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Antagonizing the α₄β₁ Integrin, but Not α₄β₇, Inhibits Leukocytic Infiltration of the Central Nervous System in Rhesus Monkey Experimental Autoimmune Encephalomyelitis

Krista G. Haanstra,* Sam O. Hofman,* Dave M. Lopes Estêvão,* Erwin L. A. Blezer,† Jan Bauer,‡ Li-Li Yang,§ Tim Wyant,∥ Vilmos Csizmadia,∥ Bert A. ‘t Hart,*∥ and Eric R. Fedýk∥

The immune system is characterized by the preferential migration of lymphocytes through specific tissues (i.e., tissue tropism). Tissue tropism is mediated, in part, by the α₄ integrins expressed by T lymphocytes. The α₄β₁ integrin mediates migration of memory T lymphocytes into the CNS, whereas the α₄β₇ integrin mediates migration preferentially into gastrointestinal tissue. This paradigm was established primarily from investigations in rodents; thus, the objective of this investigation was to determine if blocking the α₄β₁ integrin exclusively would affect migration of T lymphocytes into the CNS of primates. The effects of the dual α₄β₁ and α₄β₇ antagonist natalizumab were compared with those of the α₄β₇ antagonist vedolizumab on experimental autoimmune encephalomyelitis in the rhesus monkey. Animals received an initial i.v. bolus of placebo, natalizumab (30 mg/kg), or vedolizumab (30 mg/kg) before intracutaneous immunization with recombinant human myelin oligodendrocyte glycoprotein and then Ab once weekly thereafter. Natalizumab prevented CNS inflammation and demyelination significantly (p < 0.05), compared with time-matched placebo control animals, whereas vedolizumab did not inhibit these effects, despite saturating the α₄β₇ integrin in each animal for the duration of the investigation. These results demonstrate that blocking α₄β₇ exclusively does not inhibit immune surveillance of the CNS in primates. The Journal of Immunology, 2013, 190: 1961–1973.

The immune system is characterized by regionalization at multiple levels, and immunosurveillance in particular is characterized by the preferential migration of lymphocyte subsets through specific tissues. Naive lymphocytes primarily recirculate through secondary lymphoid organs, for example, whereas differentiated memory T cells migrate preferentially through tissues in which Ag was initially encountered, such as the skin, CNS, or gut. This adaptive response is postulated to enhance the efficiency with which the immune system responds to pathogens (1–3).

Tissue-tropic migration is mediated in part by vascular lymphocytes firmly adhering to the endothelial lumen and diapedesing into surrounding tissue. This process requires the formation of shear-resistant attachments, which are mediated by the binding of lymphocyte integrins to Ig superfamily members expressed on the endothelial lumen. Integrins are obligate heterodimers containing two distinct chains, called the α and β subunits. In mammals, 18 α and 8 β subunits have been characterized. These chains heterodimerize and create at least 24 unique integrins, many of which have distinct functions. The α₄β₁ and α₄β₇ integrins in particular are expressed by discrete subsets of memory T lymphocytes (4, 5), and these subsets exhibit distinct patterns of migration in vivo (1–3). Mechanistic investigations have demonstrated that the α₄β₁ integrin expressed on memory T lymphocytes mediates migration into the CNS, bone marrow, and skin, via firm adhesion to VCAM-1 (1, 2). In contrast, memory T lymphocytes expressing the α₄β₇ integrin preferentially migrate into the gastrointestinal tract via firm adhesion to mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) (1–3, 6, 7). These tissue-tropic mechanisms were primarily elucidated in mice, and it is unknown to what extent α₄β₇ may contribute to immune surveillance of the CNS in primates. These mechanisms also mediate inflammation. Blocking the α₄ integrins with Abs or small-molecule inhibitors reverses the clinical and pathological hallmarks of experimental autoimmune encephalomyelitis (EAE) in mice and guinea pigs (8–13). Natalizumab is an antagonist of the human α₄β₁ and α₄β₇ integrins and delays the accumulation of physical disability, as well as reducing the frequency of clinical exacerbations in patients with relapsing forms of multiple sclerosis (14). Natalizumab specifically inhibits the firm adhesion of human T cells to the inflamed spinal cord microvasculature of mice with acute EAE (15). Natalizumab also alleviates gastrointestinal inflammation in Crohn’s disease (CD) (16) and thus is a pleiotropic anti-inflammatory agent. The clinical utility of natalizumab is limited, however, by progressive multifocal leu-
koencephalopathy (PML), a life-threatening brain infection characterised by progressive damage of brain white matter at multiple locations (17–19). It is caused by recrudescence of the JC virus in immunosuppressed patients, presumably due to impaired immune surveillance of the brain by memory T lymphocytes (17–19). This theory cannot be directly tested, however, because an appropriate model of PML does not exist (17–19).

Inflammatory bowel disease (IBD) comprises a group of inflammatory conditions of the gastrointestinal tract, of which CD and ulcerative colitis (UC) are the most common forms (20). An improved therapeutic strategy for IBD could be to antagonize the α4β7 integrin exclusively, based on the premise that this would provide anti-inflammatory activity in the gastrointestinal tract without compromising immune surveillance of the CNS. Blockade of the α4β7 integrin exclusively with the Act-1 mAb induced anti-inflammatory effects and remission of disease in spontaneously colitic cotton-top tamarins (21). Vedolizumab (former versions: MLN0002, MLN02, LDP-02) is also a highly selective mAb that binds exclusively to the gut-tropic α4β7 integrin; it does not bind to other α4 or β7 integrins, such as the α4β7 integrin or the α7β7 integrin (22). It inhibits the functional activity of the α4β7 integrin by selectively antagonizing binding and adhesion to MAdCAM-1 and to the extracellular matrix glycoprotein fibronectin, but does not antagonize binding to VCAM-1 (22). It elicited anti-inflammatory effects selectively in the gastrointestinal tract of cynomolgus monkeys (23) and demonstrated statistically significant efficacy in placebo-controlled phase 2 clinical trials of patients with active UC (24) and CD (25). Therefore, vedolizumab was used in this investigation to specifically examine whether exclusive antagonism of the α4β7 integrin would compromise immune surveillance of the CNS in rhesus monkey EAE.

Materials and Methods

Natalizumab (Tysabri, Biogen Idec, Cambridge, MA) is a humanized IgG4 mAb that binds to the α4 chain of the human α4β7 and α4β1 integrins (14, 16). Vedolizumab is a humanized IgG1 version of the Act-1 mAb, which binds to the β7 chain of the human α4β7 integrin, but not to the α4β1 or α4β2 integrins (22).

Natalizumab exhibited a mean concentration producing 50% maximal binding (EC50) of 18.6 ± 11.4 ng/ml and IC50 of 70.4 ± 37.2 ng/ml for binding to rhesus monkey memory helper T lymphocytes, which is consistent with a human EC50 of 11.4 ng/ml and IC50 of 37.2 ng/ml (Table I). In contrast, vedolizumab exhibited a mean EC50 of 27.6 ± 21.3 ng/ml and IC50 of 12.2 ± 8.4 ng/ml for binding to rhesus monkey memory helper T lymphocytes, which is consistent with a human EC50 of 21.3 ng/ml and IC50 of 8.4 ng/ml for binding to human 1–125, which was produced in E. coli and purified as described previously (30). Animals were immunized on day 0 with 300 μg rMOG dissolved in 500 μl PBS, and emulsified in an equal volume of CFA (Difco Laboratories, Detroit MI).

Clinical signs were scored daily by observers blinded to the treatment, using a previously described semiquantitative scale (27, 31, 32): 0 = no clinical signs; 0.5 = loss of appetite, vomiting; 1 = substantial reduction of general condition; 2 = ataxia, sensory loss, and/or visual problems; 2.5 = incomplete paralysis of one (hemiparesis) or both sides (paraparesis); 3 = complete paralysis of one (hemiplegia) or both sides (paraplegia); 4 = complete paralysis (quadriplegia); 5 = moribund. The rMOG-induced EAE model in rhesus monkeys is characterized by acute onset and rapid disease progression, with the monkeys reaching a moribund state within 24 h. To avoid suffering, monkeys were euthanized at EAE score ≥ 2.5, or at score 2 when the animal was not expected to survive until the next day.

PK monitoring

The concentrations of natalizumab and vedolizumab in serum samples from rhesus monkeys were quantified using ELISA by a Good Laboratory Practices (GLP) methodology for vedolizumab (Questr Pharmaceutical Services, Wilmington, DE) and by a non-GLP methodology for natalizumab (Millennium Pharmaceuticals) per testing facility standard operating practice (SOP). Briefly, the assays used a goat anti-human IgG heavy and light chain, macaque-adsorbed, capture Ab (Bethyl Laboratories Montgomery, TX) and a mouse anti-human IgG4 monoclonal IgG Ab (Bethyl Laboratories) as conjugated detection Abs (Alpha Diagnostics, Owings Mills, MD). After the addition of a chromogenic HRP substrate (3,3′,5,5′-tetramethylbenzidine; Pierce Biotechnology Rockford, IL), color development was measured at 450 nm on a Wallac 1420 Victor 2 Microplate Reader (PerkinElmer, Cambridge, MA). Data were analyzed with SoftMax Pro software, version 4.8, from MindVision Software (Lincoln, NE), and all statistics were calculated using Microsoft Office Excel 2007 (Microsoft, Redmond, WA). The intensity of the color was proportional to the serum natalizumab concentration that was interpolated by four-parameter logistic regression from a standard curve ranging from 0.125 to 8.0 μg/ml. The lower limit of quantitation for natalizumab was determined to be 0.125 μg/ml, and the upper limit of quantitation was 8 μg/ml in the ELISA assay. The calculated concentration of natalizumab was within 100% ± 20% of the nominal value, and precision values of the assay parameters were ≤ 20%. An analogous quantitave ELISA assay was used for detection of vedolizumab in serum samples, via GLP methodology, per testing facility SOP (Quest Pharmaceutical Services).
**PD monitoring**

PD effects were monitored by flow cytometry. The PD assays were whole-blood competition binding assays between the therapeutic mAb administered in vivo (natalizumab or vedolizumab) and the corresponding directly labeled mAb (natalizumab–Alexa 647 or vedolizumab–Alexa 647) incubated in whole blood ex vivo. Venous blood samples were collected in K3-EDTA vacutainers [Becton Dickinson (BD), Mountain View, CA]. Whole-blood samples were washed to remove excess Ab. Samples were stained with anti-CD3, anti-CD4, CD8, CD45RA (clones SP34-2, L200, SK1, and SH9, respectively; all from BD), and CD14 (TUK4; Miltenyi) and with Alexa Fluor 647–labeled natalizumab or vedolizumab, followed by lysis of the RBCs. Samples were analyzed on an LSR-II (BD), using DIVA software (BD).

**Primate anti-human Ab monitoring**

A non-GLP evaluation of primate anti-human Ab (PAHA) to natalizumab was performed by semiquantitative ELISA of serum samples (Millennium Pharmaceuticals). Briefly, natalizumab (Biogen Idec) was immobilized to the plate. Bound anti-natalizumab Abs were detected with HRP-conjugated anti-macaque IgG, IgA, and IgM Abs (Rockland Immunochemicals, Gilbertsville, PA) after the addition of a chromogenic HRP substrate (3,3′,5,5′-tetramethylbenzidine, Pierce Biotechnology). Qualification assays were conducted, and the precision (percent coefficient of variation) of the assays was calculated to be <20% acceptance range for both intra- and interassay runs, indicating good assay reproducibility. The detection cutoff point was determined using a panel of negative control serum samples from 10 individual rhesus monkeys (LAMPIRE Biological Laboratories, Pipersville, PA). An interference test showed that the assay was specific for natalizumab. An analogous GLP evaluation of PAHA to vedolizumab was performed by semiquantitative ELISA of serum samples, per testing facility SOP (Quest Pharmaceutical Services).

**Cellular immune responses against rhMOG**

PBMCs were isolated before and once weekly after EAE induction and at the time of necropsy. At necropsy, mononuclear cells (MNCs) were isolated from the spleen. Isolated MNCs were dispensed in quadruplicate at 1 × 10^7 cells per well in 96-well round-bottom microtiter plates with 5 μg/ml rhMOG. Stimulation by Con A (5 μg/ml) was used as a positive control. MNC proliferation was assayed by the incorporation of [3H]-thymidine (cpm) in the presence of MNC proliferation without peptide. The Journal of Immunology 1963 expressed as

**Histological examination of formalin-fixed tissues**

Following MRI scanning, the formalin-fixed hemispheres were processed for histopathological examination. Three samples were excised from standardized regions of each brain and embedded in paraffin. For histochemical staining, 5- to 7-μm-thick paraffin sections were deparaffinized in xylene and transferred to 90% ethanol. H&E staining for inflammation and Klüver-Barrera staining for demyelination were performed. Immunohistochemical stainings for CD3, CD20, and MRP14 were performed as described previously (33).

**Quantification of demyelination and cells**

Klüver-Barrera–stained sections (three sections per animal) were scanned with an Agfa DuoScan Scanner at 1000 dpi resolution. Recorded images were then analyzed with Image J (version 1.44p, a public domain image processing and analysis program developed by Wayne Rasband at the National Institutes of Health, Bethesda, MD). For this procedure, white matter areas in the sections were selected with the selection tools and quantified. The same was done for demyelinated areas. Finally, demyelination was given as a percentage of total white matter. Quantification of parenchymal CD3+ T cells, CD20+ B cells, and MRP14+ macrophages in lesions was performed in consecutive sections, using an ocular morphometric grid covering an area of 4 mm² at 100-fold magnification.

**CSF sampling and processing**

CSF samples were taken prior to EAE induction, on days 4 or 5 and days 11 or 12 post EAE induction and prior to euthanasia, from sedated monkeys with a 23-gauge needle via the cisterna magna. When this method proved unsuccessful, a sample was taken via lumbar puncture. Typically, 0.5 ml clear CSF was obtained from each monkey.

**Statistical analysis**

Statistical analysis was performed using Prism 5 for Mac OS X (GraphPad, San Diego, CA). Survival curves were compared using the log-rank test (Mantel–Cox). Significance of differences between groups was calculated using the nonparametric one-way ANOVA (Kruskal–Wallis test).

**Results**

The pharmacokinetics, immunogenicity, and PD of natalizumab and vedolizumab in rhesus monkeys

Natalizumab and vedolizumab exhibited conventional pharmacokinetic properties for humanized IgGs administered to monkeys.
Substantial exposure was achieved in each animal after administration of natalizumab or vedolizumab, compared with placebo controls. All seven animals exposed to natalizumab exhibited trough levels that exceeded the EC50 for α4 integrin saturation in vitro (Table I) between days 7 and 14, and four of six animals exhibited trough levels that exceeded this EC50 between days 14 and 21 (Fig. 1A). All seven animals exposed to vedolizumab exhibited trough levels that exceeded the EC50 for α4β7 saturation in vitro (Table I) between days 7 and 21 (Fig. 1B). Higher exposures were generally achieved with vedolizumab than with natalizumab throughout the investigations (Fig. 1A, 1B). The mean concentration of vedolizumab in animals at day 14 was 557.8 ± 63.8 µg/ml, for example, whereas for natalizumab it was 184.4 ± 187.3 µg/ml. PAHA responses reduced exposure to natalizumab in some animals. All seven monkeys dosed with natalizumab developed PAHA responses by day 14 (Fig. 1C), and corresponding decreases in exposure were observed in two animals (i.e., N6 and N7, see Table II for animal demographics) on days 21 and 26 (Fig. 1A). Six monkeys dosed with vedolizumab developed PAHA responses by day 14 (Fig. 1D); however, corresponding decreases in exposure were not observed (Fig. 1B). Neutralizing activity of PAHA was identified by monitoring saturation of the target or targets expressed by T lymphocytes and monocytes by the therapeutic mAbs. Prior to exposure to therapeutic Ab, natalizumab–Alexa 647 bound to 75–92% of the total population of memory helper T lymphocytes (CD3+CD4+CD45RA−) in peripheral blood of rhesus monkeys (Fig. 1E). On day 7 post dose, this binding was completely blocked in each animal because the α4 integrins were saturated by the dosed natalizumab (Fig. 1E). A partial restoration of natalizumab–Alexa 647 binding (14–36%) was observed in animals N6 and N7 on days 14–21 (Fig. 1E), illustrating that desaturation of α4 integrins

### Table I. Binding affinities of natalizumab and vedolizumab to rhesus and human memory helper T lymphocytes

<table>
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<tr>
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<th>Natalizumab</th>
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<td>EC50 (ng/ml)</td>
<td>IC50 (ng/ml)</td>
<td>EC50 (ng/ml)</td>
<td>IC50 (ng/ml)</td>
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<tr>
<td>Rhesus</td>
<td>18.6</td>
<td>8.1</td>
<td>70.4</td>
<td>24.9</td>
</tr>
<tr>
<td>Human</td>
<td>11.4</td>
<td>ND</td>
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Competitive binding assay used the saturating concentrations of natalizumab-Alexa647 and vedolizumab-Alexa647 at 100 and 200 ng/ml, respectively.

ND, The SD could not be calculated for these donors because only two donors were analyzed. These data are nonetheless consistent with more thorough investigations of binding affinities (23, 27).

**FIGURE 1.** The pharmacokinetics, immunogenicity, and PD of natalizumab and vedolizumab in rhesus monkeys. Filled symbols represent placebo control animals (P1–P8), whereas open symbols represent animals receiving weekly i.v. doses of either natalizumab (N1–N7) or vedolizumab (V1–V7) at 30 mg/kg. Concentration of natalizumab (A) and vedolizumab (B) in serum from individual animals before (days −10 and 0) and at trough levels (days 7–26) after receiving either mAbs. Levels of PAHA in serum from individual animals before (days −10 and 0) and after (days 7–26) receiving either natalizumab (C) or vedolizumab (D). Binding of exogenous Alexa 647–labeled natalizumab (E) to the α4 integrins expressed by memory helper (CD4+/CD45RA−) T lymphocytes in peripheral blood from individual animals before (days −10 and 0) and at trough levels of dosed natalizumab (days 7–26). Binding of exogenous Alexa 647–labeled vedolizumab (F) to the α4β7 integrin expressed by gut-homing (α4β7+) memory helper T lymphocytes in peripheral blood from individual animals before (days −10 and 0) and at trough levels of dosed vedolizumab (days 7–26).
occurred in these animals. In contrast, desaturation of the α₄β₇ integrin was not observed in animals dosed with vedolizumab. Vedolizumab–Alexa 647 bound to 25–42% of total memory helper T lymphocytes (CD3⁺CD4⁺CD45RA⁻), naive and memory cytotoxic T lymphocytes (CD3⁺CD8⁺CD45RA⁻), total T lymphocytes (CD3⁺), and monocytes (CD14⁺) (data not shown).

Effects on clinical signs of EAE

In this investigation, four of eight placebo-dosed animals developed clinical signs of EAE (Table III). The mean time of onset of clinical signs of EAE was significantly shorter (p = 0.0336) for the placebo control animals, compared with animals exposed to natalizumab (Fig. 2A). In contrast, four of seven animals in the vedolizumab group developed clinical signs of EAE (Table III). The mean time of onset of clinical signs of EAE was significantly shorter (p = 0.1350), compared with the placebo-treated animals (Fig. 2B). The time to onset of clinical signs of EAE observed in this study was also compared with control group data from a previous investigation (27) (Fig. 2C). Both the historical control group and the vedolizumab group have a median of 21 d to time of onset of EAE symptoms (p = 0.3100). The placebo group has a median survival of 25 d, which is also not statistically different from that of the historical control group (p = 0.4284).

Immune responses to rhMOG

Immune responses to rhMOG were monitored to determine if antagonizing the α₄ integrins affected the generation of pathogenic cells. Peripheral blood was collected before immunization with rhMOG, once weekly thereafter, and at the time of necropsy. The anti-rhMOG proliferative responses of PBMCs were monitored weekly and in splenocytes at necropsy. An anti-rhMOG proliferative response was observed in splenocytes (Fig. 3A) and PBMCs (data not shown) from each animal. The group means for the placebo control, natalizumab, and vedolizumab groups were comparable. EAE developed in the two animals exhibiting the weakest anti-rhMOG proliferative responses at necropsy (Fig. 3A), indicating that all animals generated sufficient numbers of pathogenic cells for inducing EAE.

An Ab response to rhMOG was also detected in the serum of each animal at necropsy (Fig. 3B). The anti-rhMOG IgG means of the placebo, natalizumab, and vedolizumab groups were also comparable (Fig. 3B), and both animals exhibiting the weakest anti-rhMOG IgG response also developed EAE (Fig. 3B), illustrating that each animal had an anti-MOG response capable of inducing EAE. These data indicate that the mechanism causing the inhibition of EAE by natalizumab was not attributable to inhibiting the induction of anti-rhMOG responses.

Effects on cerebral inflammation and demyelination

Clinical signs of EAE result from lesions within brain white matter. These pathological changes were quantified by postmortem MRI of formalin-fixed brain hemispheres. Conventional T2-weighted (Fig. 4A, 4D), quantitative relaxation time (Fig. 4B, 4E), and MTR (Fig. 4C, 4F) images were obtained for each animal and used by a blinded imaging specialist (E. B.) to quantify the magnitude of cerebral lesions. Compared with white matter, lesions showed increased T2 relaxation time values and decreased MTR values. The mean semiquantitative values for lesion loads of 21 d to time of onset in brain hemispheres from the natalizumab group trended lower than those of the historical control group and the vedolizumab group have a median survival of 25 d, which is also not statistically different from that of the historical control group (p = 0.4284).
Following MRI, the formalin-fixed hemispheres were processed for histological examination. Three blocks of tissue were excised from the same regions of each hemisphere, representative sections were prepared, and cerebral demyelination (Fig. 5A–C) and inflammation (Fig. 5D–L) were quantified by an anatomic pathologist (J.B.). Four animals in the placebo control group, one animal in the natalizumab group, and three animals in the vedolizumab group exhibited demyelinating lesions, and each of these animals also exhibited clinical signs of EAE (Fig. 5M). Five animals in the placebo control group, one animal in the natalizumab group, and five animals in the vedolizumab group exhibited inflammation (Fig. 5N). The animals with the largest brain infiltrates exhibited demyelinating lesions and clinical signs of EAE, whereas two animals with mild infiltrates (P8 and V5) did not demonstrate demyelination and EAE. These data indicate that the animals P8 and V5 were also developing EAE; however, they may have been euthanized prior to demyelination and the development of clinical signs of EAE because they were time-matched comparators to an animal already exhibiting clinical signs of EAE. The composite group mean score for demyelination (Fig. 5M) and inflammation (Fig. 5N) was nonetheless significantly lower ($p = 0.0098$) in the natalizumab group than in the placebo group, whereas the vedolizumab composite group mean score was not significantly different from those for the placebo group. Collectively, these data indicate that natalizumab inhibited inflammation and the development of demyelinating lesions, whereas vedolizumab had no effect.

The cerebral lesions contained high numbers of neutrophilic polymorphonuclear granulocytes (PMN), some eosinophilic PMNs, and mononuclear leukocytes (Fig. 5D–F), which is characteristic of the rhesus monkey EAE model (27). The mononuclear leukocytes were qualitatively similar between groups and consisted of CD3+ T lymphocytes (Fig. 5D–F), a few CD20+ B lymphocytes (Figure 5G–I), and numerous MRP14++ macrophages (Fig. 5J–L). Quantification of immunohistochemical staining revealed that cerebral sections from animals exposed to natalizumab contained lower levels of CD3+ T lymphocytes, MRP14++ macrophages, and CD20+ B lymphocytes than did comparable sections from animals exposed to placebo or vedolizumab (Table IV). The infiltrates of the two animals that did not demonstrate demyelination and EAE (P8 and V5) contained proportionately more CD3+ T lymphocytes and fewer MRP14+ macrophages (Table IV), indicating that T cells may arrive at the site of a potential lesion prior to macrophages, PMNs, and demyelination. Taken together, these data demonstrate that natalizumab inhibited cerebral inflammation and demyelination, whereas vedolizumab did not; thus, it can be inferred that the $\alpha_4\beta_1$ integrin mediates the inflammation and formation of cerebral lesions in EAE.

**Infiltration of the CSF by leukocytes**

Leukocytes migrate into the CSF of monkeys developing EAE, and the level of various subsets was measured as an additional assessment of immune surveillance of the CNS. Serial CSF samples were collected before and after exposure to placebo control, natalizumab, or vedolizumab. A relative increase in CSF leuko-

![FIGURE 2. Survival curves of time to onset of clinical signs of EAE.](http://www.jimmunol.org/)

(A) Natalizumab ($n = 7$) delayed the onset of clinical signs of EAE, compared with coupled placebo ($n = 4$) in phase 1 of the investigation ($p = 0.0336$). (B) Vedolizumab ($n = 7$) did not delay the onset of clinical signs of EAE, compared with coupled placebo ($n = 4$) in phase 2 of the investigation ($p = 0.1350$). (C) Time to onset of clinical signs of EAE observed in this study was compared with historical controls (27). Animals in the placebo and vedolizumab groups developed EAE at rates that were similar to those of the historical controls ($p = 0.3100$ and $p = 0.4284$, respectively).

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![FIGURE 3. Exposure to natalizumab or vedolizumab did not affect the induction of anti-MOG responses in vivo.](http://www.jimmunol.org/)

(A) Proliferative responses of splenocytes from each animal pulsed with rhMOG ex vivo. Proliferation was calculated by subtracting background counts per minute in an unstimulated culture from the total counts per minute of stimulated cultures for each animal. (B) Anti-MOG IgG responses in vivo. The serum anti-MOG IgG responses were calculated using a positive serum.
cytes from baseline levels was observed in animals that developed clinical signs of EAE primarily at the time of necropsy. Elevations in CSF leukocytes were observed in eight of nine animals that developed EAE, and no elevations were observed in animals that did not develop EAE (Fig. 6A–C). Three placebo control animals (P1, P3, and P4), one natalizumab animal (N2), and four vedolizumab animals (V1, V2, V4, and V6) exhibited elevations in CSF leukocytes and EAE (Fig. 6A–C). Collectively, CSF samples taken from animals without overt neurological deficit (EAE score < 2) contained a median of 0.00 (range 0.00–0.07) × 10^9/l leukocytes (n = 68). Samples taken from animals with an EAE score ≥ 2 (n = 9) contained a significantly (p < 0.0001) higher number of leukocytes, with a median of 0.15 (0.10–0.44) × 10^9/l. The absence of CSF infiltrates in animals exposed to natalizumab indicates that natalizumab blocks migration of leukocytes into the CSF. Conversely, the presence of infiltrates in animals exposed to vedolizumab demonstrates that this Ab does not block migration of leukocytes into the CSF.

To determine the phenotype of leukocytes infiltrating the CSF in diseased animals, the relative percentages of leukocyte subsets in the CSF of animals exhibiting signs of EAE were compared with relative percentages of subsets in peripheral blood of the same animal (Fig. 6D–H). The relative percentage of CD3+ T cells is higher in CSF than in peripheral blood (Fig. 6D), indicating that the CSF infiltrate contains disproportionately more T cells than other leukocyte subsets. The CD4/CD8 ratio is generally similar in peripheral blood and CSF (Fig. 6E), indicating that proportional amounts of CD4+ and CD8+ cells migrated into the CSF. The relative percentage of CD14+ monocytes is also generally similar in CSF compared with blood (Fig. 6F), suggesting this subset is also migrating into the CNS. In contrast, the relative percentages of CD3+CD16+ NK cells and CD20+ B lymphocytes are lower in the CSF than in peripheral blood (Fig. 6G, 6H), indicating that disproportionately fewer of these subtypes are migrating into the CSF. These collective data thus illustrate that helper (CD3+CD4+) and cytotoxic (CD3+CD8+) T cells and monocytes (CD14+) migrated preferentially into the CSF, whereas B lymphocytes (CD20+) and NK cells (CD3+CD16+) did not migrate as readily into the CSF in animals developing EAE.

Similar to infiltration of the CSF by leukocytes, anti-rhMOG Abs were found only in the CSF of animals with EAE, although not all animals with EAE had Abs in the CSF (data not shown). Moreover, anti-rhMOG Abs were not found in CSF samples taken at time points prior to necropsy (data not shown).

Elevation of leukocytes in the vasculature corresponds to inhibition of EAE

The MRI and histology analyses demonstrated that natalizumab blocked migration of leukocyte subsets into the CNS. A consequence of this mechanism of action, in conjunction with continued homeostatic production, would be the accumulation of these subsets within the vasculature of these animals. Exposure to natalizumab induced significant (p < 0.05) elevations in the level of mature WBCs in the vasculature of animals, within 5 d of initial exposure (the shortest duration examined), compared with pre-exposure baselines and with time-matched placebo controls (Fig. 7A). This leukocytosis occurred without significant changes in RBC indices (data not shown) or neutrophils (Fig. 7B). The natalizumab-induced leukocytosis consisted of significant (p < 0.05) elevations in monocytes (Fig. 7C), eosinophils (Fig. 7D), and lymphocytes (Fig. 7E), compared with pre-exposure baselines and with time-matched placebo controls. The lymphocytosis consisted of elevations in total T lymphocytes, total and memory helper T lymphocytes, total and memory cytotoxic T lymphocytes, and total B lymphocytes, but not NK cells (Fig. 7F). Each animal exposed to natalizumab exhibited elevations in these subsets, and the one animal that developed EAE (N2) and exhibited CNS infiltrates showed the smallest overall elevation in vascular lymphocytes (data not shown). These data indicate that exposure to natalizumab sequesters specific subsets of leukocytes in the vasculature and that this effect may explain the inhibition of EAE.

In contrast to natalizumab, vedolizumab did not affect levels of total leukocytes, monocytes, eosinophils, or lymphocytes, compared with pre-exposure baselines and with time-matched placebo controls, in any animal (Fig. 7A–E). Moreover, exposure to vedolizumab did not affect levels of lymphocyte subsets that were elevated by natalizumab, including memory helper T lymphocytes, memory cytotoxic T lymphocytes, and B lymphocytes (Fig. 7F). These data demonstrate that natalizumab elicited a broader PD profile than did vedolizumab and this difference is consistent with the distinct effects of these Abs on the development of EAE in these animals.
Discussion

The use of therapeutics can be limited by adverse events associated with modulating a pleiotropic target. The utility of natalizumab in multiple sclerosis and CD indications, for example, is limited by an association with PML, a severely debilitating, often fatal opportunistic infection of the brain caused by reactivation of latent JC virus (17). The anti-inflammatory activity of natalizumab in multiple sclerosis is attributed to blocking transmigration of leukocytes, including T lymphocytes, across the endothelium into inflamed parenchymal tissue of the brain (Tysabri, U.S. package insert, 2011). It has also been postulated that this mechanism of action predisposes patients to PML because it could also block immune surveillance for reactivated virus by protective memory T lymphocytes (17–19). This theory remains largely untested, however, because an appropriate model of PML does not exist (17).

EAE is an experimental model of immune surveillance of the CNS that resembles some aspects of multiple sclerosis and is often used to assess potential perturbations of immune surveillance resulting from pharmacological intervention. In one version of this model, effector memory T lymphocytes, across the endothelium into inflamed parenchymal tissue of the brain (Tysabri, U.S. package insert, 2011). It has also been postulated that this mechanism of action predisposes patients to PML because it could also block immune surveillance for reactivated virus by protective memory T lymphocytes (17–19). This theory remains largely untested, however, because an appropriate model of PML does not exist (17).

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result in only placebos exhibiting EAE (i.e., phase 1). No effect would result in one to two placebos exhibiting EAE (e.g., phase 2), based on the unbalanced pairing of placebo to mAb-exposed animals (Table III). Conversely, a stimulatory effect would result in all mAb-exposed animals exhibiting EAE. Therefore, the difference in the incidence of EAE between placebos in each phase is primarily attributable to natalizumab having a strong inhibitory effect on the development of EAE, an effect not shared by vedolizumab. This conclusion is supported by the observation that the time of EAE onset in the placebo animals and historical controls described by Kerlero de Rosbo et al. (27) was not significantly different (Fig. 2C). Half of the animals exposed to placebo (four of eight) and vedolizumab (four of seven) developed clinical signs of EAE and, moreover, did so with similar kinetics (Fig. 2). The time to onset of EAE symptoms of the historical controls and the vedolizumab group were also not significantly different (Fig. 2C). These data contrast with those of the natalizumab group, in which 14% of animals (one of seven) developed clinical signs of EAE (Fig. 2A, 2C). It can thus be inferred that blocking the \( \alpha_\beta_1 \) integrin mediates immune surveillance of the CNS in mice.

The primary objective of the investigation was to determine if vedolizumab decreased infiltration of the brain by leukocytes, as assessed by histopathological examination, and achieving this required comparing time-matched control and therapeutic samples from euthanized animals. Animals were consequently grouped together and the entire group was euthanized, once the indicator animal had developed clinical signs of EAE. A limitation of this experimental design was that it confounded assessment of the onset of clinical signs for treatment groups because nonindicator animals were euthanized before they developed EAE. Animals P8 and V5 are specific examples, neither of which exhibited clinical signs of EAE or demyelination at necropsy, but did demonstrate T cell infiltration of the brain (Table IV).

In addition, a consequence of the experimental design is that a strong inhibitory effect in one phase, but not in the other, would cause the incidence of disease in the placebo animals in both phases to differ. This difference results from euthanizing all animals in a group once one exhibits clinical signs of EAE (Table III). For example, a strong inhibitory effect by a mAb would

<table>
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<th>Treatment Group</th>
<th>Animal</th>
<th>EAE Score at Necropsy</th>
<th>Demyelination</th>
<th>CD3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CD20&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MRP14&lt;sup&gt;b,c&lt;/sup&gt;</th>
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<sup>b</sup>Percentage of demyelinated area in the scored blocks.

<sup>c</sup>Cells counted at the rim of the lesion.

<sup>d</sup>MRP14<sup>b</sup> stains macrophages.
The clinical manifestations of EAE are associated with inflammation and demyelinating lesions in histological sections of brain white matter. Comparable levels of inflammation and demyelination were observed in the white matter of animals exposed to placebo control or vedolizumab, but significantly less ($p < 0.01$) was observed in animals exposed to natalizumab (Fig. 5). These data are consistent with similar reductions in brain inflammation in postmortem analyses of brain tissue from patients with multiple sclerosis and a patient with PML exposed to natalizumab (39).

Levels of leukocyte subsets in the CSF of rhesus monkeys were also measured as an independent assessment of immune surveillance of the CNS. An increase in the level of CSF leukocytes took place concurrently with the onset of clinical signs of EAE in this investigation, and animals in the placebo and vedolizumab groups exhibited higher levels than animals in the natalizumab group (Fig. 6). These data are consistent with clinical studies demonstrating that a single dose of vedolizumab does not alter CD4+ and CD8+ T lymphocyte levels or ratio in the CSF of healthy volunteers (40). These data contrast with those from multiple sclerosis patients exposed to natalizumab; they exhibit significantly lower levels of leukocytes and CD4+ and CD8+ T lymphocytes in CSF (41). These data collectively demonstrate that infiltration of the primate CNS by leukocytes in general is mediated by the $\alpha_4\beta_1$ integrin and not the $\alpha_4\beta_7$ integrin.

Immunohistochemical analysis of the lesions revealed that a prominent feature of the lesions of animals with EAE is acute inflammation and demyelination, with mostly granulocytes in the core. At the rim, T cells and macrophages are found, but no B cells (Fig. 5). No significant differences between the groups were found with regard to the type of infiltrating cells. It is noteworthy that the two animals with inflammation but without demyelination or EAE (P8 and V5) may represent earlier stages of lesion formation in the pathogenesis of EAE. Presumably, memory T lymphocytes are the initial type of cells arriving at a site of lesion formation. These
cells recognize endogenous MOG and initiate an autoimmune reaction, which triggers an inflammatory cascade that subsequently recruits neutrophils and monocytes, culminating in a necrotic lesion. It is important to note that neutrophils do not express α4β7 or α4β1; thus, natalizumab does not inhibit their recruitment directly. It is likely that neutrophil recruitment is inhibited indirectly by natalizumab, perhaps by preventing generation of chemotactic stimuli, which result from immune surveillance by memory T cells. Although the clinical features of EAE are also determined by lesions in the spinal cord, the clear dichotomy of demyelination in the brain of animals with EAE, and no demyelination in animals without EAE, suggests that this is a very acute process. This is unlike the marmoset EAE model, in which demyelination can be found in animals without clinical EAE (42, 43).

Dose-dependent increases in the level of mature lymphocytes in the vasculature upon exposure to natalizumab, without concomitant elevations of more immature forms, has been attributed to inhibiting extravasation of lymphocytes from the circulation into parenchymal tissues (26, 44). The blockade of CNS infiltration in monkeys exposed to natalizumab (Fig. 5) was accompanied by a significant (<0.05) increase in the absolute level of leukocyte subsets in peripheral blood of these animals (Fig. 7). Natalizumab did not significantly affect levels of neutrophils (Fig. 7B), which is consistent with the lack of expression of α4 integrins by these leukocytes (4, 5, 22, 29). Rather, the natalizumab-induced leukocytosis consisted of significant (<0.05) elevations in monocytes, eosinophils, and lymphocytes, including total T lymphocytes, total and memory helper T lymphocytes, total and memory cytotoxic T lymphocytes, and total B lymphocytes (Fig. 7). These effects are consistent with expression of α4 integrins by these subsets (4, 5, 22, 29). Of interest, exposure to natalizumab did not affect levels of NK cells (Fig. 7), despite expression of both α4β1 and α4β7 (4, 22, 29). Similar overall results have been observed in healthy cynomolgus and rhesus monkeys exposed to natalizumab (26). These data illustrate that the expression pattern of these integrins is not an accurate predictor of potential functional effects of corresponding antagonists (1–3). Finally, these effects in monkeys are consistent with the vascular leukocytosis, lymphocytosis, monocyctosis, basophilia, and eosinophilia observed in multiple sclerosis and IBD patients exposed to natalizumab (45, 46), and further illustrate that elevation of vascular leukocyte levels is a useful biomarker of the scope of PD activity of integrin antagonists.

A fundamental difference in blocking both α4 integrins versus the α4β7 integrin exclusively is the proportion of the total leukocyte population that is affected, given that the α4β7 integrin is more widely expressed than the α4β1 integrin (4, 5, 22). The population of leukocytes affected by natalizumab in this investigation was indeed larger and more diverse in composition than that affected by vedolizumab. Exposure to natalizumab elevated ~40% of the total leukocyte population in the vasculature, whereas vedolizumab did not affect these subsets of leukocytes (Fig. 7). Vedolizumab does elevate a gut-homing (α4β7 high) subpopulation of memory (CD45RA−) T lymphocytes in cynomolgus monkeys, which represents ~1% of vascular leukocytes (23). Similar data have emerged from clinical trials. Natalizumab induced significant leukocytosis, lymphocytosis, monocyctosis, basophilia, and eosinophilia in CD patients (16, 47), whereas vedolizumab did not affect levels of total leukocytes, lymphocytes, monocytes, basophils, or eosinophils in peripheral blood of CD or UC patients (24, 25). The relatively broad target population and PD effects of natalizumab may thus explain the pleiotropic effects observed to date, including those in the bone marrow (48, 49), the central and peripheral nervous systems (50), the upper and lower GI tract (16, 47, 51), the liver, the upper and lower respiratory system, the urinary system, the musculoskeletal system, and the skin (Tysabri, U.S. package insert, 2011).

Overall, these data illustrate that blocking the α4β1 integrin sequesters a larger and more diverse population of leukocytes in the vasculature, and consequently impairs immunosurveillance more broadly, than blocking the α4β7 integrin. This investigation in particular demonstrates that blocking the α4β7 integrin exclusively does not impair immune surveillance of the CNS in rhesus EAE. This same therapeutic approach (i.e., blocking α4β7) elicited anti-inflammatory activity in the gastrointestinal tract of colitic monkeys (21) and in UC (24) and CD (25) patients. Thus, targeting the α4β7 integrin exclusively may provide efficacy in UC and CD patients without impairing immune surveillance of the CNS.
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References


