Decrease in the Numbers of Dendritic Cells and CD4⁺ T Cells in Cerebral Perivascular Spaces Due to Natalizumab

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Objective: To extend our studies on the prolonged and differential effect of natalizumab on T lymphocyte numbers in the cerebrospinal fluid, we investigated the number and phenotypes of leukocytes and the expression of major histocompatibility complex (MHC) classes I and II in cerebral perivascular spaces (CPVS). We hypothesized that natalizumab reduces the number of antigen presenting cells in CPVS.

Design: A case-control study in which inflammatory cell numbers in the CPVS of cerebral tissue were assessed by immunohistochemical staining.

Subjects: A patient with multiple sclerosis (MS) who developed progressive multifocal leukoencephalopathy (PML) during natalizumab therapy. Controls included location-matched cerebral autopsy material of patients without disease of the central nervous system, patients with MS not treated with natalizumab, and patients with PML not associated with natalizumab therapy.

Results: The absolute number of CPVS in the patient with MS treated with natalizumab was significantly lower than in the control groups owing to extensive destruction of the tissue architecture. The expression of MHC class II molecules and the number of CD209⁺ dendritic cells were significantly decreased in the CPVS of the patient with MS treated with natalizumab. No CD4⁺ T cells were detectable.

Conclusions: Our observations may explain the differential and prolonged effects of natalizumab therapy on leukocyte numbers in the cerebrospinal fluid.

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Our group recently tested the hypothesis that treatment with natalizumab interferes with CNS immune surveillance.¹² We demonstrated that natalizumab therapy decreases the number of all lymphocyte sub-sets in the cerebrospinal fluid (CSF) in patients with relapsing-remitting MS.¹³¹⁴ Two unexpected observations were also made: (1) The number of CD4⁺ T cells within the CSF was affected 10 times more than the number of CD8⁺ T lymphocytes. (2) In addition, we demonstrated that CD4⁺ T cells remained depressed, even 6 months after cessation of natalizumab therapy. Thus, the biological half-life of natalizumab significantly outlasts its pharmacological half-life.
In the present study, we speculated that natalizumab, in addition to blocking the entry of effector cells into the CNS by blocking VLA-4, reduces the number of antigen presenting cells (APCs) and the expression of major histocompatibility complex (MHC) class II in cerebral perivascular spaces (CPVS). Class II MHC is an absolute requirement for reactivation and retention of CD4+ T cells in peripheral tissues. A reduced number of APCs and diminished expression of MHC class II may explain the preferential and prolonged effect of natalizumab on CD4+ T cell numbers in the CNS. We examined autopsy cerebral tissue from 1 of the 2 previously reported patients who died of PML following natalizumab therapy, using immunohistochemistry. Location-matched cerebral autopsy material of a patient without disease of the CNS, 4 patients with MS not treated with natalizumab, and 2 patients with PML not associated with natalizumab were used as controls. A total of 1720 microscopic visual fields were evaluated. The absolute number of CPVS in the patient with MS who developed PML while taking natalizumab was significantly less than in all of the control groups owing to extensive destruction of the tissue architecture. Also, the expression of MHC class II molecules, the number of CD209+ dendritic cells (DC), and the number of CD4+ T cells were significantly reduced.

We conclude that prolonged natalizumab therapy reduces the number of CD209+ DCs and the expression of MHC class II in CPVS. This observation may explain the dramatic and prolonged effect of natalizumab on CD4+ T cell numbers in the CSF. The effect of natalizumab on APCs in CPVS may have significant implications for immune responses to self and foreign antigens.

**METHODS**

**PATIENTS**

Four patient groups were analyzed based on diagnosis and treatment history. Specifically, 1 patient had Burkitt lymphoma without any detectable CNS involvement, 4 patients had MS and had never received natalizumab therapy, 2 patients had PML not associated with natalizumab, and 1 patient had MS and developed PML during natalizumab therapy. The patient with MS who developed PML during natalizumab therapy died 38 days after his last dose of natalizumab. Thus, the drug was still having biological effects at the time of death. Patient characteristics are shown in the Table. Corresponding brain areas were carefully selected for each case (Figure 1). The area that was evaluated in all patients included periventricular white matter and basal ganglia from the left cerebral hemisphere. Approximately 50% of the tissue obtained from the patients with MS who developed PML during natalizumab therapy included an area that was normointense on magnetic resonance imaging, whereas the remainder of the tissue was hyperintense. In patients with PML not associated with natalizumab treatment, most of the examined tissue was clearly affected by the disease. In these patients, tissue from the right cerebral hemisphere had to be used. In all 4 patients with MS, there was evidence of chronic demyelination throughout the tissue sections. A total of 1720 visual fields were evaluated. The minimum number of visual fields per single stain per patient was 25. Of 1720 visual fields were evaluated. The minimum number of visual fields per single stain per patient was 25. All sections were read by 1 unblinded (M. del P.M.) and 2 blinded examiners (P.D.C. and R.W.). All cases included in this study were provided by the University of Colorado Health Sciences Center School of Medicine Department of Pathology Brain Bank. The postmortem interval between death and tissue preparation was less than 24 hours. In 1 case, the paper records of the autopsy were not available. All procedures were approved by the institutional review board at the University of Colorado Health Sciences Center School of Medicine.

**HISTOLOGICAL ANALYSES**

Immunohistochemistry was performed on formalin-fixed 5-µm paraffin sections using a biotin-avidin-peroxidase technique and visualized with diaminobenzidine. The following human-specific primary antibodies were used: CD209 (clone 120507; R&D Systems, Minneapolis, Minnesota), CD20 (clone L26; Dako, Glostrup, Denmark), CD68 (clone PG-M1; Dako), CD4 (clone 4B12; Novoceastra, Newcastle upon Tyne, England), CD8 (clone C8/144B; Dako), HLA-DR, -DP, and -DQ (WR18; AbD Serotec, Oxford, England), and β2 microglobulin (polyclonal; Dako). Immunohistochemical stains were carried out on an automated BenchMark XT (Ventana, Tucson, Arizona) apparatus. Forty fields from corresponding sections stained with each of these antibodies were evaluated.

Cerebral perivascular spaces were identified by morphological structure and MHC class I (β2 microglobulin) staining. Perivascularly located single positive cells were counted per visual field. Owing to the large number and confluence of MHC

**Table. Patient Characteristics**

<table>
<thead>
<tr>
<th>Patient No./Sex/Age, y</th>
<th>Diagnosis</th>
<th>Date of Diagnosis, y</th>
<th>Pharmacotherapies</th>
<th>Date of Death, y</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/57 Burkitt lymphoma</td>
<td>2005</td>
<td>NA</td>
<td>None</td>
<td>2006</td>
<td>Respiratory failure and septic shock</td>
</tr>
<tr>
<td>2/M/61 RRMS</td>
<td>NA</td>
<td>None</td>
<td>None</td>
<td>1994</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>3/F/53 MS</td>
<td>1965</td>
<td>ACTH, prednisolone, azathioprine</td>
<td>1994</td>
<td>MS comorbidities</td>
<td></td>
</tr>
<tr>
<td>4/F/66 MS</td>
<td>1984</td>
<td>NA</td>
<td>NA</td>
<td>1992</td>
<td>MS comorbidities</td>
</tr>
<tr>
<td>5/F/NA MS</td>
<td>1994</td>
<td>Seizure</td>
<td></td>
<td>1995</td>
<td>Seizure</td>
</tr>
<tr>
<td>7/M/55 HIV and PML</td>
<td>2000</td>
<td>Natalizumab, Interferon beta-1a (Avonex)</td>
<td>2005</td>
<td>PML</td>
<td></td>
</tr>
<tr>
<td>8/R/39 Myelogenous leukemia and PML</td>
<td>12 Microglia and PML</td>
<td>1994</td>
<td>Seizure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9/F/46 RRMS and PML</td>
<td>2000</td>
<td>Natalizumab, Interferon beta-1a (Avonex)</td>
<td>2005</td>
<td>PML</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ACTH, adrenocorticotropic hormone; HIV, human immunodeficiency virus; MS, multiple sclerosis with unknown clinical phenotype; NA, information not available; PML, progressive multifocal leukoencephalopathy; RRMS, relapsing-remitting MS.

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class I–positive cells, automated image analysis was performed. Specifically, total MHC class I integrated density (area / mean chromogen intensity) was calculated from 25 visual fields per sample using ImageJ Color Deconvolution software (National Institutes of Health, Bethesda, Maryland). Images were acquired at original magnification 40 using the AxioVision Ac Software (Carl Zeiss, Oberkochen, Germany) on a Zeiss Axiophot microscope model.

MAGNETIC RESONANCE IMAGING

A fast-spin echo inversion recovery sequence was performed 12 days before the death of the patient with PML. The repetition time was 10 002 milliseconds; echo time, 145 milliseconds; inversion time, 2200 milliseconds.

STATISTICAL ANALYSIS

Correlations between continuous and categorical variables were assessed using the Mann-Whitney U test. The means of 2 normally distributed samples were compared by t tests. P values less than .05 were considered significant. The standard error of the mean is shown.

RESULTS

CHARACTERIZATION OF NATALIZUMAB-ASSOCIATED PML BY A SIGNIFICANT DECREASE IN CPVS

The absolute number of CPVS in the tissue of a patient with MS who developed PML during natalizumab therapy was significantly decreased compared with brain tissue from a control patient with nonneurological diseases, patients with MS not treated with natalizumab, and patients with PML not associated with natalizumab therapy (Figure 2). There was no difference between brain tissue that appeared affected by PML vs areas that appeared not to be involved in the patient with MS who had received natalizumab.

ASSOCIATION OF NATALIZUMAB THERAPY WITH DECREASED EXPRESSION OF MHC CLASS II AND INCREASED EXPRESSION OF MHC CLASS I IN CPVS

Expression of MHC class II in the CPVS of a patient who developed PML during natalizumab therapy was significantly reduced in the tissue of a patient with MS who developed PML while taking natalizumab compared with that of patients with MS not treated with natalizumab and patients with PML not associated with natalizumab therapy (Figure 3A and B). In contrast, expression of MHC class I in the CPVS was significantly upregulated in a patient with MS who developed PML during natalizumab therapy compared with controls (Figure 3A and C).
REDUCTION OF APCs IN CPVS BY NATALIZUMAB THERAPY

In light of the observed decrease in MHC class II expression in the CPVS of a patient with MS who developed PML during natalizumab therapy, we examined the frequency of APCs that express these molecules on their surface. The number of CD209+ DC in brain sections of a patient with MS who developed PML while taking natalizumab was significantly decreased compared with brain tissue from a control patient with nonneurological diseases, tissue from patients with MS not treated with natalizumab, and patients with PML not associated with natalizumab therapy (Figure 4A and B). The number of CD20+B cells (Figure 4A and C) and CD68+ macrophages (Figure 4A and D) were also decreased compared with the other patient groups, but the observed differences were not statistically significant (Figure 4C and D).

DECREASED NUMBER OF CD4+ AND CD8+ T CELLS IN CPVS DUE TO NATALIZUMAB THERAPY

In contrast to the control groups, no CD4+ T cells were detectable in the CPVS of a patient with MS who developed PML during natalizumab therapy (Figure 5A and B). The number of CD8+ T cells in the CPVS of brain tissue from a control patient with nonneurological diseases was similar to the number of CD8+ T cells in a patient with MS who developed PML during natalizumab therapy (Figure 5A and C). However, there were fewer CD8+ T cells in the CPVS of a patient with MS who developed PML during natalizumab therapy than in patients with MS not treated with natalizumab or patients with PML not associated with natalizumab therapy (Figure 5A and C).

COMMENT

Multiple sclerosis is an inflammatory demyelinating disorder of the CNS of unknown etiology. It is thought that aberrant immune responses to self or foreign antigens initiate and perpetuate the disease’s activity.13-15 Macroscopically, MS lesions are mostly confined to CNS white matter and are found most frequently in periventricular areas of the brain.13 Most inflammatory infiltrates consist of CD4+ and CD8+ T cells, B cells, plasma cells, macrophages, and DC.16

Within the CNS, 2 compartments play a critical role in antigen presentation: the brain parenchyma and the CPVS. Within the parenchyma, microglia cells appear to have a critical function in initiation of inflammatory responses.17 The second CNS compartment that plays a perhaps more important role in antigen presentation are CPVS, or so-called Virchow-Robin spaces.18 There is now

Figure 3. A, Representative fields of histopathological examination. B and C, Major histocompatibility complex (MHC) class II– and I–positive cells per visual field (VF), respectively. Control patients had nonneurological diseases and healthy central nervous system tissue. MS indicates patients with multiple sclerosis not treated with natalizumab; PML, patients with progressive multifocal leukoencephalopathy not associated with natalizumab therapy; Natalizumab PML, a patient with MS who developed PML during natalizumab therapy; IntDen, International Density Standard. The standard error of the mean is shown. Images were acquired at magnification ×40.
Figure 4. A, Representative fields of histopathological examination. B-D, Number of CD209\(^+\) dendritic cells (DC), CD20\(^+\) B cells, and CD68\(^+\) macrophages, respectively, within the visual field (VF). Control patients had nonneurological diseases and healthy central nervous system tissue. MS indicates patients with multiple sclerosis not treated with natalizumab; PML, patients with progressive multifocal leukoencephalopathy not associated with natalizumab therapy; Natalizumab PML, a patient with MS who developed PML during natalizumab therapy. The standard error of the mean is shown. Images were acquired at magnification \( \times 40 \).
abundant evidence that hematopoietically-derived APCs, including macrophages and DCs, reside and present antigen in CPVS and that cells in this compartment play a crucial role in the initiation and perpetuation of CNS autoimmune disease. Macrophages and B lymphocytes are also competent APCs that are abundantly present in the CPVS. There is some evidence of the kinetics of APC turnover in CPVS. Using radiation bone marrow chimeras, Lassmann et al demonstrated that meningeal and perivascular monocytes are replaced over the course of several weeks by hematogenous cells under normal conditions, and this turnover is accelerated in experimental autoimmune encephalomyelitis. Another group of investigators showed an ongoing migration of macrophages from the peripheral blood into the CPVS. The exact mechanisms by which hematopoietic APCs gain access to CPVS in experimental autoimmune encephalomyelitis have not been studied. As VLA-4 is expressed on all myeloid and lymphoid cells, it is likely that this integrin plays a critical role in the egress of APCs from the blood into CPVS.

Natalizumab therapy reduces the number of CPVS-associated bone marrow–derived APCs within the CPVS. In contrast, MHC class I is expressed ubiquitously, including by astrocytes, endothelial cells, and pericytes, cells that constitute the CPVS that do not migrate to the brain from the peripheral blood and that are likely not affected by pharmacological VLA-4 antagonists. Up-regulation of MHC class I may reflect an increase in soluble proinflammatory mediators in the setting of an infection. Our observation may explain the prolonged effect of natalizumab on the reduction of CD4+ and CD8+ T cells in the CSF and the more pronounced effect on CD4+ T lymphocytes. Regarding the number of CPVS, the number of leukocytes, and the expression of MHC molecules in the tissue of the patient with MS who developed PML while taking natalizumab, there was no difference in areas that appeared normointense on magnetic resonance images and tissue that appeared hyperintense.

One important question is how many doses of natalizumab are required to significantly reduce the number of APCs in the CPVS and to impair the presentation of self and foreign antigens to T cells. In the absence of longitudinal immunohistopathologic CNS evaluations, which will not be feasible in human patients, it will be impossible to provide an accurate assessment. Based on clinical observations made in patients with relapsing-remitting MS treated with natalizumab, we propose the following sequence of events: activated leukocytes are capable of adhering to the endothelium of blood vessel walls and migrating into the CNS. In healthy brain and spinal...
cord, cells that do not persist as CNS autoantigens or foreign antigens are not being presented to them in the context of MHC (Figure 6A). During inflammation, antigen-specific T cells do persist for longer periods of time and may play an important role in the amplification of an immune response (Figure 6B). The rapid onset of the beneficial clinical effects of natalizumab observed in clinical trials may be due to an immediate decreased migration of CD4+ and CD8+ T cells into the brain and spinal cord (Figure 6C). Long-term uninterrupted natalizumab therapy may lead to significant reduction of antigen presenting cells within the cerebral perivascular spaces including CD20+ B cells, CD68+ macrophages, and CD209+ dendritic cells (Figure 6D).

Figure 6. Activated leukocytes are capable of adhering to the endothelium of blood vessel walls and migrating into the central nervous system (CNS) (A). However, these cells do not persist in the brain and spinal cord if a CNS autoantigen or a foreign antigen is not being presented to them in the context of major histocompatibility complex (A). During inflammation, antigen-specific T cells do persist for longer periods of time and may play an important role in the amplification of an immune response (B). The rapid onset of the beneficial clinical effects of natalizumab observed in clinical trials may be due to an immediate decreased migration of CD4+ and CD8+ T cells into the brain and spinal cord (C). Long-term uninterrupted natalizumab therapy may lead to significant reduction of antigen presenting cells within the cerebral perivascular spaces including CD20+ B cells, CD68+ macrophages, and CD209+ dendritic cells (D).
Currently estimated at 0.1%,6 our observations suggest that long-term natalizumab therapy may significantly impair cellular immune responses within the CNS. In this setting, the risk of CNS infections, including PML, may be significantly increased. As the discontinuation of natalizumab alone may not be sufficient to prevent a catastrophic outcome in such a setting,30 a treatment paradigm that allows for treatment holidays and reconstitution of CPVs APCs may need to be considered.

Fortunately, our study was limited by the number of patients with MS who developed PML while taking natalizumab and the considerable difficulties obtaining cerebral tissue from those patients for histopathological investigation. In addition, no brain tissue of patients with MS who are taking natalizumab and who did not develop PML was available to us. While we cannot generalize our observations regarding the effects of natalizumab on cellular numbers and composition in CPVs, we believe that our observations are an important contribution to further understand the effects of VLA-4 antagonism on the immune system within the CNS.

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REFERENCES