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A routine-qualified flow cytometric method for the identification of multiple sclerosis patients with a reduced therapeutic effectiveness of natalizumab

Abstract

Background: Natalizumab-neutralizing antibodies (NABs) occur in 9% of natalizumab-treated multiple sclerosis (MS) patients. Loss of clinical and biological efficacy in patients with persisting NABs requires termination of natalizumab treatment. Because high-titer NABs are strongly associated with persistence of NABs, we investigated if determination of natalizumab saturation levels of α-4 integrins by flow cytometry has the potential to identify patients early with NABs.

Methods: Cell-bound natalizumab and natalizumab saturation of α-4 integrins on T cells were detected by flow cytometry using a monoclonal anti-human IgG4 antibody. Peripheral blood mononuclear cells were enriched from venous blood collected at the start (baseline) and every 4 weeks immediately before subsequent infusions until up to 9 months from natalizumab-treated patients with NABs (n=4) and at the start and after 1 (n=15), 2 (n=14), 3 (n=9), 6 (n=7), and 9 months (n=3) from natalizumab-treated patients without NABs. Natalizumab saturation levels (in %) of T cells were determined by relating median fluorescence intensities (MFIs) of in vivo bound natalizumab to MFIs after in vitro incubation with saturating amounts of natalizumab. Determination of serum NABs was performed by ELISA.

Results: In patients without NABs, the median natalizumab saturation level of T cells over 9 months was 75% (confidence interval of 95%: 72–78%). In two of the four patients with NABs, the natalizumab saturation level of T cells only reached approximately 50% after the first infusion and further declined to baseline levels with the second infusion. Low-titer NABs were measured after the first infusion and development of persistent high-titer NABs led to termination of natalizumab treatment after 6 months. In another two patients, the natalizumab saturation level of T cells was 74% and 68% after the first infusion, temporarily decreased to approximately baseline levels and re-increased after approximately 6 months. Transient NABs were detected after 2 and 3 months, which resolved after 5 and 6 months.

Conclusions: Monitoring the natalizumab saturation level on T cells is a fast and reliable method to identify patients with a reduced treatment effect due to NABs. Both high- and low-titer NABs were equally effective in reducing cellular natalizumab saturation levels. We were able to show that monitoring the natalizumab saturation levels by flow cytometry, is a sensitive method for detecting a prolonged NAB-mediated reduced treatment effect because NABs are apparently effective longer than suggested by the detection limit of ELISA.

Keywords: biomarker; flow cytometry; multiple sclerosis; natalizumab; neutralizing antibodies.

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Introduction

Natalizumab (Tysabri) is the first humanized monoclonal antibody approved for the treatment of relapsing remitting multiple sclerosis (MS). This humanized antibody binds directly to the α-4 subunit of the adhesion molecule

(AM) VLA-4 (Very Late Antigen-4, α-4-β-1; CD49dCD29) on immune cells. The antibody-mediated blocking of the interaction of VLA-4 with its ligands VCAM-1 (vascular cell adhesion molecule-1) on the endothelial cells, prevents the transmigration of immune cells across the blood-brain barrier into the central nervous system (CNS) [1, 2].

The treatment of MS patients with natalizumab showed a significant reduction in the relapse rate, reduced numbers of new or expanding T2 lesions on the MRI and a slowing of the disease's progression [3, 4]. However, the opposite side of this impressive therapeutic effect are life-threatening complications, such as progressive multifocal leukoencephalopathy (PML). Furthermore, occurrence of neutralizing antibodies (NAB) against natalizumab can lead to allergic reactions and a reduced effectiveness of Tysabri therapy [5, 6]. NAB were detected in 9% (n=57 patients) of patients according to the AFFIRM study, of which 3% were transiently and 6% were consistently positive. In transiently NAB positive MS patients, the full effectiveness of therapy was again reached after about 6 months of treatment (time until patients were NAB negative again). In case of persistent NAB, a reduced clinical effectiveness, an increased incidence with infusion-related, and undesired events and relapses are to be expected [5, 7, 8]. In one study, we were able to show that NAB could not be detected temporarily shortly after the infusion, because NAB and natalizumab neutralize each other. This was also demonstrated by a short-term reduction in the α-4 integrin level (CD49d) on immune cells and by the reduction of the sVCAM-1 level in the serum. But this effect, triggered by the natalizumab infusion, disappeared after a few days: α-4 integrin and sVCAM-1 levels recovered and NAB were detected again [7].

In patients with NAB the effect of natalizumab is not given over the entire period, and there is an increased risk of disease progression. It is important to develop a method that allows for an early identification of such patients, who, therefore, can be closer monitored during infusions and changes in the treatment can be undertaken if necessary.

Studies have shown that a flow cytometric monitoring of the CD49d expression on T lymphocytes might a possible biomarker for monitoring the effectiveness of natalizumab [9, 10]. However, other studies indicated that natalizumab saturation levels of immune cells are a more robust parameter than CD49d expression levels for drawing conclusions about the success of natalizumab treatment [11, 12]. In the current study, we examined whether analyzing the natalizumab saturation level on T lymphocytes by flow cytometry represents a sensitive method to early identify patients with a reduced therapeutic success and increased probability of infusion-related side effectiveness due to NAB. The flow cytometric results about natalizumab saturation level were compared with the findings of serological NAB testings by ELISAs.

Materials and methods

Patients

Nineteen patients (13 women, 6 men, aged 18–65 years, median: 46 years) with clinically defined relapsing-remitting MS according to the revised McDonald criteria of 2010 [13] were included in this study. Of the 19 patients, 4 patients (4 women, median: 47 years) had developed NAB. All patients received the standard dose of 300 mg of natalizumab (intravenous infusion) every 4 weeks. Venous blood was collected from MS patients without NAB prior to the start of natalizumab treatment and after 4 (n=15), 8 (n=14), 12 (n=9), 24 (n=7) and 36 (n=3) weeks – each time directly prior to the next infusion. Venous blood was collected from the 4 MS patients with NAB every 4 weeks, each time directly prior to the next infusion. The study was approved by the local Ethics Committee (Ethics Committee of Salzburg 415-E774/6-2007), and all patients gave their written consent.

Sample processing

Mononuclear cells (MNC) were isolated from venous blood using Vacutainer CPT tubes (Becton Dickinson AG, Basel, Switzerland) according to the instructions of the company and diluted to a concentration of 1×10^6 cells/mL for the determination of the cellular natalizumab saturation level. For determination of in vivo bound natalizumab 100 µL cell suspension were stained with the respective antibodies (for cell-bound natalizumab: anti-human IgG4 (α-huIgG4), clone HP6025, FITC, and for the T-cells: CD3, clone UCHT1, ECD). The cells were then washed and fixed with a 1% formaldehyde solution. For the determination of 100% natalizumab saturation, the cells were incubated with saturating amounts of natalizumab (10 µg/mL) prior to staining with the above mentioned antibodies. Natalizumab saturation levels of T cells were analyzed using a 5-color flow cytometer (Cytomics FC500, Beckman Coulter,

Vienna, Austria). The natalizumab saturation level (in %, also see formula) of the T cells was then determined by relating the median fluorescence intensity (MFI) of in vivo bound natalizumab to the MFI of in vitro saturated immune cells (10 µg/mL, representing 100% natalizumab saturation) [12].

Formula:

$$\text{Natalizumab saturation level (\%)} = \frac{\text{MFI in vivo bound natalizumab}}{\text{MFI in vitro saturated natalizumab}} \times 100$$

Serum NAB were determined by the national reference laboratory (Department of Neurology, Innsbruck) and classified as low and high NAB titers in case of positive ELISA results [14].

Statistics

The mean of the MFI and the 95% confidence interval were calculated using Microsoft Excel (Microsoft Office 2007, Redmond, VA, USA).

Results

We compared natalizumab saturation levels of T cells as measured by flow cytometry with the ELISA results on serum NAB (Figure 1 and Table 1).

Figure 1 shows that patients without NAB (point-like marker symbol; after 1 month n=15, after 2 months n=14, after 3 months n=9, after 6 months n=7, and after 9 months n=3 patients) exhibit an average natalizumab saturation level of 75% over a period of 9 months following the start of therapy (95% confidence interval, ranging from 72% to 78%). In contrast, the natalizumab-saturation curves of the four patients with NAB differ significantly from the natalizumab-saturation curves of patients without NAB.

Two of the four patients with NAB (patients 1 and 4) showed a low NAB titer 1 month after the first infusion and natalizumab saturation levels of only 47% and 51%, respectively (diamond-shaped and cross-shaped marker symbols). Prior to the third infusion, natalizumab saturation levels of both patients even were as low as pre-treatment baseline levels. Patient 4 (cross-shaped marker symbol) initially only had a low NAB titer after 2 months, and developed a high NAB titer 1 month later. In the other

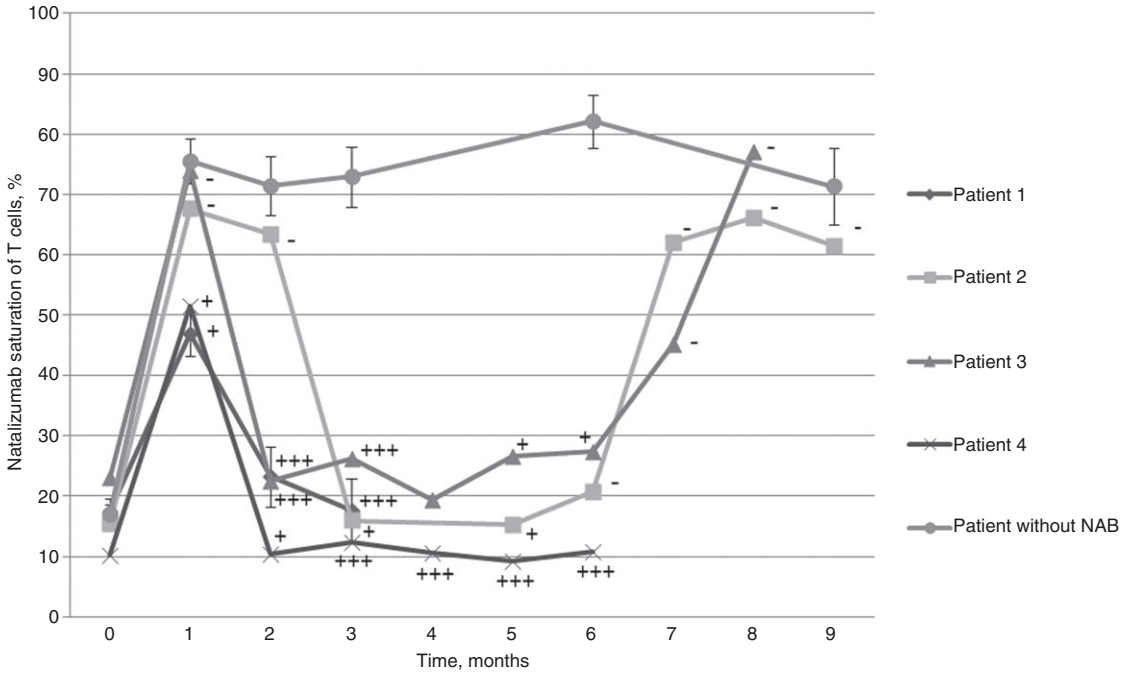

Figure 1 Natalizumab saturation of T lymphocytes (in %) from the flow cytometric analysis correlates with the NAB titers from ELISAs before and after the natalizumab treatment.

Average natalizumab saturation degree of CD3+ T cells of patients without NAB before natalizumab therapy, after 1 month (n=15), 2 (n=14), 3 (n=9), 6 (n=7) and 9 (n=3) months (• marker symbol). Course of natalizumab saturation in patients with persistent NAB (patient 1 with ◆ marker symbol and patient 4 with × marker symbol) and patients with transient NAB (patient 2 with ■ marker symbol and patient 3 with ▲ marker symbol). 95% confidence interval as error indicator for patients without NAB; –, negative NAB titer; +, low positive NAB titer; +++, high positive NAB titer.

Table 1 Flow cytometric analyses of natalizumab saturation (in %) of T lymphocytes were correlated with the NAB titers from ELISAs before and after natalizumab treatment.

Time, months	Pat. without NAB		Pat. with NAB							
	Mean NS, %	NAB	Patient no. 1		Patient no. 2		Patient no. 3		Patient no. 4	
			NS, %	NAB	NS, %	NAB	NS, %	NAB	NS, %	NAB
0 (Baseline)	17	×	17	×	15	×	23	×	10	×
1	76	–	47	+	68	–	74	–	51	+
2	71	–	23	+++	63	–	22	+++	10	+
3	73	–	18	+++	16	+	26	+++	12	+++
4	×	×	×	+++	×	×	19	×	11	+++
5	×	×	×	×	15	+	27	+	9	+++
6	82	–	Discontinuation of treatment		21	–	27	+	11	+++
7	×	×			62	–	45	–	Discontinuation of treatment	
8	×	×			66	–	77	–		
9	71	–			62	–				

Pat., Patients; NS, Natalizumab saturation; NAB, Neutralizing antibodies against natalizumab; Baseline, before start of therapy; ×, no measurement; –, negative NAB titer; +, low positive NAB titer; +++, high positive NAB titer.
Average natalizumab saturation degree of CD3+ T cells of patients without NAB before natalizumab therapy, after 1 month (n=15), 2 (n=14), 3 (n=9), 6 (n=7) and 9 (n=3) months. By comparison, the course of natalizumab saturation in patients with persistent NAB (patient 1 and patient 4) and patients with transient NAB (patient 2 and patient 3).

patient (diamond-shaped marker symbol) high NAB titer could be detected already after the second infusion. In both patients, the persistently high NAB titers led to the discontinuation of natalizumab treatment after about 6 months.

Patient 3 (triangular marker symbol) did not exhibit any abnormality for both the natalizumab saturation level (74%) and the NAB titer (negative) after the first infusion. After the second infusion, however, the natalizumab saturation dropped to pre-treatment levels. At the same time, the serological analysis revealed high-titer NAB. By contrast, patient 2 (square marker symbol) showed slightly diminished natalizumab saturation levels after the first and second infusions (68% after the first infusion, 63% after the second), while ELISA results for serum NAB were still negative. After the third infusion, natalizumab saturation levels dropped to the pre-treatment level, and high-titer NAB were detected by ELISA. In these two patients NAB were transient. The natalizumab saturation levels of immune cells recovered after 5 to 6 months, and the ELISA test for NAB was negative again. Interestingly, cellular natalizumab saturation levels were still decreased compared to patients without NAB but ELISA results already were negative. Pretreatment natalizumab saturation levels of about 17% (standard deviation of 3%) reflect background noise (due to the anti-human IgG4 antibody).

Discussion

Natalizumab is an important and highly effective therapy in the treatment of patients with active relapsing-remitting MS. However, the excellent therapeutic effect is overshadowed by side effects, such as PML, NAB, allergic reactions, as well as a slightly increased susceptibility to infection in some cases [5, 6]. About 6% of natalizumab-treated patients develop persistent NAB [5, 8], and have to discontinue the treatment for this reason. It is important to have a biomaker which is informative if the treatment can be effective. This study, and previous studies from our group, have shown that the natalizumab saturation level of immune cells can be such a biomarker [11, 12]. We demonstrated the importance of regular 4-weekly infusion intervals by showing that patients experience strong fluctuations in their cellular natalizumab saturation levels in case of prolonged infusion intervals of 6–8 weeks [12]. Furthermore, we demonstrated in a patient with NAB that freshly infused natalizumab transiently neutralized NAB to the end that serum NAB were no longer detectable but natalizumab saturation levels of immune cells had recovered. This implies that natalizumab can unfold its therapeutic effect during this period. This effect, however, is temporary and dependent on the dose [7].

In the current study, we have demonstrated that natalizumab saturation levels of immune cells determined

by flow cytometry is an appropriate method to detect the presence of NAB in the blood of natalizumab-treated patients. As exemplified in patient 2, analyzing the cellular natalizumab saturation level can yield an early indication about the development of NAB. This was demonstrated by slightly decreased natalizumab saturation levels of lymphocytes from patient 2 whereas NAB test results still were below the detection limit. This finding corroborated the observation that in both patients with transient NAB (patients 2 and 3), the cellular natalizumab saturation levels were still reduced, despite NAB had disappeared according to the negative ELISA results.

Another observation was that high or low serum NAB titers do not necessarily correlate with natalizumab saturation levels of immune cells. Patient 4, for example, had low titer NAB after 2 months which apparently were sufficient to vastly neutralize natalizumab, since the natalizumab saturation level of lymphocytes corresponded to those of untreated patients. Patient 2, too, exhibited low or even negative NAB titers combined with low natalizumab saturation levels until after about 6 months when natalizumab saturation levels increased again to similar values of patients without NAB. These data show that neither the natalizumab saturation level of lymphocytes nor the extent of NAB titers allow for conclusions about persistence or transience of NAB. The determination of natalizumab saturation levels of T lymphocytes by flow cytometry is a well-suited method to identify patients with an increased risk to develop NAB. It allows to focus more attention on them including the option for early considerations about an alternative treatment since they are at an increased risk of further disease progression due to the NAB-mediated reduced treatment effect. However further studies are warranted to confirm the suspected connection between therapeutic effectiveness (blocking of α-4 integrin) and the clinical therapeutic effect.

Interestingly, still another possible clinical application emerges from very recent data [15]. A collective of five patients gave rise to the idea that determining the natalizumab saturation level of lymphocytes from patients who are to be switched from natalizumab to fingolimod could be useful to identify the optimal time-point for starting the sequential therapy. In this context, there is an urgent need for a biomarker for the optimal duration of a treatment interruption between the two immunosuppressive agents. On the one hand, an accumulating immunosuppressive effect with consecutive opportunistic infections should be avoided. On the other hand, it is important to ensure that patients with a highly active disease progression are given adequate disease-modifying protection. In this case, too, future studies will clarify whether the flow cytometric analysis of natalizumab saturation levels of lymphocytes can be used routinely for this specific issue.

Conflict of interest statement

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