Immunological Markers of Optimal Response to Natalizumab in Multiple Sclerosis

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Objective: To explore cell subsets and molecules that changed specifically in patients with multiple sclerosis (MS) who had an optimal response to natalizumab. Natalizumab is a monoclonal antibody that inhibits the migration of activated immune cells to the central nervous system. It shows high efficacy in modifying the natural history of MS and induces freedom of disease activity in about 40% of treated patients with MS.

Design: Prospective study of intrathecal immunoglobulin synthesis and cerebrospinal fluid lymphocyte subsets in patients with MS before and 1 year after beginning treatment with natalizumab. We monitored clinical and magnetic resonance imaging activity during a median time of 2 years.

Setting: Two tertiary hospitals from the Spanish National Health Service.

Patients: A total of 23 patients with MS.

Main Outcome Measures: The differences between patients free of disease activity and patients with active disease during treatment.

Results: Of the 23 patients, 10 (43.5%) remained free of disease activity during follow-up. The remaining 13 patients (56.5%) had relapses or new lesions despite natalizumab therapy. We did not find differences in demographic variables or clinical data between both groups prior to natalizumab therapy. All patients showed a decrease in cerebrospinal fluid CD4 cells regardless of their response to treatment. Conversely, only patients free of disease activity showed a decrease in local IgM and, to a lesser extent, in IgG synthesis. They also showed lower percentages of B cells, particularly of CD5 and plasmablast subsets that virtually disappeared after treatment with natalizumab.

Conclusion: These data indicate that inhibition of intrathecal antibody synthesis is associated with a complete therapeutic response to natalizumab in patients with aggressive MS.

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Multiple sclerosis (MS) is the most frequent chronic demyelinating disease of the central nervous system and a major cause of disability in young adults. It is considered to be an autoimmune disease in which activated lymphocytes enter the central nervous system and trigger an inflammatory cascade that leads to recruitment of other immune cells. This results in demyelination and axonal loss that leads to irreversible neurological deficits. Treatment with interferon beta or glatiramer acetate, the first disease-modifying therapies used to treat MS, improve the natural history of the disease and delay the onset of permanent neurological deficits. Nevertheless, their beneficial effect is partial because they only reduce the relapse rate by about 30%, and their effect on disability progression is more controversial. Natalizumab is a humanized monoclonal antibody directed against the α4 chain of the adhesion molecule VLA-4. It was approved by the US Food and Drug Administration for the treatment of relapsing-remitting MS in 2004. It decreases the relapse rate by up to 68% and the rate of progression of disability by 42%, reducing magnetic resonance imaging (MRI) activity by up to 92%. Post hoc analyses of the pivotal trial (ie, the Natalizumab Safety and Efficacy in Relapsing-Remitting Multiple Sclerosis study [AFFIRM]) showed that the proportion of patients with no new or enlarg-
ing MRI-detected lesions during the study period was greater in the natalizumab group than in the placebo group.³ Therefore, disease remission seems an attainable goal for patients with MS who are treated with this new agent, something that the other disease-modifying therapies could not achieve in many cases.⁴ It has been shown that treatment with natalizumab disrupts the migration of lymphocytes to the central nervous system.² It induces a persistent decrease in the number of T cells, B cells, and plasma cells present in cerebrospinal fluid (CSF).⁵,⁶ Additional mechanisms may also play a role in the beneficial effects of natalizumab; for example, treatment with natalizumab induces variations in serum and CSF cytokine profiles and may have an effect on T-cell activation.⁷,⁸ The aim of our study was to ascertain whether any of these mechanisms are associated with freedom of disease activity in MS. This would provide clues to understanding key points of the mechanism of action of this drug and could provide biomarkers to predict response and to prevent adverse effects to natalizumab.

## METHODS

### PATIENTS

Our study was approved by the ethical committees of Hospital Ramón y Cajal, Madrid, and Hospital Virgen Macarena, Sevilla, Spain. Written informed consent was obtained from all patients before entry into the study. A total of 23 patients diagnosed with MS according to the diagnostic McDonald criteria⁹ were included.

## PATIENT TREATMENT AND FOLLOW-UP

Patients received natalizumab at a dose of 300 mg by intravenous infusion every 4 weeks. They were examined every 3 months, with an additional neurological assessment in cases of relapse. Relapses were defined as per Poser et al.¹⁰ Exacerbation was defined as a worsening of neurological impairment or an appearance of a new symptom or abnormality attributable to MS, lasting at least 24 hours, and preceded by stability for at least 1 month. The Expanded Disability Status Scale (EDSS) score was determined at each visit. When EDSS assessment was performed during a relapse, a new EDSS score was recorded a month later, when the neurological situation had stabilized. An MRI scan of the brain was performed within a month of treatment initiation and 1 and 2 years after starting treatment with natalizumab. The MRI scans were performed in 1.5-T scanners with standard head coils. A slice thickness of 5 mm and a field of view of 24 cm were acquired to obtain contiguous axial sections that covered the entire brain from the foramen magnum to the vertex. The following sequences were performed: T1-weighted imaging, axial fluid-attenuated inversion recovery T2-weighted imaging, axial T2-weighted imaging, axial proton density T2-weighted imaging, and T1-weighted imaging with gadolinium.

## SAMPLES

Samples of CSF and blood were obtained from patients prior to and 1 year after starting natalizumab therapy. Paired serum and CSF samples obtained from patients at Hospital Virgen Macarena in Sevilla, Spain, were aliquoted and stored at ~80°C until assayed. For patients at Hospital Ramón y Cajal in Madrid, Spain, fresh CSF samples (4-6 mL) were centrifuged at 500g for 15 minutes, and the cellular pellet processed. Then, these CSF and serum samples were aliquoted and stored at ~80°C until quantitative and oligoclonal band studies were performed.

Oligoclonal bands were analyzed by isoelectric focusing and immunoblotting, as previously described.¹¹-¹³ IgM, IgG, and albumin were quantified in serum and CSF samples by nephelometry in a Siemens nephelometer. IgG and IgM indexes were calculated as previously described.¹⁴,¹⁵It has been defined that IgG and IgM indexes higher than 0.77 and 0.1, respectively, reflect intrathecal IgG and IgM synthesis. The CSF CXCL13 levels were quantified by use of an enzyme-linked immunosorbent assay (R&D Diagnostics) following the manufacturer’s instructions.

## LABELING OF CELLS AND FLOW CYTOMETRY

The following monoclonal antibodies were used: control mouse isotypes IgG1-phycoerythrin (PE), IgG1-PerCP-Cy5.5, IgG1-allophycocyanin (APC), anti–CD19 PerCP-Cy5.5, anti–CD38-APC, anti–CD4 PerCP-Cy5.5, anti–CD3–PE, anti–CD8-APC, and anti–CD45-fluorescein isothiocyanate (FITC) were from BD Biosciences; and anti–CD5–PE was from Beckman Coulter. The CSF cells were washed, resuspended in 100 µL of phosphate-buffered saline, divided in 3 identical aliquots, and labeled with optimal concentrations of anti–CD45-FITC, and the isotype controls conjugated with PE, PerCP, and APC or with anti–CD45-FITC, anti–CD5–PE, anti–CD19 PerCP-Cy5.5, and anti–CD38–APC or with anti–CD45-FITC, anti–CD3–PE, anti–CD4

## Table 1. Clinical Characteristics of Patients Classified According to Their Response to Natalizumab Treatment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients Free of New Disease Activity</th>
<th>Patients With Ongoing MS Disease Activity</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>35.6 (2.9)</td>
<td>38.7 (2.6)</td>
<td>.38</td>
</tr>
<tr>
<td>Sex, No. of patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>10</td>
<td>.65</td>
</tr>
<tr>
<td>Disease duration, y</td>
<td>12.3 (3.4)</td>
<td>8.9 (1.4)</td>
<td>.77</td>
</tr>
<tr>
<td>EDSS score at entry</td>
<td>4.5 (0.6)</td>
<td>5.5 (0.6)</td>
<td>.19</td>
</tr>
<tr>
<td>Relapses 2 y before treatment, No.</td>
<td>3.2 (0.6)</td>
<td>2.9 (0.4)</td>
<td>.77</td>
</tr>
<tr>
<td>T2 lesions at entry, No.</td>
<td>28.7 (5.4)</td>
<td>34.0 (6.9)</td>
<td>.77</td>
</tr>
<tr>
<td>Washout period, d</td>
<td>118.7 (31.3)</td>
<td>140.3 (23.6)</td>
<td>.92</td>
</tr>
<tr>
<td>Duration of treatment, mo</td>
<td>24.7 (1.1)</td>
<td>24.3 (1.1)</td>
<td>.92</td>
</tr>
<tr>
<td>Increased EDSS score after treatment, No. of patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td></td>
<td>.48</td>
</tr>
<tr>
<td>No</td>
<td>10</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Relapses during treatment, No. of patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td></td>
<td>.01</td>
</tr>
<tr>
<td>No</td>
<td>10</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>New MRI-detected lesions during treatment, No. of patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>No</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: EDSS, Expanded Disability Status Scale; MRI, magnetic resonance imaging; MS, multiple sclerosis.
We studied 23 consecutive patients with MS who underwent natalizumab treatment. All but 1 initiated this therapy after the failure of treatment with first-line immunomodulatory drugs with a variable time of washout. All patients had high inflammatory activity before treatment. The mean (SE) number of relapses in the 2 years before starting natalizumab was 3.04 (0.37) (median, 3; range, 1-7; interquartile range [IQR], 2-4). The mean (SE) number of T2 lesions before treatment was 31.7 (4.5) (median, 3; range, 1-7; IQR, 9-43). Nineteen patients showed both IgG and IgM oligoclonal bands restricted to CSF, 2 patients showed IgG but not IgM bands, and another 2 patients lacked IgG and IgM oligoclonal bands in CSF. Patients were treated with natalizumab for a mean (SE) of 24.48 (0.75) months (median, 24 months; range, 15-31 months; IQR, 24-24 months). No patient had anti-natalizumab antibodies. We divided the patients into 2 groups according to their response to treatment. The first group (13 patients [56.5%]) comprised those individuals who showed disease activity during natalizumab treatment; this disease activity was defined as experiencing any of the following events (alone or in combination): onset of MS relapses; sustained increased EDSS score for more than 3 months; or new or enlarging T2 hyperintense or enhancing lesions on MRI scan despite natalizumab therapy. Two patients from this group had an increased EDSS score and new MRI-detected lesions, 4 patients had new relapses and new MRI-detected lesions, 2 patients had new relapses, and 5 patients had revealed only new T2 lesions on MRI scans during natalizumab treatment. The second group (10 patients [43.5%]) included patients who were free of disease activity because they did not experience any of those disease activity–related events.

As shown in Table 1, differences in response to natalizumab were not associated with demographic or clinical variables at entry. However, we found an association between response to treatment and laboratory data. Table 2 shows the results of CSF samples obtained before and 1 year after treatment initiation. All patients free of new disease activity had IgG and IgM bands restricted to CSF before treatment. After treatment with natalizumab, they all experienced a reduction in IgG and IgM indexes. In all cases, the IgM index decreased below 0.1, which is the cutoff value that indicates intrathecal IgM synthesis. Moreover, in 70% of patients free...
of new disease activity, the oligoclonal IgM bands disappeared after the second lumbar puncture (P = .003) (see the representative example in Figure 1A). In addition, the oligoclonal IgG pattern also changed in some of these patients, with some bands disappearing in the second CSF sample (Figure 1C). The decrease in intrathecal immunoglobulin synthesis was not so homogeneous in patients with continued disease activity following natalizumab treatment. Only 50% of them showed a decrease in IgG index. They had no changes in IgG oligoclonal patterns (Figure 1D). Particularly striking was the decrease in IgG index. They had no changes in IgG oligoclonal bands after natalizumab treatment who had IgM bands that none of the 9 patients with continued disease activity following natalizumab treatment who had IgM bands after treatment had no IgM oligoclonal bands after the second lumbar puncture (see the representative example in Figure 1B). In addition, only 1 of these 9 patients had an IgM index below 0.1 after natalizumab treatment.

**Figure 2** shows overall changes in immunoglobulin indexes in each group. In the group of patients with ongoing disease activity during treatment, natalizumab treatment was not associated with significant changes in IgM index (mean [SE], 0.29 [0.09] before treatment vs 0.15 [0.03] after treatment; P = .97) or IgG index (mean [SE], 1.05 [0.14] before treatment vs 0.99 [0.14] after natalizumab; P = .75). Conversely, we found clear differences between patients free of new disease activity. The mean (SE) IgM index decreased from 0.19 (0.05) before treatment to 0.06 (0.01) after treatment (P = .001). The mean (SE) IgG index changed in these patients from 1.11 (0.17) to 0.68 (0.06), exhibiting a trend (P = .052), although not statistically significant, toward decreased levels.

To further investigate the association between down-regulation of B-cell response and freedom of disease activity in response to natalizumab, we explored B-cell subsets in the 2 serial CSF samples that we were able to obtain from 6 patients in each group. All these patients had oligoclonal IgM bands in their first CSF samples. Results are shown in **Table 3**. Patients free of disease activity showed a decrease in the percentage of total B cells (P = .01) and of CD5+ (P = .007), CD5− (P = .01), and CD38+ (plasmablasts; P = .009) B-cell subsets. Moreover, CD5+ B cells and plasmablasts virtually disappeared from the CSF samples after natalizumab therapy. Patients who had ongoing disease activity during natalizumab treatment did not exhibit significant decreases in the percentage of total CSF B cells or in the percentages of the CD5+, CD5−, or CD38− B-cell subsets. The reduction in B-cell numbers was not associated with a reduction in CXCL13 levels. This chemokine was no longer detectable in CSF samples after natalizumab treatment in all cases, independent of the presence or absence of ongoing activity of the disease. Overall, the mean (SE) measured CXCL13 levels before and after natalizumab treatment were 17.9 (8.9) and 0.0 (0.0) pg/mL, respectively (P = .002). With respect to CSF T cells (Table 3), both groups experienced a clear decrease in the percentage of CD4+ T cells, regardless of their response to natalizumab (patients with ongoing disease activity, P = .008; patients free of new disease activity, P = .002) and an increase in CD8− T-cell percentage (patients with active disease, P = .004; patients free of new disease activity, P = .002).

**COMMENT**

Previous studies explored different immune mechanisms associated with the effect of natalizumab treatment. Treatment with natalizumab is known to modify leukocyte subsets in CSF, as reflected by decreasing CD4+ T-cell and B-cell counts and by increasing CD8− T-cell percentages. In addition, it has been reported that a good response to natalizumab is associated with a decrease in the levels of neurofilament light chains, a putative marker of axonal damage, in CSF. However, little is known about the mechanisms associated with freedom from ongoing disease activity during natalizumab treatment. Identifying these mechanisms will contribute to a further understanding of the key immunological processes that contribute to active disease in patients with MS.

We studied additional CSF samples in a cohort of 23 patients with active MS who were treated with natalizumab and followed prospectively for a median time of 2 years. Of these 23 patients, 22 did not respond to first-line immunomodulatory therapies. Treatment with natalizumab decreased the rate of disability progression and reduced by 90% the number of relapses in the overall group. In addition, 10 patients (43.5%) remained entirely free of new disease activity; that is, they did not have new MS relapses or increased EDSS scores, nor did they develop new brain MRI-detected lesions during natalizumab therapy. These percentages were similar to those...
obtained in the pivotal trial by Polman et al. We studied intrathecal immunoglobulin synthesis and lymphocyte subsets in CSF samples obtained before and 1 year after starting natalizumab treatment. Of note, although patients were selected to receive natalizumab by clinical criteria of active disease, most of them (19 of 23 patients) exhibited intrathecal IgM synthesis before natalizumab treatment. This is consistent with prior reports that local IgM synthesis is associated with an aggressive MS course.

Of the 13 patients with active disease despite natalizumab therapy, 9 (69.2%) showed intrathecal IgM synthesis before treatment. They did not experience a significant decrease in IgG or IgM indexes after receiving natalizumab. In fact, the IgM index remained above 0.1 (the cutoff value that indicates intrathecal IgM synthesis) in 90% of these patients after treatment. This may be due to the relatively high CSF IgM indexes that many of these patients exhibited before treatment initiation, which perhaps could not be fully inhibited during 1 year of treatment. Four active patients did not exhibit oligoclonal IgM bands. Two of them also lacked IgG bands. They exhibited low values of IgG and IgM indexes before natalizumab treatment, and these low

Table 3. Intrathecal Lymphocyte Subsets at Entry Into Study and After Natalizumab Treatment, Classified According to Their Response to Natalizumab

<table>
<thead>
<tr>
<th>CSF Sample</th>
<th>Patients Free of New Disease Activity</th>
<th>Patients With Ongoing MS Disease Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At Entry</td>
<td>After Treatment</td>
</tr>
<tr>
<td>B cells</td>
<td>2.45 (0.31)</td>
<td>0.56 (0.20)</td>
</tr>
<tr>
<td>CD5⁺ B cells</td>
<td>0.70 (0.08)</td>
<td>0.11 (0.08)</td>
</tr>
<tr>
<td>CD5⁻ B cells</td>
<td>1.74 (0.30)</td>
<td>0.45 (0.21)</td>
</tr>
<tr>
<td>Plasmablasts</td>
<td>1.06 (0.38)</td>
<td>0.0 (0.0)</td>
</tr>
<tr>
<td>CD4⁺ T cells</td>
<td>58.40 (2.09)</td>
<td>34.53 (4.35)</td>
</tr>
<tr>
<td>CD8⁺ T cells</td>
<td>24.61 (3.94)</td>
<td>51.72 (4.46)</td>
</tr>
</tbody>
</table>

Abbreviations: CSF, cerebrospinal fluid; MS, multiple sclerosis.
values did not change perceptibly during treatment. This seems to indicate that antibodies do not play an important role in disease activity in this small group of patients.

All patients who were free of new disease activity exhibited intrathecal IgM synthesis before treatment. In contrast to patients with active disease, they all experienced a considerable decrease in the IgM index after being treated with natalizumab, and, even more, in 70% of them, the IgM bands disappeared. We also observed a moderate decrease in the IgG index and changes in the oligoclonal IgG pattern in these patients. Of note, this effect was not produced by other treatments,22–24 as IgG patterns have been considered an ongoing fingerprint of the disease in patients with MS.24

Data on B- and T-cell subsets were in line with these results. Both groups of patients experienced a considerable reduction in the percentage of CD4+ T cells during treatment, but only patients who were free of new disease activity also showed a significant decrease in percentage of B cells and plasmablasts in CSF samples. In fact, for these patients who were free of new disease activity, both the CD5+ B cells that are responsible for intrathecal IgM secretion and the plasmablasts that are strongly associated with intrathecal IgG synthesis almost disappeared from the CSF samples. Divergences in the reduction of intrathecal B cells between patients free of new disease activity and patients with ongoing disease activity following treatment were not associated with different CSF levels of CXCL13, a chemokine implied in B-cell migration. This chemokine appeared to decrease uniformly in both groups of patients. An alternative explanation for the differences in the reduction in local B cells between these 2 groups of patients may be the different expressions of VLA-4 shown by B lymphocytes depending of their activation status,25 which can affect inhibition of B-cell migration by natalizumab.8

Our results show that the reduction of CSF B-cell counts induced by natalizumab runs in parallel with the decrease in intrathecal immunoglobulin synthesis, particularly evident in patients who appear to respond optimally to natalizumab. Most intrathecal immunoglobulins are synthesized by plasmablasts and plasma cells in MS.26 These cells express high levels of VLA-4,27 which is crucial for anchoring them to their microenvironment.27 Natalizumab’s action on these cells may contribute to the reduction of intrathecal immunoglobulin synthesis.

These data suggest that downregulation of local antibody synthesis within the central nervous system may contribute to an optimal response to natalizumab in patients with MS who have an aggressive disease course. However, it cannot be completely ruled out that a reduction in disease activity due to natalizumab might result in a decreased intrathecal humoral response. Further research is necessary to better understand the mechanisms undergoing optimal immune responses to natalizumab treatment.

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