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Antagonizing the $\alpha_4\beta_1$ Integrin, but Not $\alpha_4\beta_7$, 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. Fedyk*

The immune system is characterized by the preferential migration of lymphocytes through specific tissues, primarily through tissues in which the immune system responds to pathogens (1–3). Tissue tropism is mediated, in part, by the $\alpha_4$ integrins expressed by T lymphocytes. The $\alpha_4\beta_1$ integrin mediates migration of memory T lymphocytes into the CNS, whereas the $\alpha_4\beta_7$ 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 $\alpha_4\beta_1$ integrin exclusively would affect migration of T lymphocytes into the CNS of primates. The effects of the dual $\alpha_4\beta_1$ and $\alpha_4\beta_7$ antagonist natalizumab were compared with those of the $\alpha_4\beta_7$ 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 $\alpha_4\beta_7$ integrin in each animal for the duration of the investigation. These results demonstrate that blocking $\alpha_4\beta_7$ exclusively does not inhibit immune surveillance of the CNS in primates.

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koencephalopathy (PML), a life-threatening brain infection charac-
terized 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). Inflamatory bowel disease (IBD) comprises a group of in-
flammatory 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 αβ or β7 integrins, such as the α4β7 integrin or the α6β4
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-inflam-
atory effects selectively in the gastrointestinal tract of cyno-
molgus 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 exclu-
sive antagonism of the α4β7 integrin would compromise immune
surveillance of the CNS in rhesus monkey EAE.

Materials and Methods

Abs
Natalizumab (Tysabri, Biogen Idec, Cambridge, MA) is a humanized IgG4
mAb that binds to the α4 chain of the human α4β7 integrin (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 α6β4 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 con-
sistent with a human EC50 of 11.4 ng/ml and IC50 of 37.2 ng/ml (Table I)
and with a previous investigation (20). 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 37.2 ng/ml and IC50 of 8.4 ng/ml (Table I). In contrast,
natalizumab did not bind to marmoset, rabbit, rat, or mouse lymphocytes
data not shown), and vedolizumab did not bind to guinea pig, rat, or
mouse lymphocytes (data not shown).

Animals
Naive, adult rhesus monkeys (Macaca mulatta) were randomly selected
from the purpose-bred colony of the Biomedical Primate Research Centre
and housed under conventional, non-specific pathogen-free conditions.
Animals were included only after a complete physical, hematological, and
biochemical checkup had been performed. During the study, monkeys
were pair housed and remained under intensive veterinary care. The daily
diet consisted of commercial food pellets for nonhuman primates (Sniff,
Soest, Germany), supplemented with rice, raisins, peanuts, and fresh fruit.
Drinking water was provided ad libitum. Ethically responsible use of
nonhuman primates was further ensured by modeling the pharmacody-
amic (PD) responses of cynomolgus monkeys to natalizumab and vedo-
limzumab and then extrapolating the minimum number of rhesus monkeys
required per treatment group to yield statistically significant differences in
PD responses after exposure to natalizumab and vedolizumab. This mod-
eling predicted that statistically significant PD results would require at
least seven animals per treatment group. This experimental design, all
study protocols, and experimental procedures were reviewed and approved
by the Biomedical Primate Research Centre’s Ethics Committee, in ac-
cordance with Dutch law on animal experimentation.

Experimental design

The rhesus monkey recombinant human myelin oligodendrocyte glyco-
protein (rhMOG) EAE model (27) was specifically chosen because T cells
are required for induction of the disease (28), the expression profiles of
rhesus monkey α4β7 and α4β1 integrins are similar to those of humans
(29), it is the only EAE model in which both natalizumab and vedolizumab
are pharmacologically active, and finally, all animals develop disease in
this model (27).

Twenty-two animals were stratified over three groups, ensuring comparable
(i.e., no significant differences in group means) age, weight, and sex distri-
bution (Table II). Placebo animals (n = 8) were administratively coupled in
a 1:1 (doublet) or 1:2 (triplet) ratio with natalizumab- (n = 7) or vedo-
limzumab-treated (n = 7) animals before EAE induction (Table III). The
experiment was executed in two phases. The objective of the first phase was
to determine if antagonizing the α4 integrins would inhibit development of
EAE. Seven natalizumab-treated animals were randomly coupled with four
placebo-treated animals, yielding 4 groups (Table III). When one animal of
a doublet or triplet was diagnosed with EAE score ≥ 2 (the indicator ani-
mal), the remaining coupled animals were euthanized either the same day or,
at maximum, 48 h later to ensure time-matched comparators for subsequent
postmortem analyses of samples. The objective of the second phase was to
determine if specifically antagonizing the α4β7 integrin would also inhibit the
development of EAE. This phase was conducted identical to the first
phase, except that vedolizumab-treated animals (n = 7) were coupled with
four additional placebo-treated animals (Table III). Natalizumab and vedo-
limzumab were dosed at 30 mg/kg on days 0, 7, 14, and 21, and placebo
animals received an equivalent volume of saline.

EAE induction and monitoring

EAE was induced using rhMOG encompassing the extracellular domain,
amino acids 1–125, which was produced in E. coli and purified as de-
scribed previously (30). Animals were immunized on day 0 with 300 µg
rhMOG 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 =
complete 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 rhMOG-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 (Quest Pharmaceutical
Services, Wilmington, DE) and by a non-GLP methodology for nata-
limab (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 Labora-
tories Montgomery, TX) and a mouse anti-human IgG monoclonal Ab
conjugated to horseradish peroxidase (Alpha Diagnostics, Owings Mills,
MD). After the addition of a chromogenic HRP substrate (3,3′,5,5′-
4-tetramethylbenzidine; Pierce Biotechnology Rockford, IL), color
development was measured at 450 nm on a Wallac 1420 Victor 2 Micro-
plate 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
2003 (Microsoft, Redmond, WA). The intensity of the color was pro-
portional 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 quantita-
tive ELISA assay was used for detection of vedolizumab in serum samples,
via GLP methodology, per testing facility SOP (Quest Pharmaceutical
Services).

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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 run, indicating good assay reproducibility.

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 × 10^5 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 (1:10000; cat. no. A9794; Sigma-Aldrich, Zwijndrecht, The Netherlands). OD values were converted to arbitrary units using the same positive control on all plates as reference.

Humoral immune responses against rhMOG

Serum samples were collected prior to EAE induction, once weekly thereafter, and at necropsy. CSF samples obtained as described below and the sera were tested by ELISA in 96-well microtiter plates for the presence of Abs against rhMOG. Plates were coated with rhMOG (5 μg/ml) and incubated overnight at 4°C. After washing and blocking with PBS/1% BSA, the wells were incubated in duplicate with 1:100 or 1:1000 diluted sera or CSF. Bound rhesus monkey Abs were detected with alkaline phosphatase–labeled goat–anti-human IgG (1:2000; cat. no. AH11305; Invitrogen) or alkaline phosphatase–labeled goat–anti-human IgM (1:10000; cat. no. A9794; Sigma-Aldrich, Zwijndrecht, The Netherlands). Conjugate binding was quantified with p-nitrophenyl phosphate (Sigma-Aldrich). OD values were converted to arbitrary units using the same positive control on all plates as reference.

Postmortem magnetic resonance images

Postmortem magnetic resonance images (MRI) of formalin-fixed hemispheres were recorded on a 9.4-T horizontal-bore nuclear magnetic resonance spectrometer (Varian, Palo Alto, CA), equipped with a quadrature coil (RAPID Biomedical, Rimpau, Germany). Formalin-fixed hemispheres were submerged in a nonmagnetic oil (Fomblin; perfluorinated polyether, Solvay Solexis, Weeps, The Netherlands) to prevent unwanted susceptibility artifacts. On a sagittal scout image, 111 contiguous coronal slices of 0.75 mm covering the complete hemisphere were defined, with the following characteristics: field of view = 55 × 55 mm; matrix = 256 × 256; voxel volume = 34.6 × 10^{-3} mm^3; two transitions. The following MRI datasets were collected to analyze the size (qualitative), spatial distribution, and characteristics of white matter lesions:

1) T2 relaxation images. These maps were calculated by a monoexponential fit of five spin echo images with increasing echo times (TE). Repetition time = 7500 ms; TE = 12.5+4 × 12.5 ms. The sequence image in this sequence, that is, TE = 25 ms, was selected as the T2-weighted image. T2-weighted and T2 relaxation time images are highly sensitive for changes in water distribution, such as the occurrence of vasogenic edema induced by inflammation.

2) Magnetization transfer ratio (MTR) images were calculated from two T1-weighted spin echo images with and without an MT-saturation pulse. Repetition time = 4250 ms; TE = 20 ms; MT-pulse: 8.19 ms gaussian-shaped pulse, nominal flip angle 1000°, offset −9.4 kHz. MTR values thus represent reduction of the MR signal as a result of the off resonance saturation pulse. Reduction of the MTR value of a tissue occurs when the density of tissue macromolecules decreases, such as by demyelination or when the tissue water content increases (inflammation).

The presence of white matter lesions was semiquantiatively graded between 0 (no lesions in white matter structures) and 10 (total white matter is affected by the lesion). The rater (E.B.) was blinded for the treatment while scoring.

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 histochimical staining, 3- to 5-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 mm^2 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.

Manual WBC and RBC counting was performed. Samples with >0.01 × 10^11/l RBC, indicative of blood contamination, were excluded from further analysis. The samples were centrifuged. Supematants were stored for determination of anti-rhMOG Abs. Pelleted cells were analyzed by FACS for expression of CD3, CD4, CD8, CD16, and CD20. The mAbs clones used are specified under Subset analysis.

Subset analysis

Venous blood samples, collected as explained above, were washed and stained for CD3, CD4, CD8, CD14, and CD45RA as described earlier for the PD monitoring. In addition, cells were stained with anti-CD16 and anti-CD20 clones 3G8 and L27, respectively; all from BD). Samples were analyzed on an LSR-II (BD), using DIVA software (BD).

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 mg/ml, for example, whereas for natalizumab it was 184.4 ± 187.3 mg/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+CD45RA2) 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>
<thead>
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</table>

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).
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−), naive and memory helper T lymphocytes (CD3+CD4+CD45RA−), total T lymphocytes (CD3+), and monocytes (data not shown) from each animal. The group means for the placebo, natalizumab, and vedolizumab groups were comparable (Fig. 3B), 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 proliferative responses at necropsy (Fig. 3A), indicating that all animals generated sufficient numbers of pathogenic cells for inducing EAE.

Effects of 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 in brain hemispheres from the natalizumab group trended lower than those of the placebo control, natalizumab, and vedolizumab groups (Fig. 4G). The mean semiquantitative values for lesion loads 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 survival 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.

In this investigation, four of eight placebo-dosed animals developed clinical signs of EAE. This finding contrasts with the natalizumab group, in which one of seven 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), and the onset of clinical signs in these animals was not significantly delayed (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).

EAE developed in the two animals exhibiting the weakest anti-rhMOG proliferative responses at necropsy (Fig. 3A), indicating 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.

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Table III. Experiment phases, groups, and individual EAE scores

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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-
number of leukocytes, with a median of 0.15 (0.10–0.44)
in the CSF of animals exhibiting signs of EAE were compared
vedolizumab demonstrates that this Ab does not block migration
CSF. Conversely, the presence of infiltrates in animals exposed to
The absence of CSF infiltrates in animals exposed to natalizumab
leukocytes (\(\text{CD}^{14}\)) monocyes is also generally similar in CSF compared with blood (Fig.

Similar to infiltration of the CSF by leukocytes, anti-rhMOG Abs were found
nievals taken from animals with an EAE

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 conse-
quence of this mechanism of action, in conjunction with
continued homeostatic production, would be the accumu-
lation 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. This leukocytosis occurred without
significant changes in RBC indices (data not shown) or neut-
rophils (Fig. 7B). The natalizumab-induced leukocytosis con-
sisted of significant \((p < 0.05)\) elevations in monocytes (Fig.
7C), eosinophils (Fig. 7D), and lymphocytes (Fig. 7E), com-
pared with pre-exposure baselines and with time-matched
placebo controls. The lymphocytosis consisted of elevations
in total T lymphocytes, total and memory helper T lympho-
cyes, total and memory cytotoxic T lymphocytes, and total
B lymphocytes, but not NK cells (Fig. 7F). Each animal ex-
posed 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
infiltrates prior to necropsy (data not shown). These data indicate that
expression 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, com-
pared 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
in elevated by natalizumab, including memory helper T lympho-
cyes, 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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Studies specifically addressing the role of \( \alpha_4\beta_1 \) integrin in EAE pathogenesis have produced inconsistent results. A seminal investigation demonstrated that the \( \alpha_4\beta_1 \) integrin was not required for the development of EAE in a SJL/N mouse model (9). Blocking the \( \alpha_4\beta_1 \) integrin exclusively or in conjunction with the \( \alpha_4\beta_7 \) integrin did not affect the development of EAE (8–13) and, moreover, in mice lacking expression of the \( \alpha_4 \) or \( \beta_7 \) integrin chains (34). Collectively, these data conclusively demonstrate that the \( \alpha_4\beta_1 \) integrin mediates the migration of leukocytes into the CNS.

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III). For example, a strong inhibitory effect by a mAb would 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 $\alpha_{4}\beta_{1}$ integrin, but not the $\alpha_{4}\beta_{7}$ integrin mediates immune surveillance of the CNS in mice.

To date, comparable investigations have not been conducted in primates. Therefore, we assessed the effects of antagonizing the $\alpha_{4}$ integrins in the rhesus monkey EAE model because this is the only established EAE model (28) in which both vedolizumab and natalizumab are pharmacologically active (Table I). This specific rhMOG protocol was chosen because all animals develop EAE (27) and generally do so prior to the development of strong neuromyelitis optica protocol was chosen because all animals develop EAE (28) in which both vedolizumab and natalizumab are pharmacologically active (Table I). This specific rhMOG protocol was chosen because all animals develop EAE (27) and generally do so prior to the development of strong neuromyelitis optica.

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

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<tr>
<th>Treatment Group</th>
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<th>Demyelination (%)</th>
<th>CD3$^a$</th>
<th>CD20$^b$</th>
<th>MRP14$^{bc}$</th>
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$^a$Percentage of demyelinated area in the scored blocks.

$^b$Cells counted at the rim of the lesion.

$^{bc}$MRP14 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 \(\alpha_4\beta_1\) or \(\alpha_4\beta_2\); thus, natalizumab does not inhibit their recruitment directly. It is likely that neutrophil recruitment is induced 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 \((p < 0.05)\) 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 \(\alpha_4\) integrins by these leukocytes (4, 5, 22, 29). Rather, the natalizumab-induced leukocytosis consisted of significant \((p < 0.05)\) 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 \(\alpha_4\) 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 \(\alpha_4\beta_1\) and \(\alpha_4\beta_7\) (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, monocytes, 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 \(\alpha_4\) integrins versus the \(\alpha_4\beta_7\) integrin exclusively is the proportion of the total leukocyte population that is affected, given that the \(\alpha_4\beta_1\) integrin is more widely expressed than the \(\alpha_4\beta_7\) 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 \(\sim 40\%\) 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 \((\alpha_4\beta_1^{high})\) subpopulation of memory (CD45RA\(^-\)) T lymphocytes in cynomolgus monkeys, which represents \(\sim 1\%\) of vascular leukocytes (23). Similar data have emerged from clinical trials. Natalizumab induced significant leukocytosis, lymphocytosis, monocytes, 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 \(\alpha_4\beta_1\) integrin sequesters a larger and more diverse population of leukocytes in the vasculature, and consequently impairs immunosurveillance more broadly, than blocking the \(\alpha_4\beta_7\) integrin. This investigation in particular demonstrates that blocking the \(\alpha_4\beta_7\) integrin exclusively does not impair immune surveillance of the CNS in rhesus EAE. This same therapeutic approach (i.e., blocking \(\alpha_4\beta_7\)) elicited anti-inflammatory activity in the gastrointestinal tract of colitic monkeys (21) and in UC (24) and CD (25) patients. Thus, targeting the \(\alpha_4\beta_7\) integrin exclusively may provide efficacy in UC and CD patients without impairing immune surveillance of the CNS.
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Disclosures
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