technique has been used as a screening analysis to detect the presence of binding antibodies to IFN-β as a step previous to determining NABs to these IFNs. Although these absorbances do not reflect antibody titres, they are a way in which to compare levels between different treatment groups.

- **Determination of NABs to IFN-β**

The assay was made as previously described [17], which is a modification of the assay described by Kawade's et al. [13]. Briefly, plates (Nunc, Roskilde, Denmark) were seeded with 100 µl of a line of human epidermal cells (HEP–2) at $1.5x10^5$ cells/ ml, incubated for 24 h at 37°C, 5% CO_2 in medium RPMI 1940 (Bio-Whittaker, Md., US) with 1% fetal calf serum. Sera collected were inactivated at 56°C for 30 min and then two-fold serial dilutions (ranging from 1:4 to 1:512) of serum were mixed with 10 IU/ml IFN-β_{1a} or 100 IU/ml IFN-β_{1b}. (In several assays performed in our laboratory to compare the specific activity of the two IFN-β preparations, 10 IU/ml IFN-β_{1a} showed the same biological effect in inhibiting the cytophatic effect of the virus as 100 IU/ml IFN-β_{1b}). These were incubated for 1 h, and then 100 µl/well was added to the monolayer culture. A standard curve with two-fold dilutions, from 20 to 1.5 IU/ml IFN-β_{1a} and from 200 to 15 IU/ml IFN-β_{1b}, as well as a virus control (without interferon) and

a cell control (without interferon or virus) were added to each plate. The plates were incubated for 24 h at 37°C, 5% CO_2. The wells were then all infected with vesicular stomatitis virus, Indiana strain, except for the cell control wells. After incubating the plates for 24 h at 37°C, 5% CO_2, the contents were discarded, and 100 µl crystal violet stain (Merck, Darmstadt, Germany) was added to each well. After 15 min, the plates were rinsed and allowed to dry. NAB levels were considered as the serum dilution producing 50% inhibition of the IFN-β action in its cytopathic effect of the virus.

- **Statistical studies**

Levene's test was carried out to study the differences in the binding antibody level in patients treated with IFN-β_{1a} or IFN-β_{1b}. The Wilcoxon test was used to assess the association between the presence of NABs and the number of relapses in the patients.

Results

- **Binding antibody determination to IFN-β**

The ELISA was carried out in parallel in each patient analysing the binding antibodies to both IFNs although each patient had only been treated with one type. We also determined the presence of binding antibodies in a healthy group (n=211), observing that some subjects present binding antibodies to IFN-β without any IFN treatment. The mean absorbance was 0.438 ± 0.539 for antibodies to IFN-β_{1a} and 0.429 ± 0.52 for antibodies to IFN-β_{1b}. Because of this range in antibody levels we selected an arbitrary value near the mean to establish the baseline. This value was an optical density of 0.5. The antibody binding studies (Fig. 1) found that 32% of the patients treated with IFN-β_{1a} had a significant level of binding antibodies while 52% of those treated with IFN-β_{1b} had antibodies (P=0.03). The optical density data from these patients are shown in Table 1.

- **Neutralising antibody determination**

The same samples in which binding antibodies to either of the IFN-β assayed were detected were also analysed for the presence of NABs. The number of positive cases depended on the type of IFN-β used in the treatment (Table 1): 14% of the patients treated with IFN-β_{1a} had NABs and 24% of those treated with IFN-β_{1b}. No association was found between the binding and NAB antibody titres (Table 1).

- **Clinical data during the treatment: relapses**

In the IFN-β_{1a} group (n=22) there were 35 relapses during the year preceding treatment (annualised relapse rate 1.60 ± 0.57) and 8 in the year following treatment (annualised relapse rate 0.37 ± 0.64), the corresponding figures in the IFN-β_{1b} group (n=31) were 57 (1.84 ± 0.58) and 14 (0.45 ± 0.96) $P < 0.0001$ for both groups. There

was no therapeutic difference between the two treatments (*P*=0.302). During the year of treatment 50% of patients receiving IFN-β_{1a} and 45% of patients receiving IFN-β_{1b} experienced no relapse, and no patient entered a chronic phase. No significant difference in Expanded Disability Status Score was seen in either group of patients. However, the period of follow-up (1 year) was too short to expect marked changes, and the number of patients was small; no further analysis or comments concerning this aspect are made, although the Expanded Disability Status Score is probably most relevant.

At the time that the samples were obtained, after 1 year of treatment, 32% of the patients treated with IFN-β_{1a} and 48% of those treated with IFN-β1b were suffering relapses. Of these patients suffering exacerbation only 2 (9%) in the IFN-β_{1a} group and 1 (3 %) in the IFN-β_{1b} group had coincident NABs (Table 2). The Wilcoxon test showed no association between the presence of NABs and an increase in the number of relapses in the patients at sample collection in either treatment group.

- **Cross-reactivity**

The ability of the antibodies induced against one IFN-β used in the treatment to react with the other was determined by ELISA, testing the serum sample of each patient against both IFN-β_{1a} and IFN-β_{1b}, independently of the treatment received. Data showed that the antibodies were able to react to the IFN-β not used in the treatment, indicating a strong cross-reactivity. This effect was observed in patients with high levels as well as those with low levels of binding antibodies (Fig. 2). This is possibly due to the great similarity in their chemical structures, which the antibodies that were induced are unable to distinguish.

Discussion

MS is probably an autoimmune disease, produced by a proinflammatory process. One of the various treatments available is IFN-β, principally as a result of its immunomodulatory effect. Several reports have demonstrated the efficacy of IFN-β in MS in decreasing the frequency of relapses and the number of lesions on magnetic resonance imaging [26, 27] as well as in slowing disease progression [8, 20]. However, there are some patients in whom this treatment is less effective. Although there are many possible reasons for this lack of efficacy in the treatment, one might be the presence of antibodies that bind with the IFN-β, thus blocking its biological activity and preventing the interaction with its receptor. These are termed NABs [2].

Patients treated with IFN-β can produce specific antibodies to it [19, 23, 24], although these antibodies may not be neutralising. The presence of antibodies to IFN-β has also been demonstrated during viral infection, autoimmune diseases, in some cases in healthy subjects and in mice [16, 24].

We observed binding antibodies to both types of recombinant IFN-β used in the treatment of MS, antibodies to IFN-β_{1a} in 32% of cases and those to IFN-β_{1b} in 52%. These data most probably show that the chemical differences between the two recombinant IFN-β molecules used in the treatment of MS patients lead to differences in their immunogenic capacity. These differences still show in the presence of NABs, as 14% of the IFN-β_{1a} patients and 24% of the IFNβ_{1b} patients had NABs. The incidence of NABs to IFN-β_{1a} in this study is higher than the incidence previosly reported (5% at 24 months by Rudick et al. 1998 [24]; 2% at 24 and 30 months in CHAMPS (Jacobs et al. 2000) [11]). This variation has also been reported by others [1] and may arise because IFN-β_{1b} is not glycosylated and can produce aggregates which have a higher antigenic capacity. This type of IFN-β also has some mutations compared with

endogenous IFN, which may be recognised by the immune system and lead to the production of specific antibodies [19]. Several reports have shown that the presence of NABs during IFN-β treatment is transitory because these antibodies disappear despite continuing treatment [17, 23]. Although the two types of IFN-β used in the treatment of MS showed differences in the levels of antibodies induced, possibly due to their chemical differences or to different doses used in the treatment with each IFN, these antibodies are able to cross-react with the other IFN-β, and against which they have not been induced [9, 14]. We found no patient in whom specific antibodies were induced that reacted only with the same type of IFN-β used in the treatment, indicating that it will probably not be useful, at least for some time, to switch to another IFN-β product when NABs develop in a patient after treatment with one type of IFN-β, as has been suggested by Antonelli et al. [4].

Although some authors report an association between the presence of NABs and the worsening of the clinical state of the patients during IFN-β treatment [23, 24, 27, 28], others have not found a significant association [3, 9, 17]. Our study agrees with the latter, since we observed that the presence of NABs and the development of a relapse occurred simultaneously in only two (9%) patients treated with IFN-β_{1a} and one (3 %) of those treated with IFN-β_{1b}. Most importantly, there were no significant differences in the annualised relapse rate between the two groups. No association therefore existed between the presence of NABs and a worsening in the clinical status of the MS patients treated with either of the two types of IFN-β used. Further research must be directed to the intriguing issue of why NABs apparently do not neutralise the activity of IFN-β in the treatment of MS, as opposed to the case with IFN-α [18] (a class I IFN which shares a great structural homology and the same receptor as IFN-β) in the treatment of other conditions.

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Table 1. Data on patients with binding and/or neutralising antibodies to IFN-β

Patient no.	Binding antibodies	Neutralising antibodies	Relapses
IFN-β_{1a} group			
1	0.609	-	-
2	0.664	-	-
3	0.563	+ (1:20)	+
4	0.919	-	-
5	1.142	+ (1:10)	-
6	1.713		-
7	0.568	+ (1:20)	+
IFN-β_{1b} group			
1	1.240	-	-
2	1.867	-	-
3	1.249	-	-
4	0.663	+ (1:80)	-
5	1.673	+ (1:80)	-
6	1.192	-	-
7	0.637	-	-
8	0.520	+ (1:320)	-
9	1.445	+ (1:20)	-
10	1.080	-	-
11	0.961	+ (1:40)	-
12	1.302	-	+
13	0.724	-	-
14	1.435	+ (1:960)	-
15	0.881	-	-
16	0.553	+ (1:80)	+
17	1.081	-	-

Table 2. Number and percentage of patients with binding and neutralising antibodies (NAB), number and percentage of patients with coincidental relapses at sampling, and patients with simultaneous relapses and NAB in the two treatments groups studied.

	Binding antibodies		NAB		Relapses at sampling		Simultaneous occurrence of relapse and NAB	
Treatment	*n*	%	*n*	%	*n*	%	*n*	%
IFN-β_{1a}	7	31.8	3	13.6	7	3.8	2	9.0
IFN-β_{1b}	16	51.6	7	23.0	15	48.4	1	3.2
Total	3	43.0	10	18.9	22	41.5	3	5.6

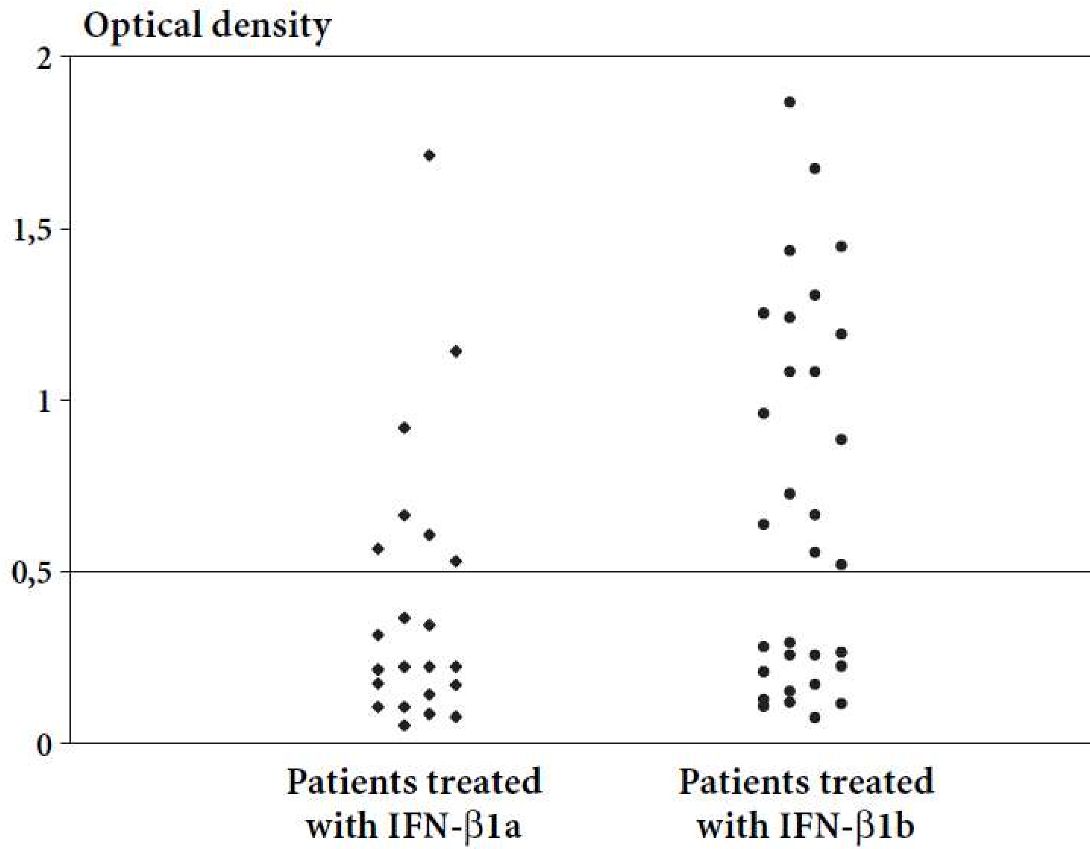
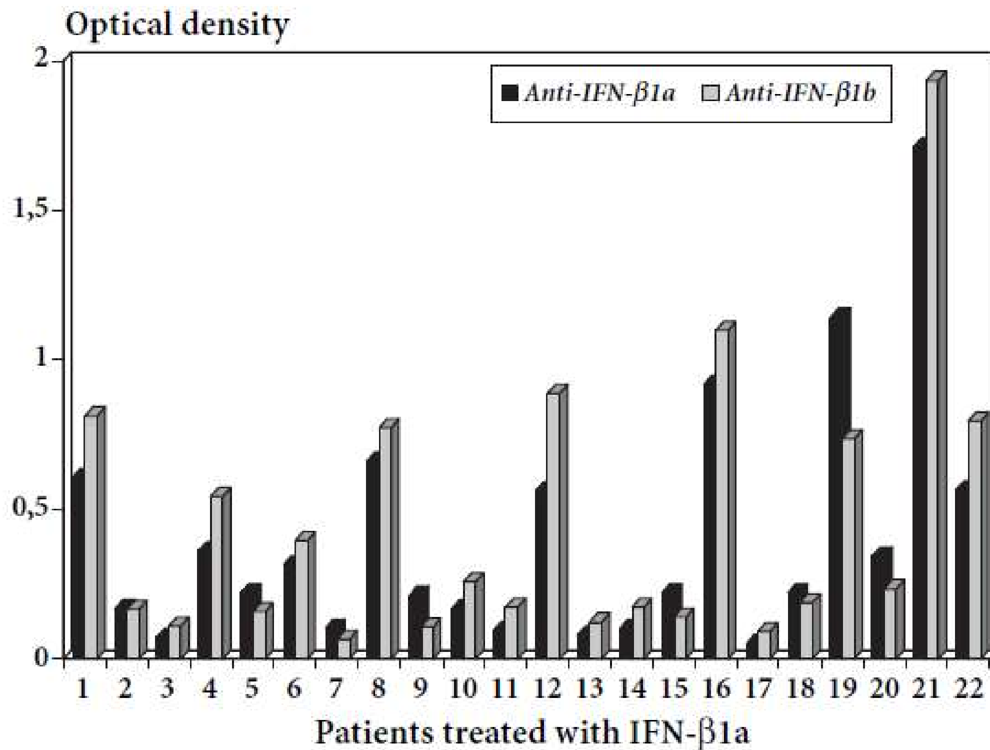


Figure 1. Patients' samples after 1 year of treatment were analysed by ELISA to determine binding antibodies to IFN-β_{1a} in patients treated with IFN-β_{1a} (*circles*) and to IFN-β_{1b} in patients treated with IFN-β_{1b} (*diamonds*).



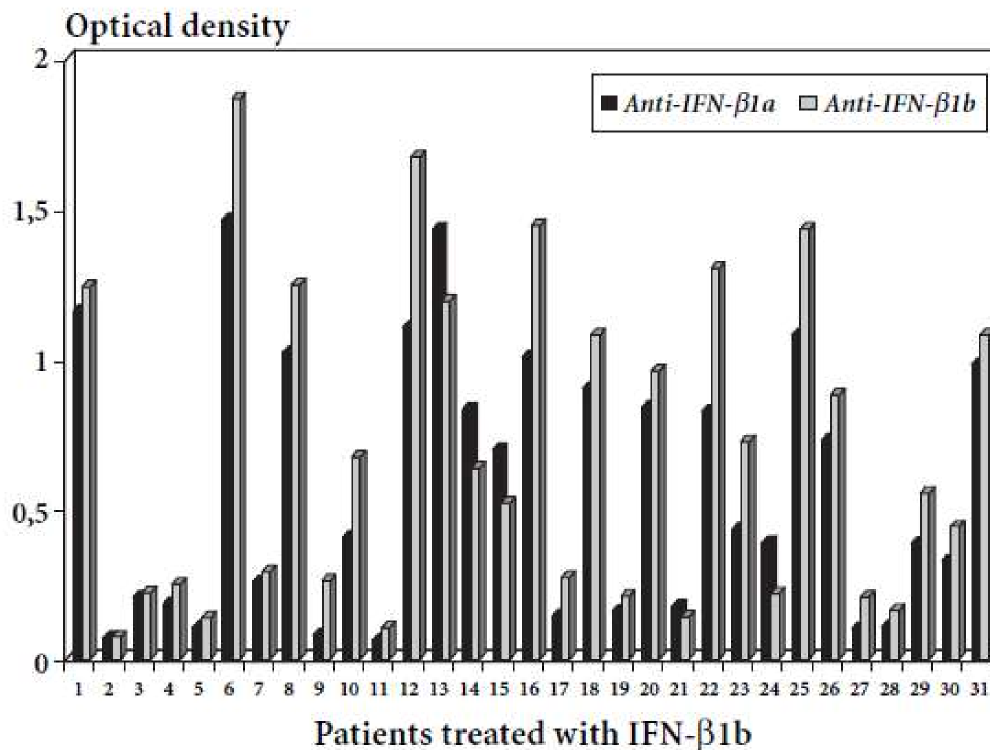


Figure 2. a Cross-reactive study. The antibody recognition of IFN-β_{1a} (*black bars*) and IFN-β_{1b} (*light bars*) was determined in the sera collected after 1 year of treatment from all patients treated with IFN-β_{1a} (n=22). **b** Cross-reactive study. The antibody recognition of IFN-β_{1a} (*black bars*) and IFN-β_{1b} (*light bars*) was determined in the sera collected after 1 year of treatment from all patients treated with IFN-β_{1b} (n=31)