

Altered CD4⁺/CD8⁺ T-Cell Ratios in Cerebrospinal Fluid of Natalizumab-Treated Patients With Multiple Sclerosis

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Background: Treatment with natalizumab, a monoclonal antibody against the adhesion molecule very late activation antigen 4, an $\alpha 4\beta_1$ integrin, was recently associated with the development of progressive multifocal leukoencephalopathy, a demyelinating disorder of the central nervous system caused by JC virus infection.

Objective: To test the effect of natalizumab treatment on the CD4⁺/CD8⁺ T-cell ratios in cerebrospinal fluid (CSF) and peripheral blood.

Design: Prospective longitudinal study.

Setting: Academic and private multiple sclerosis centers.

Patients: Patients with multiple sclerosis (MS) treated with natalizumab, untreated patients with MS, patients with other neurologic diseases, and human immunodeficiency virus–infected patients.

Main Outcome Measures: CD4⁺ and CD8⁺ T cells were enumerated in CSF and peripheral blood. The mean fluorescence intensity of unbound $\alpha 4$ integrin on pe-

ripheral blood CD4⁺ and CD8⁺ T cells was analyzed before and after natalizumab therapy.

Results: Natalizumab therapy decreased the CSF CD4⁺/CD8⁺ ratio of patients with MS to levels similar to those of human immunodeficiency virus–infected patients. CD4⁺/CD8⁺ ratios in peripheral blood in patients with MS progressively decreased with the number of natalizumab doses, but they remained within normal limits. Six months after the cessation of natalizumab therapy, CSF CD4⁺/CD8⁺ ratios normalized. The expression of unbound $\alpha 4$ integrin on peripheral blood T cells decreases with natalizumab therapy and was significantly lower on CD4⁺ vs CD8⁺ T cells.

Conclusions: Natalizumab treatment alters the CSF CD4⁺/CD8⁺ ratio. Lower expression of unbound $\alpha 4$ integrin on CD4⁺ T cells is one possible mechanism. These results may have implications for the observation that some natalizumab-treated patients with MS developed progressive multifocal leukoencephalopathy.

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MULTIPLE SCLEROSIS (MS) is an inflammatory demyelinating disorder of the central nervous system (CNS) of unknown origin.¹ Natalizumab (Tysabri; Biogen Idec Inc, Cambridge, Mass, and Elan Corp, Dublin, Ireland) is a humanized monoclonal antibody designed to bind very late activation antigen 4, which is an $\alpha 4\beta_1$ (CD49d/CD29) integrin that serves as an adhesion molecule and is expressed by all leukocytes except neutrophils. Natalizumab was intended to prevent ingress of leukocytes into the CNS² and other target tissues by physically blocking the interaction of very late activation antigen 4 with its ligands, vascular cell adhesion molecule 1³ or the CS-1 fragment of fibronectin.⁴ The clinical effectiveness of natalizumab in MS, Crohn disease, and rheumatoid arthritis was evaluated in ap-

proximately 3000 patients. Based on the results of a phase 2 clinical trial⁵ and data from 2 phase 3 studies (the AFFIRM [Natalizumab Safety and Efficacy in Relapsing Remitting Multiple Sclerosis] monotherapy trial⁶ and the SENTINEL [The Safety and

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Efficacy of Natalizumab in Combination With Interferon Beta-1a in Patients With Relapsing Remitting Multiple Sclerosis] add-on trial with interferon beta-1a [Avonex; Biogen Idec Inc]⁷), the Food and Drug Administration approved natalizumab for the treatment of relapsing forms of MS on November 23, 2004 (<http://www.fda.gov/bbs/topics/news/2004/NEW01141.html>). On February 28, 2005, the manufacturers of natalizumab announced the

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Table. Characteristics of the Patient Cohorts*

	Patients, No.	Age, Median (Range), y	Sex, F/M	Age at MS Diagnosis, Median (Range), y	EDSS Score, Median (Range)
HHUD OND controls	17	33 (16-79)	1/0.9	NA	NA
HHUD MS patient cohort	35	37 (18-59)	2.6/1	29 (16-51)	2.0 (0-5.0)
HHUD HIV patient cohort	16	40 (27-64)	1/15	NA	NA
MSCTN MS natalizumab cohort	10	49 (37-53)	10/0	38 (24-47)	3.5 (0-6.0)
UTSW MS natalizumab cohort	13	43 (31-54)	6.5/1	36 (22-50)	2.0 (1.5-4.0)
MNI MS natalizumab cohort	8	46 (32-55)	3/1	41 (27-46)	2.3 (1.0-4.5)

Abbreviations: EDSS, Expanded Disability Status Scale; HHUD, Heinrich Heine University, Düsseldorf, Germany; HIV, human immunodeficiency virus; MNI, Montreal Neurological Institute, Montreal, Quebec; MS, multiple sclerosis; MSCTN, Multiple Sclerosis Center at Texas Neurology; NA, not applicable; OND, other noninflammatory neurologic disorders; UTSW, University of Texas Southwestern Medical Center at Dallas Multiple Sclerosis Center.

*Lymphocyte composition in cerebrospinal fluid and peripheral blood was assessed in patients with MS enrolled in the SENTINEL [The Safety and Efficacy of Natalizumab in Combination With Interferon Beta-1a in Patients With Relapsing Remitting Multiple Sclerosis] and AFFIRM [Natalizumab Safety and Efficacy in Relapsing Remitting Multiple Sclerosis] trials at the UTSW and at the MSCTN. One patient with MS who received natalizumab at the UTSW after its approval was also included. Control subjects included patients with OND, patients with MS who were not treated with natalizumab, and HIV-infected patients whose cerebrospinal fluid had been examined at the HHUD within 1 year. For quantitation of unbound $\alpha 4$ integrin expression on peripheral blood T cells, patients with clinically definite relapsing-remitting MS¹² who were transitioning into the open-label phase of the natalizumab clinical trial program at the MS Clinic l'Hôpital Charles LeMoyné and the MNI were studied.

voluntary withdrawal of this agent from the market, and the use of natalizumab in clinical trials was halted after 2 patients with MS and 1 patient with Crohn disease who had received natalizumab in clinical trials were diagnosed as having progressive multifocal leukoencephalopathy (PML), a demyelinating disorder of the CNS caused by infection with the human JC polyomavirus.⁸⁻¹⁰

This study examined cerebrospinal fluid (CSF) and peripheral blood CD4⁺/CD8⁺ ratios in patients treated with natalizumab compared with controls (including patients infected with human immunodeficiency virus [HIV]). Specifics regarding absolute numbers of different lymphocyte phenotypes in CSF and peripheral blood of natalizumab-treated patients are reported elsewhere and do not include HIV-infected controls.¹¹ We demonstrate that natalizumab therapy significantly alters the CD4⁺/CD8⁺ ratio in CSF, reducing it to levels comparable with those in HIV-infected individuals, a patient group known to be at high risk for PML. In addition, we show that the expression of unbound $\alpha 4$ integrin on T cells decreases with natalizumab therapy and is significantly lower on CD4⁺ vs CD8⁺ T cells.

METHODS

PATIENTS

For analysis of CD4⁺/CD8⁺ ratios, 15 CSF and 23 blood samples from patients with MS treated with natalizumab were analyzed at study enrollment, and 13 samples were analyzed at 6-month follow-up. Control subjects included 17 patients with other, non-inflammatory neurologic diseases (headache, polyneuropathy, and normal pressure hydrocephalus), 35 patients with clinically definite relapsing-remitting MS¹² who did not receive natalizumab, and 16 HIV-infected patients. Natalizumab-treated patients with MS were recruited from the University of Texas Southwestern Medical Center at Dallas Multiple Sclerosis Center and at the Multiple Sclerosis Center at Texas Neurology. Controls were recruited from Heinrich Heine University, Düsseldorf, Germany.

Eight patients with clinically definite relapsing-remitting MS¹² who were transitioning into the open-label phase of the natalizumab clinical trial program at the Montreal Neurological Institute, Quebec, provided peripheral blood samples for quantitation of unbound $\alpha 4$ integrin. All the patients were studied dur-

ing treatment with the standard 300-mg monthly intravenous regimen of natalizumab. For each patient, blood samples were collected just before and 1 1/2 hours after each treatment.¹³ Informed consent was obtained from all patients, and the study procedures were approved by the respective institutional review boards.

CELL COUNTS

Neubauer or Fuchs-Rosenthal counting chambers were used to count total cell numbers in CSF and peripheral blood mononuclear cells by means of light microscopy. Total CSF cell count was determined manually by trypan blue exclusion or methylene blue staining; the lower limit of detection was 250 cells/mL.¹⁴

FLOW CYTOMETRY

For investigation of CD4⁺/CD8⁺ ratios, peripheral blood mononuclear cells were isolated by means of density gradient centrifugation (Ficoll-Paque; Amersham Pharmacia, Uppsala, Sweden). The CSF cells were collected by means of centrifugation. Cell staining for flow cytometry was performed according to published methods.¹⁵ A flow cytometer (FACSCalibur; BD Biosciences, San Jose, Calif) was used for data acquisition. Flow cytometry data were analyzed using a software program (CellQuestPro; BD Biosciences, or FlowJo; Tree Star Inc, Ashland, Ore). Flow cytometry data for CD4⁺/CD8⁺ ratios were reviewed by 3 of us (O.S., S.C., and B.H.).

For analysis of unbound $\alpha 4$ integrin (CD49) on CD4⁺ and CD8⁺ T cells, 3-color staining was performed by incubating whole blood with saturating amounts of directly conjugated monoclonal antibodies against CD49d, CD3, CD4, and CD8 (BD PharMingen, San Diego, Calif).¹³ Isotype-matched antibodies were used as negative controls (BD PharMingen).¹³ Flow cytometry data were acquired using a BD FACScan (Becton Dickinson, Franklin Lakes, NJ), and the mean fluorescence intensity of $\alpha 4$ integrin was analyzed by a blinded operator using FlowJo software.

DATA ANALYSIS

The Mann-Whitney test was used to compare CD4⁺/CD8⁺ ratios in CSF and peripheral blood. The strength of associations between the CD4⁺/CD8⁺ T-cell ratio in peripheral blood and the number of natalizumab doses received was analyzed using the Spearman rank correlation coefficient. The $\alpha 4$ integrin expression levels on T cells were compared using paired *t* tests. *P* < .05 is considered statistically significant.

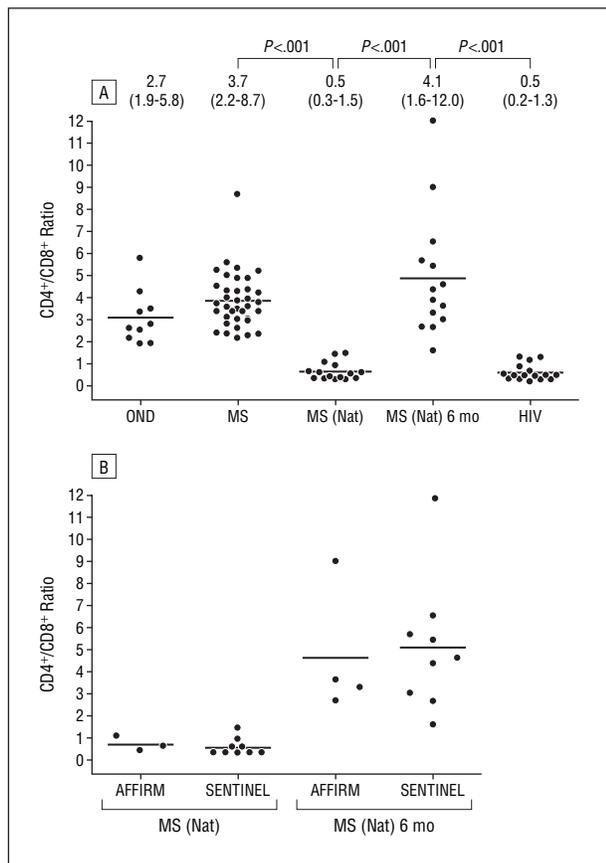


Figure 1. The CD4⁺/CD8⁺ ratios in cerebrospinal fluid (CSF). A, Natalizumab reverses CD4⁺/CD8⁺ T-lymphocyte ratios in CSF. Ratios in patients with other neurologic diseases and patients with multiple sclerosis who had not received natalizumab therapy (MS) were not statistically different from one another. In contrast, patients with MS who had received natalizumab therapy (MS [Nat]) displayed very low CD4⁺/CD8⁺ ratios, comparable with that of a human immunodeficiency virus 1-infected patient cohort (HIV). Six months after the cessation of therapy, CSF CD4⁺/CD8⁺ ratios returned to normal in patients with MS treated with natalizumab (MS [Nat] 6 mo). The normalization of CD4⁺/CD8⁺ T-cell ratios 6 months after cessation of natalizumab therapy (MS [Nat] 6 mo) was driven by a further decline in the absolute number of CD8⁺ T cells, and not by the normalization of CD4⁺ T-lymphocyte numbers in the CSF. Values are given as mean (range). B, The CD4⁺/CD8⁺ ratios in CSF of MS (Nat) patients are not affected by interferon beta therapy. No statistically significant difference in CD4⁺/CD8⁺ T-cell ratios was observed between patients enrolled in the AFFIRM [Natalizumab Safety and Efficacy in Relapsing Remitting Multiple Sclerosis] monotherapy trial and the SENTINEL [The Safety and Efficacy of Natalizumab in Combination With Interferon Beta-1a in Patients With Relapsing Remitting Multiple Sclerosis] add-on trial with interferon beta-1a within 1 month (MS [Nat]) and 6 months after the cessation of natalizumab therapy (MS [Nat] 6 mo). Horizontal bars indicate means. OND indicates other noninflammatory neurologic disorders.

RESULTS

Participant characteristics are given in the **Table**. All patients with MS had relapsing-remitting disease. The duration of MS was similar in natalizumab-treated patients with MS and in controls.

The CSF CD4⁺/CD8⁺ ratios in patients with other, non-inflammatory neurologic diseases and patients with MS who had not received natalizumab were comparable with published data (**Figure 1A**).¹⁶⁻¹⁹ In contrast, CSF CD4⁺/CD8⁺ ratios in patients with MS who had received natalizumab were very low (**Figure 1A**) and were not significantly different from those in HIV-infected controls

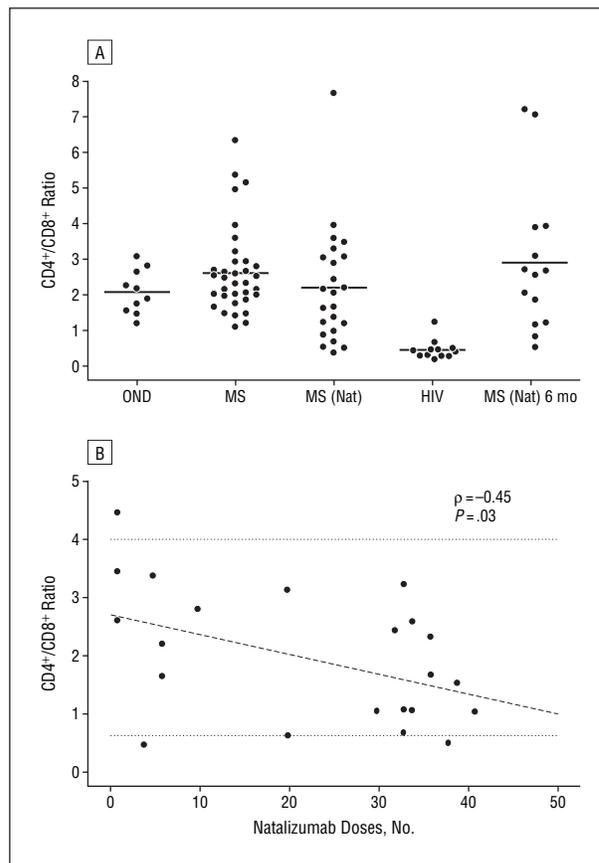


Figure 2. CD4⁺/CD8⁺ ratios in peripheral blood (PB). A, CD4⁺/CD8⁺ T-cell ratios in PB of natalizumab-treated patients with multiple sclerosis (MS) are normal. Unlike in human immunodeficiency virus (HIV) 1-infected patients, CD4⁺/CD8⁺ ratios in PB of patients with other noninflammatory neurologic disorders (OND), MS patients and patients with MS treated with natalizumab (MS [Nat]) were comparable with published patient cohorts.^{7-11,16} Reanalysis of T-lymphocyte subsets 6 months after the cessation of natalizumab therapy (MS [Nat] 6 mo) revealed that CD4⁺/CD8⁺ T-cell ratios remained normal. Horizontal bars indicate means. B, Long-term natalizumab therapy alters the composition of lymphocytes in PB. The PB CD4⁺/CD8⁺ T-cell ratios decreased with increasing numbers of natalizumab doses (diagonal dotted line). Dotted horizontal lines indicate the upper and lower limits of normal CD4⁺/CD8⁺ T-cell ratios in PB.¹⁶

($P = .8$). There were no statistically significant differences in CD4⁺/CD8⁺ T-cell ratios between patients enrolled in the AFFIRM trial and those enrolled in the SENTINEL trial (**Figure 1B**). A patient who received a single dose of natalizumab had a CSF CD4⁺/CD8⁺ ratio as low as a patient who received 41 doses. When examined 6 months after the cessation of natalizumab therapy, the CSF CD4⁺/CD8⁺ ratio had normalized (**Figure 1A**).

Peripheral blood CD4⁺/CD8⁺ ratios in patients with other neurologic diseases, untreated patients with MS, and natalizumab-treated patients with MS were within normal limits. As expected, CD4⁺/CD8⁺ ratios were low in HIV-infected patients (**Figure 2A**). Peripheral blood CD4⁺/CD8⁺ ratios decreased significantly with increasing numbers of natalizumab doses but remained within normal limits (**Figure 2B**). Six months after the cessation of natalizumab therapy, CD4⁺/CD8⁺ T-cell ratios in peripheral blood remained normal (**Figure 2B**).

The expression of unbound $\alpha 4$ integrin on CD4⁺ and CD8⁺ T cells decreased significantly after natalizumab

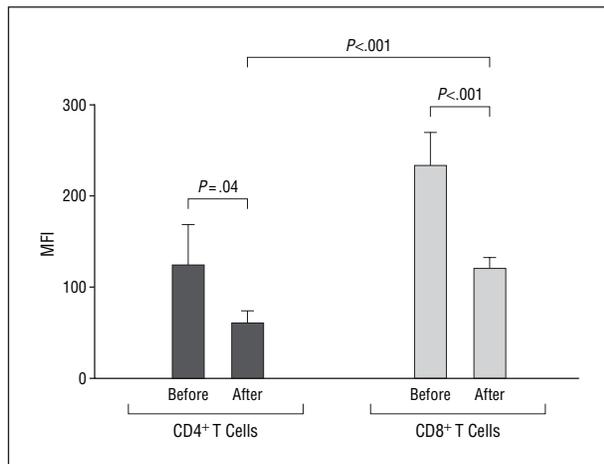


Figure 3. Mean fluorescence intensity (MFI) of unbound $\alpha 4$ integrin on CD4⁺ and CD8⁺ T cells before and after natalizumab infusion in patients with multiple sclerosis. Natalizumab treatment resulted in significantly decreased levels of available surface expression of unbound $\alpha 4$ integrin in CD4⁺ and CD8⁺ T cells. After natalizumab infusion, levels of unbound $\alpha 4$ integrin were significantly higher on CD8⁺ T cells compared with CD4⁺ T cells. Error bars represent SD.

therapy (**Figure 3**). CD4⁺ T cells expressed significantly less unbound $\alpha 4$ integrin before and after natalizumab therapy than CD8⁺ T cells (Figure 3).

COMMENT

The occurrence of PML in 3 of approximately 3000 natalizumab-treated patients was unexpected. Progressive multifocal leukoencephalopathy is a demyelinating CNS disorder caused by active infection of oligodendrocytes by JC virus.²⁰ JC virus is presumably acquired in childhood, and the seroprevalence of antibodies to the virus is 80% to 90% in adults.²¹ JC virus remains latent in the host, although consensus has not been reached regarding the site of latency, and peripheral blood lymphocytes, particularly B cells, kidney, and brain, have all been suggested.²²⁻²⁸ Progressive multifocal leukoencephalopathy results when the virus reactivates, and this most often occurs in patients with underlying immunodeficiency, particularly those infected with HIV.²⁹ It was for this reason that we compared CD4⁺/CD8⁺ ratios in CSF and peripheral blood of natalizumab-treated patients with MS with those in HIV-infected patients.

Although the patient numbers were small, the data indicate that natalizumab therapy results in a striking decrease in the CSF CD4⁺/CD8⁺ ratio. The biological significance of a decreased CSF CD4⁺/CD8⁺ ratio in the context of a cellular immune response against foreign antigens, such as JC virus, is unknown. In patients with AIDS, CD4⁺/CD8⁺ ratios have long been used as a disease marker. Our data show that CD4⁺ and CD8⁺ T cells expressed significantly less unbound $\alpha 4$ integrin before and after natalizumab therapy and that the effect on CD4⁺ T cells was greater than on CD8⁺ T cells. This suggests that the differential surface expression of unbound $\alpha 4$ integrin on CD4⁺ and CD8⁺ T cells may contribute to the differential effect of natalizumab on these lymphocyte subsets; probably only the unbound (available) fraction of very late activation antigen 4 on the cell surface will facilitate the migration of these cells into tissues. The total amount of

unbound $\alpha 4$ integrin is one possible explanation for altered CD4⁺/CD8⁺ ratios in CSF. Another is that a threshold of total cell surface expression of unbound $\alpha 4$ integrin is required for effective migration of leukocytes from peripheral blood into target tissues and that it differs for CD4⁺ T cells vs CD8⁺ T cells.

Alteration of the CD4⁺/CD8⁺ ratio in CSF by natalizumab may provide information on the pathogenesis of MS. Although antigen-specific CD4⁺ T cells have long been implicated in the pathogenesis of MS, several recent studies^{30,31} demonstrated clonal and oligoclonal accumulation of CD8⁺ T cells in the CNS of patients with MS. However, the initiation and perpetuation of most antigen-specific CD8⁺ T-cell responses requires the help of CD4⁺ T cells.³² The clinical efficacy of natalizumab and the present data may suggest that CD4⁺ T cells do indeed play a critical role in the inflammatory cascade of MS.

The decrease in CD4⁺/CD8⁺ ratios in peripheral blood with increasing doses of natalizumab may reflect a differential sequestration of CD4⁺ and CD8⁺ T cells into various tissues. The reduced CD4⁺/CD8⁺ ratios in peripheral blood across time do not correlate with CD4⁺/CD8⁺ ratios in CSF, which were unaffected by the total number of natalizumab doses. Prolonged treatment with natalizumab may affect the circulation of leukocytes from peripheral tissues into the blood and may explain this discrepancy. Although the mechanism remains undefined at present, the implications for immune control of CNS infections are potentially important and suggest that prolonged, uninterrupted natalizumab therapy may eventually alter systemic cellular immune responses.

Interpretation of these data should not lead to the conclusion that natalizumab therapy is the biological equivalent of an infection with HIV. These data point out at least 1 significant difference between natalizumab-treated patients with MS and HIV-infected patients: although CD4⁺/CD8⁺ ratios in peripheral blood may be low in all stages of HIV infection, they remain within normal limits in patients who receive a limited course of natalizumab therapy.

CONCLUSION

Treatment of patients with MS with natalizumab leads to an immediate decrease in the CD4⁺/CD8⁺ ratio in CSF but not in peripheral blood. Although patients with HIV had similarly low CSF CD4⁺/CD8⁺ ratios compared with natalizumab-treated patients, patients with HIV also had low peripheral blood CD4⁺/CD8⁺ ratios. The biological implications of a low CSF CD4⁺/CD8⁺ ratio are not defined. However, the differential effect of natalizumab on specific lymphocyte subsets in the CNS, as reflected by a differential decrease in $\alpha 4$ integrin on CD4⁺ compared with CD8⁺ T cells, and alteration of the CSF CD4⁺/CD8⁺ ratio could pose an increased risk of opportunistic infections in natalizumab-treated patients.

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