The Development of Experimental Autoimmune Encephalomyelitis in the Mouse Requires α4-Integrin but Not α4β7-Integrin

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Abstract

Because monoclonal antibodies (mAbs) directed against α4-integrin and VCAM-1 inhibit the development of experimental autoimmune encephalomyelitis (EAE) in vivo, it has been concluded that the successful therapeutic effect is due to interference with α4β1/VCAM-1–mediated interaction of autoaggressive T cells with the blood-brain barrier. A possible role for α4β7-integrin, or interference with other T cell mediated events during the pathogenesis of EAE, has not been considered. We have compared the effects of mAb therapy on the development of EAE in the SJL/N mouse, using a large panel of mAbs directed against α4, β7, the α4β7-heterodimer, and against VCAM-1. Although encephalitogenic T cells express both α4-integrins, mAbs directed against the α4β7-heterodimer or against the β7-subunit did not interfere with the development of EAE. In contrast, mAbs directed against α4 and VCAM-1 inhibited or diminished clinical or histopathological signs of EAE. Our data demonstrate for the first time that α4β7 is not essential for the development of EAE. Furthermore, our in vitro studies suggest that the therapeutic effect of anti–α4-treatment of EAE might also be caused by inhibition of antigen-specific T cell proliferation. (*J. Clin. Invest.* 1998. 102:2096–2105.)

Key words: α4-integrin • α4β7-integrin • experimental autoimmune encephalomyelitis • vascular cell adhesion molecule-1 • endothelium

Introduction

It has become evident that cell adhesion molecules (CAMs)[1] on the surface of leukocytes and endothelial cells are actively involved in the recruitment of specific leukocyte subsets into different tissues (1, 2). Lymphocytes which are recruited into the CNS during inflammation express a unique adhesion molecule phenotype (3, 4). Considering the structural and functional uniqueness of the blood-brain barrier (BBB) endothelium, the question has been posed whether differentiation of the BBB endothelium includes the expression of BBB-specific CAMs. In fact, there is no evidence for BBB-specific CAMs to date, but rather for suppression of the expression of E- and P-selectin (5). A series of studies performed by us and others has demonstrated that ICAM-1 and VCAM-1 are upregulated on cerebral vessels during inflammatory conditions of the CNS such as experimental autoimmune encephalomyelitis (EAE; 6–8). EAE is a $CD4^+$ T cell–mediated autoimmune disease of the CNS, which because of its clinical and histopathological features is considered the prototype model for the human disease multiple sclerosis. In EAE, the upregulation of ICAM-1 and VCAM-1 on the BBB precedes the perivascular infiltration and the onset of disease (9 and Schulz, M., and B. Engelhardt, unpublished observations), suggesting that their expression is a prerequisite for inflammatory cell entry into the CNS. Evidence for the functional importance of cerebral endothelial expression of ICAM-1 and VCAM-1 is provided by studies using a modified Stamper-Woodruff frozen section assay (10). These studies demonstrate that in vitro lymphocytes bind to inflamed cerebral vessels, which have increased levels of ICAM-1 and VCAM-1 via interaction with LFA-1/Mac-1 and α4-integrin, respectively (7, 11). The functional relevance of ICAM-1 induced on cerebral vessels during EAE for the recruitment of inflammatory cells across the BBB in vivo is still a matter of debate (12, 13). In contrast, there is accumulating evidence that, in vivo, α4-integrin/VCAM-1 interactions play a pivotal role in the recruitment of inflammatory cells across the BBB during EAE. Intravenous application of anti–α4-integrin antibodies reduces cellular infiltration of the CNS and inhibits the development of EAE (11, 14) and most importantly can reverse the ongoing disease process in the guinea pig (15). Furthermore, it seems that the ability of autoantigen specific T cell clones to induce EAE at least in part correlates with their expression levels of α4-integrin (16, 17). The general conclusions from these studies were that anti–α4-integrin mAbs interfere with α4β1/VCAM-1–mediated recruitment of inflammatory cells across the BBB.

However, two alternative possibilities for the mechanism of action of anti-α4 antibodies have not yet been considered. First, it has not yet been examined whether the second α4-integrin, α4β7, might be involved. Second, interference of the anti-α4 antibodies with the pathogenesis of EAE could be due to blocking T cell–mediated events unrelated to migration. This is supported by the result that mAb R1.2, although a poor inhibitor of α4-integrin–mediated lymphocyte homing in vivo (18), has been shown to interfere with the development of EAE (17).

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Received for publication 12 June 1998 and accepted in revised form 28 October 1998.

1. *Abbreviations used in this paper:* aEAE, active EAE; APC, antigen-presenting cell; BBB, blood-brain barrier; CAM, cell adhesion molecule; EAE, experimental autoimmune encephalomyelitis; tEAE, transfer EAE.

J. Clin. Invest.

0021-9738/98/12/2096/10 $2.00
Volume 102, Number 12, December 1998, 2096–2105
http://www.jci.org

Therefore, the aim of this study was to determine the roles of α4β1, α4β7, and VCAM-1 in the pathogenesis of EAE considering an inhibitory effect in lymphocyte recruitment into the CNS and also their possible interference with other T cell–mediated events involved in the pathogenesis of EAE.

Methods

mAbs. The rat anti–mouse mAbs used in this study are summarized in Table I (19–26). The mAbs were purified from serum-free culture supernatant of hybridoma cells by ammonium-sulfate precipitation and dialyzed against sterile PBS. Purity of mAb preparations was confirmed by SDS-PAGE. Endotoxin levels of mAb preparations were routinely determined by Fresenius (Tanusstein, Germany) and were below detection levels (< 0.6 EU/ml).

Induction of EAE. Female SJL/N mice were obtained from Bomholdgård Breeding (Ry, Denmark) between 3 and 4 wk of age. Active EAE (aEAE) and passive transfer EAE (tEAE) were induced exactly as described before (5, 7).

T lymphocyte lines. PLP-specific T cell lines were established from the popliteal and inguinal lymph nodes of SJL/N mice immunized subcutaneously with 20 μg of PLP peptide (amino acids 139–151) in complete Freund's adjuvant containing 60 μg/ml *Mycobacterium tuberculosis* H37Ra exactly as described (5). All the established lines contain $CD4^+$ T cells only and recognize their antigen in the molecular context of MHC class II. The encephalitogenic potential of the T cell lines was tested after the second round of antigen-specific stimulation by intravenous injection of 3×10^6 freshly activated T cell blasts into syngeneic naive recipients. Antigen specificity was tested in proliferation assays (see below).

Proliferation assay. T cells were seeded in 96-well plates at 2×10^4 cells/well in RPMI 1640 supplemented with 10% FCS (PAA, Linz, Austria), 10 U/ml penicillin/streptomycin, 2 mM L-glutamine, nonessential amino acids, 1 mM sodium pyruvate, and 5×10^{-5} M β-mercaptoethanol (Sigma, Munich, Germany) together with 5×10^5 irradiated syngeneic spleen cells as antigen-presenting cells (APCs). The specific antigen PLP or the irrelevant control antigen protein-purified derivate of *M. tuberculosis* (PPD) was added at 10 μg/ml. ConA was added at 2.5 μg/ml. After 48 h of incubation, tritiated thymidine (^{3}H-dT) was added to the cultures (1 μCi/well) and the incubation was continued for another 16 h. Cultures were harvested on filter paper using a cell harvester (Inotech, Berthold, Bad Wildbad, Germany) and incorporation of ^{3}H-dT was measured by liquid scintillation counting. For the antibody inhibition studies, 20 μg/ml of antibodies was present throughout the assay in wells where T cells were restimulated with PLP. All tests were performed in triplicates. Assays were repeated three to five times for each T cell line.

Treatment of EAE. For treatment of EAE, mAbs were injected intravenously. To determine the serum levels of circulating rat IgG during an EAE experiment, serum was taken daily from two to three animals per group. Serum levels of injected mAbs were either determined by FACS® analysis using α4-integrin–positive T cells and VCAM-1–positive bEnd5 (27, 28) or by anti–rat Ig ELISA. In case of aEAE, a single intravenous injection of 400 μg mAb per mouse was given on day 11, which is the day before the expected onset of clinical aEAE in control animals. In aEAE animals, serum levels of mAb injected at day 11 after immunization remained stabile for four consecutive days in the case of DATK 32, Fib 30, Fib 504, and 6C7.1. In contrast, serum levels of PS/2, R1.2, 4B12, MK2.7, and 9B5 started to drop already 2 d after mAb injection (data not shown). The differences in circulation times of the different mAbs cannot be attributed to their respective isotypes, therapeutic efficacies, or the antigen they recognize. In case of PS/2 and R1.2, loss of detectable serum levels of antibody correlated with the 2–3-d delay of clinical onset of aEAE, which indicates that the short circulation times of the mAbs are enough to provide an inhibitory effect on aEAE. In the case of tEAE, 400 μg mAb/per mouse was injected intravenously together with the encephalitogenic T cells and mAb injection was repeated on day 4 as serum detection levels of all the mAbs used in this study (see Table III) dropped at that day (data not shown).

Flow cytometry. Flow cytometric analysis was performed as previously described (3, 5). Data were analyzed using FACScan® (Becton Dickinson, Heidelberg, Germany) and the Cellquest software. For analysis, light scatter gates were drawn to include either all live cells or lymphocytes only, as indicated in Results.

Adhesion assay on endothelial cells. Adhesion assays have been carried out exactly as described (28). Assays comparing adhesion of encephalitogenic versus nonencephalitogenic T cell lines to TNF-α (16 h, 5 nM) stimulated bEnd5 were repeated six times, whereas direct comparison of T cell adhesion of one individual T cell line to stimulated versus unstimulated bEnd5 was repeated four times.

Adhesion assay on VCAM-1. Recombinant human VCAM-1 was kindly provided by Dr. R. Lobb (Biogen Inc., Boston, MA). VCAM-1 or the control protein BSA was coated (each 10 μg/ml) in PBS onto 21-field (4 mm diameter) glass slides at 4°C in humidity overnight. Fields were washed twice with PBS and blocked with 100% calf serum for 1 h at 20°C. T cell blasts were resuspended in RPMI/25 mM

Table I. Rat Anti–mouse mAbs Used in This Study

Antibody	Reactivity	Isotype	Source	Reference
9B5	Isotype control	IgG2a	E.C. Butcher, Stanford, CA	22
R1/2	α4	IgG2b	B. Holzmann, Munich, Germany	21
PS/2	α4	IgG2b	P. Kincade, Oklahoma City, OK	24
5-3	α4	IgG2b	P. Altevogt, Heidelberg, Germany	26
9EG7	β1	IgG2a	D.V.	23
DATK-32	α4β7	IgG2a	E.C. Butcher	19
Fib 30	β7	IgG2a	E.C. Butcher	19
Fib 504	β7	IgG2a	E.C. Butcher	19
MK2.7	VCAM-1	IgG1	E.C. Butcher	25
6C7.1	VCAM-1	IgG1	U.S. and D.V.	Unpublished
4B12	VCAM-1	IgG2a	U.S. and D.V.	Unpublished
2A11	VCAM-1	IgG2a	U.S. and D.V.	Unpublished
MJ7/18	Endoglin	IgG2a	E.C. Butcher	20
GK1.5	CD4	IgG2b	ATCC, Rockville, MD	ATCC
ER-TR2	MHC class II	IgG2b	BMA Biomedicals AG, Augst, Switzerland	BMA
GoH3	α6	IgG2a	PharMingen, Hamburg, Germany	PharMingen

Hepes and 5% calf serum (10^6 cells/ml). 20 μl of cell suspension (2×10^4 cells/field) was incubated for 30 min at room temperature under shear (50 rpm on a rotating platform, model KS250 basic; IKA Laboratories, Staufen, Germany). Nonadherent cells were washed off by dipping the slides twice into PBS. Adherent cells were fixed in 2.5% glutaraldehyde in PBS for 2 h. For antibody inhibition assays, T cell blasts or slides were preincubated with mAb at 20 μg/ml for 15 min at room temperature and washed; adhesion assays were performed as described. Samples were done in triplicates. Assays were analyzed by video assisted light microscopy (NIH Image software) and bound cells per predefined field were determined by counting five fields per well. Assays were repeated five times.

Results

Establishment of PLP-specific T cell lines. T cell lines specific for the encephalitogenic peptide amino acids 139–153 of PLP were established from SJL/N mice. 10 of those T cell lines are described here. As determined by FACS® analysis, all established T cell lines were $CD4^+$ $CD8^-$ Th1 cells (data not shown) and recognize their specific antigen in a MHC class II–restricted way (see below). 8 out of 10 T cell lines were encephalitogenic as tested by transfer of freshly activated T cell blasts into syngeneic naive recipients (data not shown). After the second antigen-specific restimulation in vitro, the PLP-specific T cell lines showed a strong specific proliferation in response to PLP (Table II). The amount of the proliferative response to PLP in comparison to either spontaneous proliferation or to proliferation in the presence of control antigens did not correlate with the encephalitogenic potential of the respective PLP-specific T cell lines (Table II). Encephalitogenicity was retained up to the fourth antigen-specific restimulation in vitro. After later restimulations, induction of EAE required the injection of higher numbers of T cell blasts; most of the PLP-specific T cell lines lost their encephalitogenic potential after the sixth restimulation.

α4β1 and α4β7 are both expressed on encephalitogenic and nonencephalitogenic PLP-specific T cell lines. It has been proposed that surface expression of α4-integrin correlates with the encephalitogenic potential of murine PLP- or MBP-specific T cell clones (16, 17). However, it has not been addressed if this is due to the expression of α4β1- or α4β7-integrins on the autoaggressive T cells. Therefore, we investigated the surface expression of α4β1 and α4β7 on our PLP-specific T cell lines by FACS® analysis using a large panel of mAbs directed against the integrin subunits α4, β1, β7, and the integrin-heterodimer α4β7 (Table I). As only freshly activated T cell blasts but not resting T cells are able to transfer EAE, we focused on investigating the surface expression of α4β1- and α4β7-integrins on the freshly activated T cell blasts for each PLP-specific T cell line. Unexpectedly, T cell blasts from all PLP-specific T cell lines showed surface expression of the integrin subunits α4, β1, β7, and the α4β7-heterodimer, irrespective of their encephalitogenic potential (Fig. 1 and data not shown). In fact, one of the nonencephalitogenic T cell lines, namely SJL.PLP3, showed the highest surface levels for the investigated integrins (Fig. 1). Direct comparison of the surface expression of the integrin subunits α4, β1, β7, and the α4β7-heterodimer on T cell blasts with that on resting T cells from the same line revealed that generally T cell blasts expressed much higher levels of α4, β1, β7, and α4β7 than did resting T cells (Fig. 1). At the same time, surface expression of the α6-integrin subunit, which was expressed mostly at low levels on the PLP-specific T cell lines, was not upregulated on T cell blasts in comparison to resting T cells (data not shown). Neither the surface levels of α4, β1, β7, or α4β7, nor the degree of their regulation, nor the percentage of $\alpha4\beta7^{high}$ or $\beta7\text{-integrin}^{high}$ T cell blasts within an individual T cell line could be correlated to the encephalitogenic potential of the PLP-specific T cell line. With the exception of the nonencephalitogenic T cell lines SJL.PLP3 and SJL.PLP8, injection of 3×10^6 T cell blasts of each of the different PLP-specific T cell lines into naive recipients resulted in EAE of the same clinical severity (data not shown). In summary, our data show that α4β1 and α4β7 are both expressed on PLP-specific T cell lines and are significantly upregulated upon antigen-specific stimulation. Neither the expression levels of α4-integrins nor the percentage of α4β7-positive T cells within a PLP-specific T cell line allowed any prediction of the encephalitogenic potential of the individual T cell line.

PLP-specific T cell lines can use α4β1 and α4β7 to bind to brain endothelium in vitro. The mere surface expression of α4β1- and α4β7-integrin on PLP-specific T cell blasts does not allow us to draw conclusions about their involvement in the disease process in EAE. To define whether both α4-integrins can contribute to adhesion of PLP-specific T cell blasts to brain endothelial cells we performed in vitro adhesion assays

Table II. Antigen Specificity of T Cell Lines in the Second In Vitro Restimulation

T cell line	No antigen	PLP	PPD	ConA
SJL.PLP1	$106.4 + 0.8 \times 10^3$*	$131.2 + 9.8 \times 10^3$	$94.3 + 45.0 \times 10^3$	$145.1 + 11.9 \times 10^3$
SJL.PLP2	$3.5 + 1.1 \times 10^3$	$40.8 + 25.2 \times 10^3$	$9.6 + 3.2 \times 10^3$	$64.0 + 2.3 \times 10^3$
SJL.PLP3	$16.7 + 1.9 \times 10^3$	$215.5 + 10.0 \times 10^3$	$55.3 + 1.4 \times 10^3$	$180.0 + 64.6 \times 10^3$
SJL.PLP4	$24.8 + 1.7 \times 10^3$	$150.4 + 6.1 \times 10^3$	$78.6 + 13.2 \times 10^3$	$186.1 + 6.1 \times 10^3$
SJL.PLP5	$7.1 + 0.8 \times 10^3$	$12.0 + 1.5 \times 10^3$	$6.5 + 1.9 \times 10^3$	$9.5 + 1.5 \times 10^3$
SJL.PLP6	$10.3 + 1.7 \times 10^3$	$115.9 + 0.7 \times 10^3$	$23.4 + 0.3 \times 10^3$	$12.6 + 0.7 \times 10^3$
SJL.PLP7	$10.1 + 1.9 \times 10^3$	$114.6 + 5.2 \times 10^3$	$19.0 + 0.4 \times 10^3$	$27.2 + 3.3 \times 10^3$
SJL.PLP8	$2.5 + 0.1 \times 10^3$	$27.9 + 1.9 \times 10^3$	$3.2 + 0.4 \times 10^3$	$19.1 + 1.4 \times 10^3$
SJL.PLP9	$30.5 + 2.9 \times 10^3$	$94.2 + 3.0 \times 10^3$	$25.0 + 1.9 \times 10^3$	$137.6 + 14.5 \times 10^3$
SJL.PLP10	$79.1 + 1.4 \times 10^3$	$106.3 + 9.9 \times 10^3$	$26.7 + 6.2 \times 10^3$	$115.1 + 17 \times 10^3$

*Proliferation measured as incorporation of [^{3}H]thymidine is shown. The numbers represent mean cpm ±SD of triplicates. Shaded T cell lines are nonencephalitogenic.

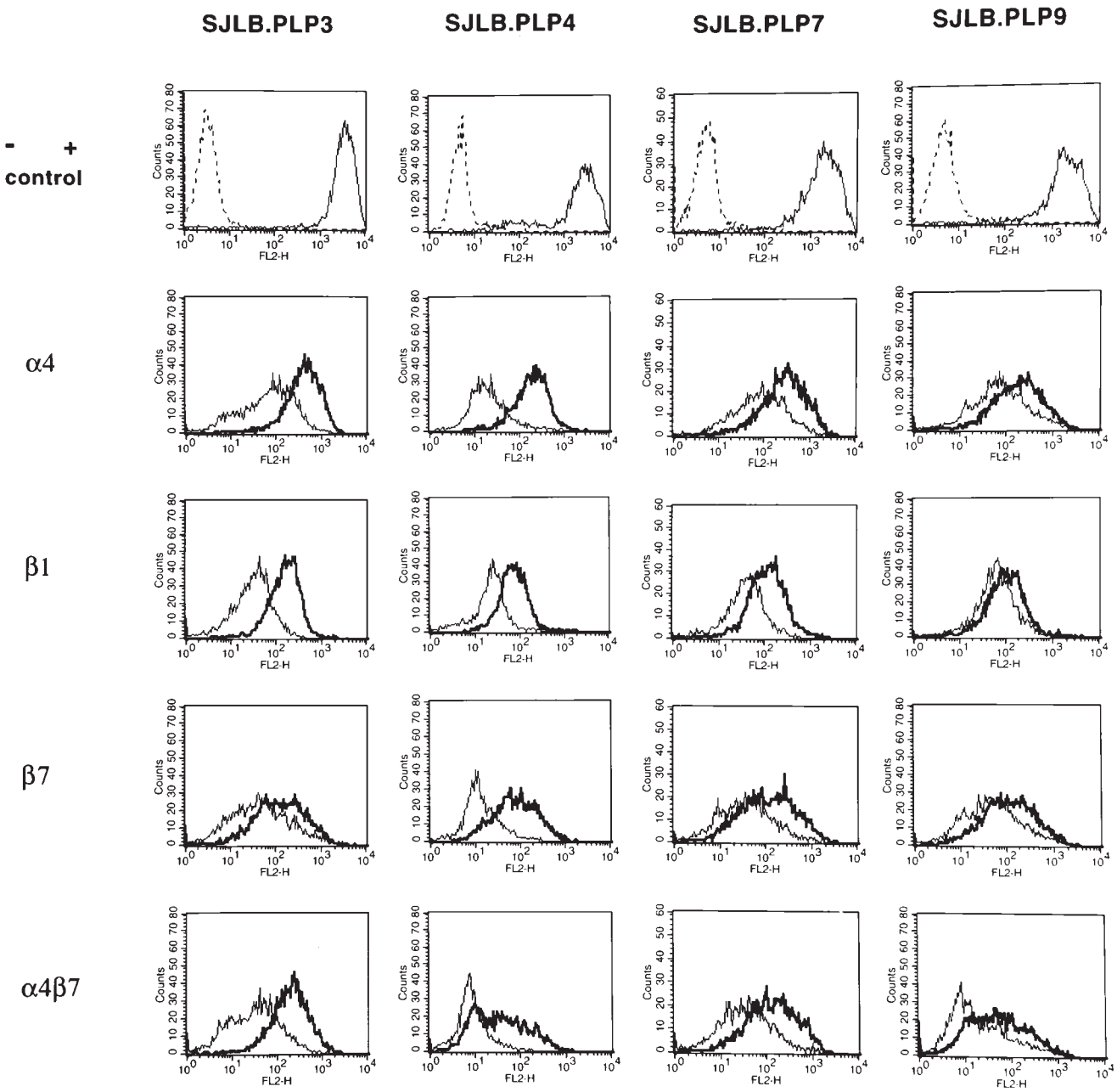

Figure 1. Expression of α4β1 and α4β7 on PLP-specific T cell lines. Expression of the α4-, β1-, and β7-integrin subunits and the α4β7-heterodimer on PLP-specific T cell lines as determined by FACS® analysis. The top panel overlays show negative staining with an isotype-matched control mAb (*dashed line*, − control) and positive staining with anti-Thy 1.2 (*straight line*, + control). Overlays for α4-, β1-, β7-, and α4β7-integrin show surface staining on freshly activated T cell blasts (*thick line*) in comparison to resting T cells (*thin line*).

with the brain endothelial cell line bEnd5. Upon stimulation with TNF-α bEnd5 express high levels of the α4-integrin ligand VCAM-1, whereas unstimulated bEnd5 express very low levels of VCAM-1 or no detectable VCAM-1 on their surface (27, 28).

T cell blasts of both encephalitogenic and nonencephalitogenic PLP-specific T cell lines adhered to unstimulated bEnd5. Pretreatment of T cell blasts with a panel of different mAbs directed against the α4-integrin subunit, against β7, or against the α4β7-heterodimer slightly inhibited their adhesion to unstimulated bEnd5 (Fig. 2; data not shown). Pretreatment of unstimulated bEnd5 with a panel of anti–VCAM-1 resulted in either no significant inhibition of T cell adhesion or in an inhibitory effect that was even smaller than that observed with anti–α4-integrin mAbs. This indicated that the α4-integrin–mediated adhesion of PLP-specific T cell blasts to bEnd5 is not mainly mediated via endothelial VCAM-1. Stimulation of bEnd5 with TNF-α increased the number of encephalitogenic and nonencephalitogenic PLP-specific T cell blasts binding to bEnd5 (Fig. 2; data not shown). Pretreatment of encephalitogenic and nonencephalitogenic T cell blasts with a panel of mAbs directed against the α4-integrin subunit dramatically inhibited their adhesion to stimulated bEnd5 (Fig. 2; data not shown). Antibodies directed against β7 or against the het-

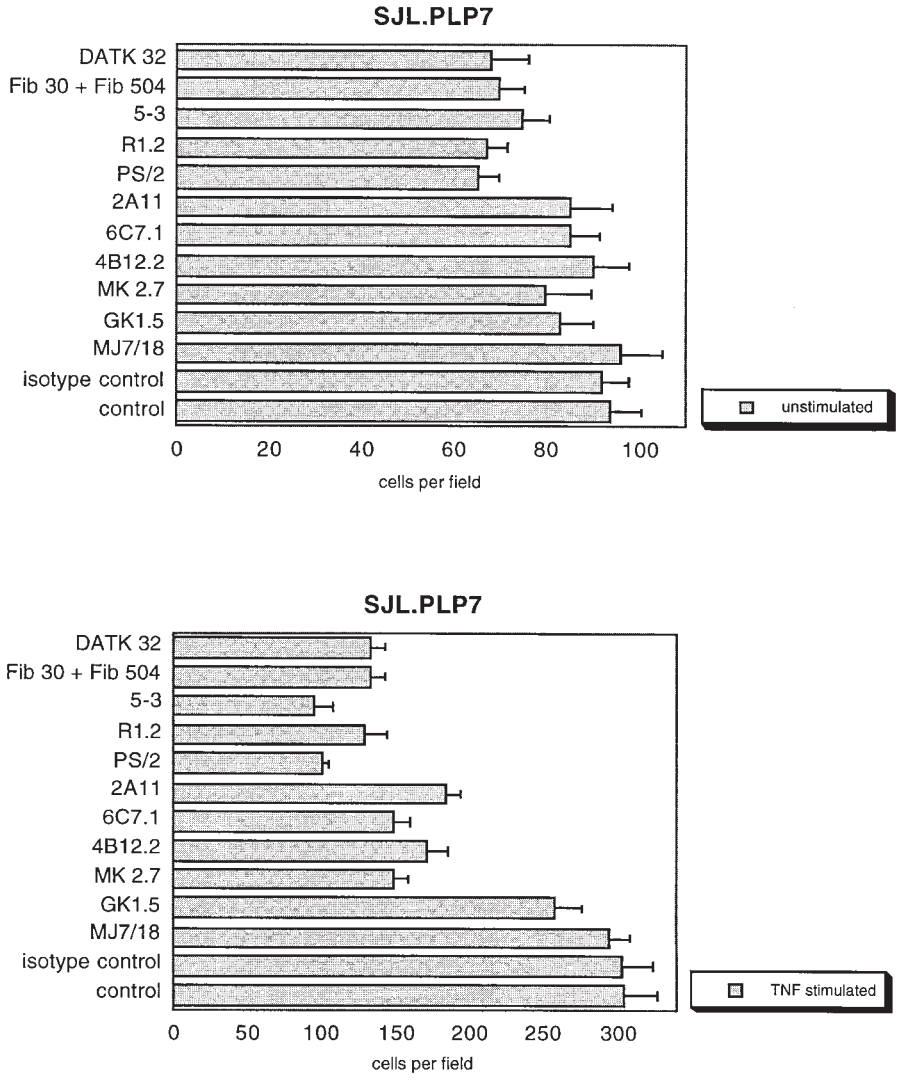

Figure 2. Adhesion of PLP-specific T cell lines to unstimulated and TNF-α–stimulated brain endothelium. One representative experiment comparing the adhesion of freshly activated T cell blasts from the encephalitogenic T cell line SJL.PLP7 to untreated and TNF-α treated bEnd5 is shown. Whereas α4-integrins do not play a major role in adhesion of the T cells to unstimulated bEnd5, adhesion of SJL.PLP7 to stimulated bEnd5 could be inhibited significantly by mAbs directed against α4-integrin and to a slightly lesser degree by mAbs directed against β7- and α4β7-integrin. Preincubation of stimulated bEnd5 with anti–VCAM-1 mAbs also significantly reduced adhesion of SJL.PLP7. Bars represent mean±SD (n = 3).

erodimer α4β7 were also inhibitory, although to a slightly lesser degree than the anti-α4 mAbs (Fig. 2). Since in addition to α4β1 and α4β7 no other α4-integrin is known to date, it can be concluded that PLP-specific T cell lines can adhere via α4β1 and α4β7 to stimulated brain endothelium in vitro irrespective of their encephalitogenic potential. Pretreatment of bEnd5 with anti–VCAM-1 mAbs resulted in a reduced adhesion of PLP-specific T cell blasts to stimulated bEnd5 cells (Fig. 2). The fact that anti–VCAM-1 mAbs inhibited adhesion of PLP-specific T cell blasts to bEnd5 to a slightly lesser degree than did anti–α4-integrin mAbs points to a minor role for other α4 ligands in adhesion of T cell blast to bEnd5. Since the α4β7 ligand MAdCAM-1 is not expressed on bEnd5, another possible α4-integrin ligand on bEnd5 is fibronectin. Taken together, our data demonstrate that whereas α4-integrins play a minor role in mediating adhesion to unstimulated brain endothelium, they play a major role in mediating adhesion of PLP-specific T cell blasts to stimulated endothelium in vitro. In the latter case, both α4β1 and α4β7 can contribute to this adhesion mainly via endothelial VCAM-1.

PLP-specific T cell lines can use α4β1 and α4β7 to bind to VCAM-1 in vitro. To substantiate our observation that α4β1- and α4β7-integrin are used by PLP-specific T cell blasts to bind directly to VCAM-1, we performed adhesion assays on purified recombinant human VCAM-1. PLP-specific T cell lines readily bound to VCAM-1 (Fig. 3). Adhesion was mediated exclusively via α4-integrin since anti–α4-integrin mAbs completely inhibited T cell binding to VCAM-1. mAbs directed against β7 and the α4β7-heterodimer partially inhibited T cell binding to VCAM-1, implying that α4-integrin–mediated binding to VCAM-1 was mediated by α4β1 plus α4β7-integrin (Fig. 3).

mAbs directed against α4 and VCAM-1, but not against β7 or α4β7, influence the development of tEAE. To determine which α4-integrin is involved in the pathogenesis of EAE transferred by freshly activated PLP-specific T cell blasts in vivo, we asked whether antibodies directed against α4, β7, or the α4β7-heterodimer were able to influence the development of the disease. All animals from the control group injected with an isotype-matched control mAb and the groups treated with anti-β7 or anti-α4β7 lost weight and developed clinical EAE. In contrast, mice treated with the anti–α4-integrin mAb PS/2 were protected from disease development until day 11 after T cell transfer (Fig. 4) when animals were killed for histopathological examination. Inflammatory infiltrates were detected in the brains and spinal cords of all diseased mice, whereas clinically healthy mice from the PS/2 group showed no inflammatory infiltrates (data not shown). The development of mild clinical

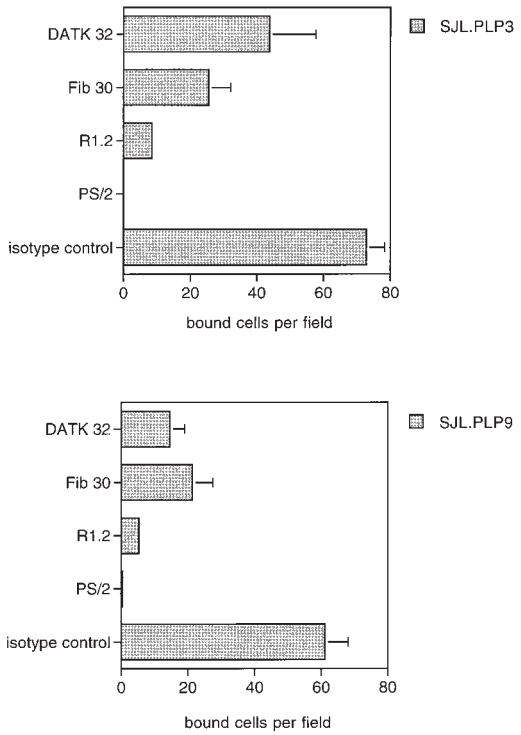

Figure 3. Adhesion of PLP-specific T cell lines to VCAM-1. One representative experiment comparing the adhesion of T cell blasts from the encephalitogenic T cell line SJL.PLP9 with adhesion of T cell blasts from the nonencephalitogenic T cell line SJL.PLP3 to recombinant VCAM-1 is shown. Adhesion to VCAM-1 was completely inhibited in the presence of mAbs directed against α4 and partially in the presence of mAbs against β7- and α4β7-integrin. Bars represent mean±SD ($n = 3$).

symptoms within the PS/2 group was paralleled by the presence of small infiltrates in the spinal cord parenchyma. These data demonstrate that α4-integrin but not β7-integrins are required for the development of tEAE.

To substantiate this finding and to exactly define the involvement of α4-integrins and their ligand VCAM-1 in the pathogenesis of EAE, we set up large scale experiments which allowed the direct comparison of the therapeutic effects of a large panel of mAbs directed against these molecules. Table III summarizes one representative large scale experiment where tEAE was induced with the encephalitogenic T cell line SJL.PLP9. The same results were reproduced when inducing tEAE with the encephalitogenic T cell line SJL.PLP4 (data not shown). Three different anti–α4-integrin mAbs, PS/2, R1.2, and 5-3, were clearly protective in tEAE, since they delayed the onset of clinical disease, diminished the severity of clinical tEAE, and reduced the duration of the clinical disease. Again, neither treatment with anti–β7- nor anti–α4β7-integrin mAbs showed any influence on the development of tEAE. Three different mAbs directed against VCAM-1 also showed an inhibitory effect on the development of tEAE as they delayed the disease onset. However, neither of the anti–VCAM-1 mAbs showed a significant influence on the severity or the duration of the disease as did the anti–α4-integrin mAbs. The difference in therapeutic efficacy between anti-α4 mAbs and anti–VCAM-1 mAbs was further substantiated by the observation that the delay in disease onset achieved by treatment with anti-α4 mAbs was significantly larger than the delay achieved by treatment with anti–VCAM-1 mAbs (Table III). Thus, in tEAE anti–VCAM-1 treatment was less effective when compared with anti–α4-integrin treatment.

mAbs directed against α4-integrin and VCAM-1 ameliorate aEAE. We next asked whether our observations that α4-mediated interaction with VCAM-1 is involved in the pathogenesis of EAE held true in aEAE, where the autoaggressive T cells are activated in vivo. A single injection of different anti–α4-integrin mAbs or anti–VCAM-1 mAbs on day 11 after immunization, which is one day before the expected onset of clinical aEAE in this model, significantly delayed the onset of aEAE and additionally diminished the mean severity of the clinical disease (Table IV). Control mAbs had no influence on the development of aEAE. Thus, α4-integrin and VCAM-1 are critically involved in the pathogenesis of aEAE.

Anti–α4-integrin mAbs interfere with the antigen-induced proliferation of PLP-specific T cell lines. In contrast to the anti–α4-integrin mAb PS/2, the anti–α4-integrin mAb R1.2 has been reported to be ineffective in inhibiting lymphocyte homing in vivo (18). The confirmation of this observation in the SJL/N mouse by us (Engelhardt, B., unpublished data) suggested that the protective effects of anti–α4-integrin mAbs in EAE might be based on different inhibitory mechanisms in vivo. Therefore, we investigated whether anti–α4-integrin mAbs interfere with antigen-induced proliferation of PLP-specific T

Table III. Effect of mAbs on the Development of tEAE Mediated by SJLB.PLP9

Treatment	Control mAb	DATK-32	Fib 30	PS/2	R1.2	5-3	MK2.7	2A11.11	6C7.1
Number of animals	7	7	7	6	7	6	7	7	7
Number of animals with clinical disease	7	7	7	6	7	6	7	7	7
Mean day of onset of tEAE	9.4±0.5	9.7±0.5	9.8±0.7	14.5±1.9*‡	13.3±0.5*‡	13.5±1.9*‡	10±0‡§	11.1±0.4*‡	12.7±0.5*‡
Mean severity of clinical disease	1.9±0.2	1.9±0.2	1.9±0.2	0.7±0.4*	1.4±0.3§	1.1±1	1.1±0.4‖	Killed early	1.8±0.7
Mean duration of clinical tEAE	11.1±1.5	11.2±1.8	11±2.1	7.2±3.6‖	12.2±3	6.8±3§	11±0.7	Killed before complete recovery	12.8±5.2

According to the Student's *t* test the differences are *$P < 0.0001$ = extremely significant; §$P < 0.001$ = very significant; ‖$P < 0.01$ = significant when compared to the values of control mAb. ‡When the values of each anti-α4 treatment group (PS/2, R1.2, 5-3) marked with ‡ were compared to the values of either anti–VCAM-1 treatment group (MK2.7, 2A11.11, 6C7.1) also marked with ‡, they were calculated to differ at least significantly with the exception of 5-3 in comparison to 6C7.1.

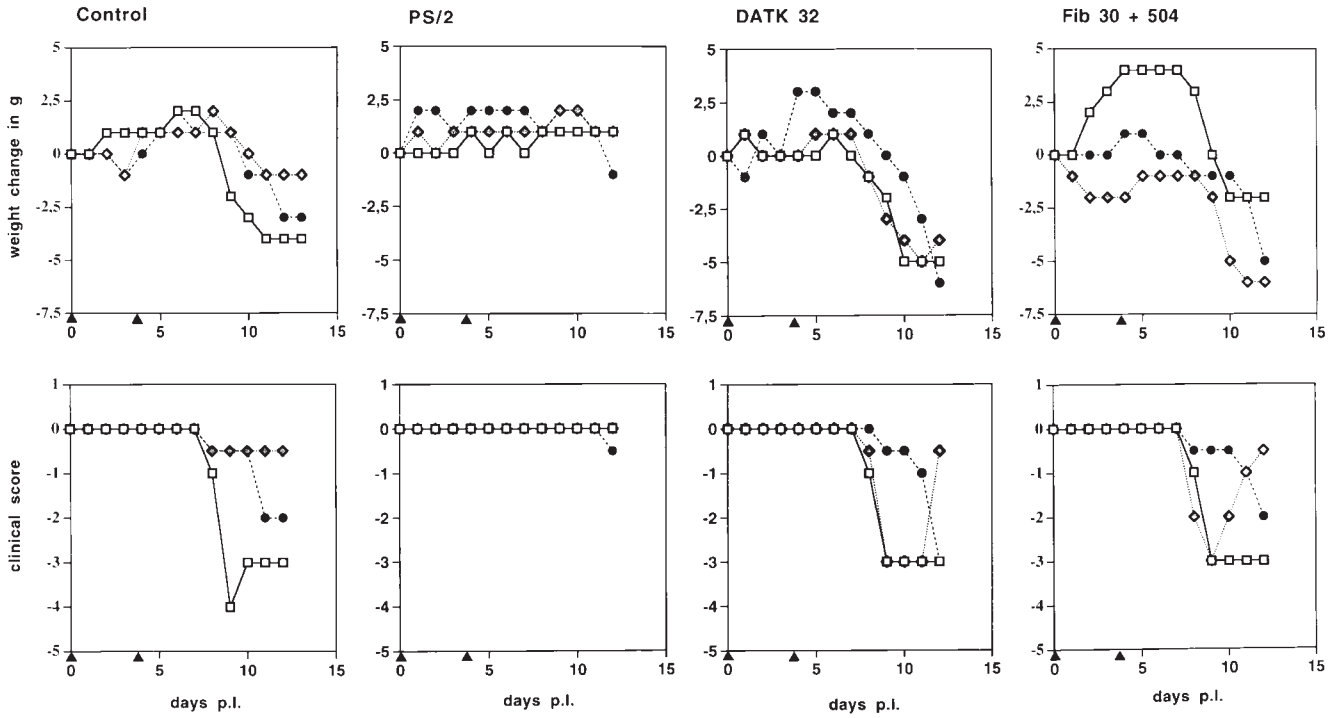

Figure 4. Inhibition of tEAE by mAbs directed against α4- but not against β7- or α4β7-integrin. Three representative animals from each treatment group of $n = 7$ mice are shown in the graphs. One line represents one mouse using the same symbol for an individual mouse in the two graphs shown for each treatment group. Arrowheads indicate the days of mAb injection. Whereas animals from the control group, the anti-α4β7 (DATK 32) group, and the anti-β7 (Fib-30 + 504) group showed weight loss and clinical disease, the animals from the PS/2 group showed no development of tEAE until day 11 after immunization (*p.I.*).

cells in vitro. In cocultures with irradiated syngeneic APCs, resting PLP-specific T line cells only proliferate in the presence of their specific antigen PLP or in the presence of the T cell mitogen ConA (Fig. 5). The anti-α4 mAb R1.2 did interfere with the antigen-specific induced proliferation of all PLP-specific T cell lines (Fig. 5; data not shown). This was not true in the same way for the anti-α4 mAb PS/2, which only in the case of some PLP-specific T cell lines interfered with PLP-induced proliferation, and mostly to a lesser degree than did R1.2 (Fig. 5). Both mAbs also seemed to reduce the ConA-stimulated proliferation of some PLP-specific T cell lines; however, the observed reduction in proliferation was not significant in every assay (Fig. 5). As expected, mAbs directed against CD4 or MHC class II, used as positive inhibitory controls, completely inhibited the antigen-induced proliferation of the $CD4^+$ MHC class II restricted PLP-specific T cell lines, whereas the addition of isotype-matched control mAbs did not significantly alter the PLP-induced proliferation (Fig. 5). mAbs directed against β7-integrin, the α4β7-heterodimer, or against β1 had no influence on PLP-induced proliferation (Fig. 5). Additionally, none of the anti–VCAM-1 mAbs inhibited the PLP-induced proliferative response of the PLP-specific T cell lines. The inhibitory effect of R1.2 and PS/2 on PLP-induced proliferation was not due to a general interference with T cell proliferation since IL-2–dependent proliferation of PLP-specific T cell lines was not altered by the presence of any of the above mentioned mAbs (Fig. 5). In fact, the presence of R1.2 and PS/2 did not result in any appreciable change of these T cell cultures. Taken together, this suggests that the anti–α4-integrin mAb R1.2 and to a lesser degree PS/2 specifically interfere with the antigen-induced proliferation of PLP-specific T cell lines.

Discussion

mAbs directed against α4-integrin have been shown to successfully interfere with the development of EAE in different animal models (11, 14, 15, 17). Since the anti–α4-integrin treat-

Table IV. Effect of Single Injections of mAbs Day 11 after Immunization on the Development of aEAE

Treatment	Saline	Control mAb	MJ7/18	PS/2	R1.2	6C7.1
Number of animals	7	16	3	20	16	4
Number of animals with clinical disease	6	16	3	14	14	3
Mean day of onset of aEAE	12.6±1.5	12.3±0.6*	12.8±0.3*	14.1±0.8‡	14±0.5‡	14.3±0.6‡
Mean severity	3.75±0.35	3.5±0.8*	Killed early	2±1.2‡	1.65±1.2‡	Killed early

*No significant difference to saline group according to the Student's *t* test; ‡$P < 0.0001$, these differences are considered to be extremely significant, when compared to the values of the control mAb or saline groups.

ment proved to substantially decrease inflammation in the CNS, its therapeutic success has been attributed to inhibition of α4β1/VCAM-1–mediated recruitment of inflammatory cells across the BBB. A role for the α4β7-integrin in EAE has not been considered to date. To delineate which of the two known α4-integrins is involved in the pathogenesis of EAE, we established 10 individual $CD4^+$ PLP T cell lines from SJL/N mice. All PLP-specific T cell lines showed surface expression of both α4β1- and α4β7-integrins. Both α4-integrins were significantly upregulated on the surface of freshly activated T cell blasts, suggesting that modulation of α4-integrin surface levels might be relevant for the trafficking or the pathogenicity of encephalitogenic T cells. Neither the expression level of α4β1 nor that of α4β7 correlated with the disease-inducing activity of the PLP-specific T cell lines. In fact, the highest α4-integrin expression was observed on one of the nonencephalitogenic T cell lines. This is in contrast to previous findings where high surface expression of α4-integrin has been correlated with the EAE-inducing capacity of autoaggressive T cells (17). The apparent discrepancy of our findings with this earlier report (17) could be due to the fact that the former study compared individual T cell clones from a single T cell line. However, our findings are substantiated by a study from Kuchroo et al. (16) that demonstrated PLP-specific T cell clones, which despite high surface levels for α4-integrin were not encephalitogenic. Thus, high surface levels of α4-integrins do not allow us to predict the encephalitogenic potential of the respective PLP-specific T cell line.

To define whether both α4-integrins might be involved in T cell recruitment into the CNS, we asked whether PLP-specific T cell lines can bind via both α4-integrins to brain endothelium in vitro. Using a large panel of inhibitory mAbs, we could demonstrate that in vitro both α4β1 and α4β7 significantly contribute to the adhesion of PLP-specific T cell blasts to activated brain endothelium. Using several anti–VCAM-1 mAbs we defined endothelial VCAM-1 as the major ligand. Since

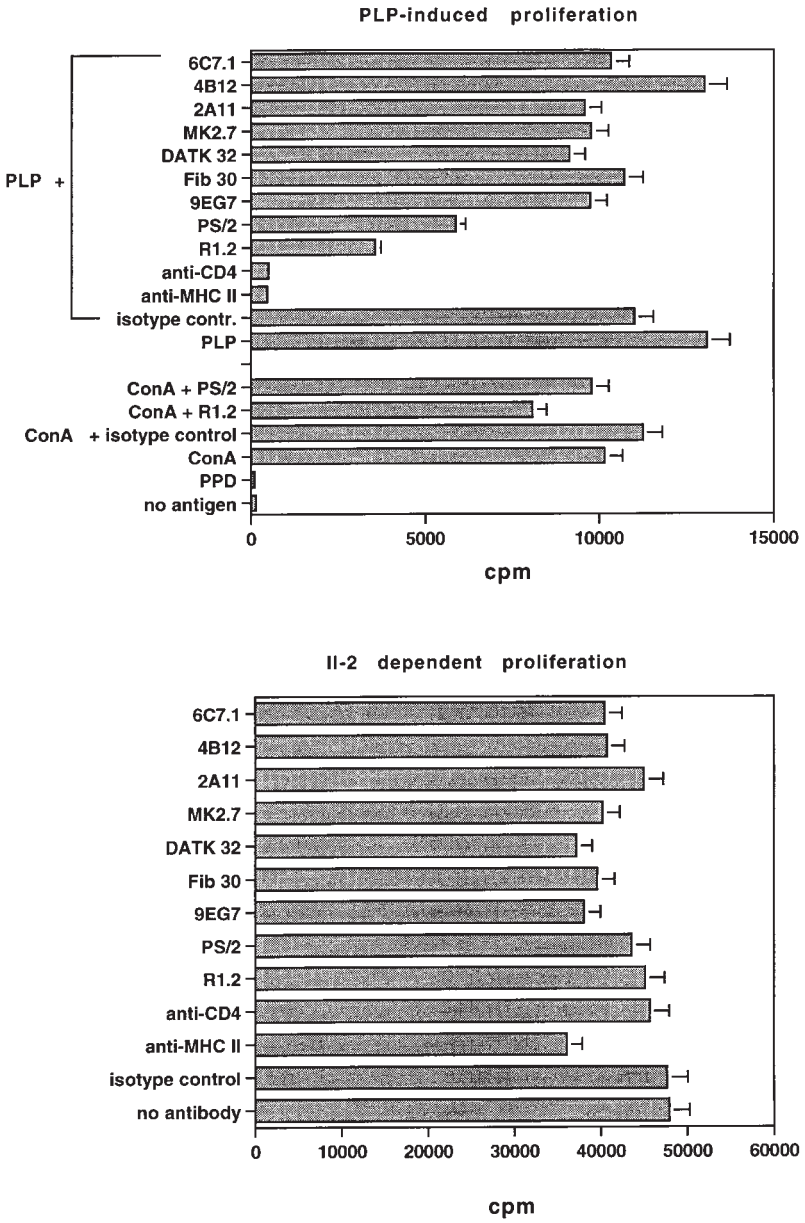

Figure 5. Anti–α4-integrin mAbs interfere with antigen-induced but not with IL-2–dependent proliferation of PLP-specific T cell lines. One representative experiment showing the proliferative response of the T cell line SJL.PLP4 is shown. (*Top*) In the presence of irradiated syngeneic spleen cells as APCs, SJL.PLP4 proliferated in response to the specific antigen PLP and in the presence of the T cell mitogen ConA; however, there was no proliferation in the absence of antigen or in the presence of the irrelevant antigen PPD. In some but not all assays, ConA-stimulated proliferation seemed to be slightly reduced in the presence of R1.2. Antibodies directed against CD4 and MHC class II completely inhibited the antigen-induced proliferation of the PLP-specific T cell line. The anti–α4-integrin mAb R1.2 and, in the case of this T cell line to a lesser degree, PS/2 partially inhibited this proliferative response. Antibodies against β1-, β7-, or the α4β7-heterodimer had no effect, nor did mAbs directed against VCAM-1. (*Bottom*) Antigen-independent proliferation of SJL.PLP4 in IL-2–containing medium. None of the above mAbs interfered with the IL-2–dependent proliferation. Proliferation is shown as incorporation of ^{3}H-dT. Values represent triplicates ±SD.

anti–VCAM-1 mAbs always inhibited adhesion to a slightly lesser degree than did the antiintegrin mAbs, other α4-integrin ligands such as fibronectin (29, 30) might play a minor role in adhesion of PLP-specific T cell blasts to brain endothelial cells. However, binding studies to recombinant VCAM-1 emphasized that PLP-specific T cell blasts can bind to VCAM-1 via both α4β1 and α4β7. Taken together, our in vitro studies revealed no obvious difference in the functional status of α4β1 and α4β7 on encephalitogenic versus nonencephalitogenic PLP-specific T cell lines. Furthermore, all T cell lines used both α4-integrins for adhesion mainly via VCAM-1 to stimulated brain endothelium in vitro.

Although our in vitro investigations indicated that both α4β1 and α4β7 can mediate T cell adhesion to stimulated brain endothelium, we could not demonstrate any role for β7 or α4β7 in the pathogenesis of EAE. Neither mAbs directed against β7 nor against the α4β7-heterodimer showed any influence on the development of EAE. However, the very same mAbs have been demonstrated to successfully inhibit α4β7-mediated lymphocyte homing into mucosal lymph nodes in healthy animals (18) and to inhibit spontaneous autoimmune diabetes (31). Although our data cannot exclude that β7-integrins can participate in EAE pathogenesis, they demonstrate clearly for the first time that β7-integrins are not required for the development of EAE. Lack of a role for α4β7 in EAE correlates with our previous findings that inflammatory T cells present in the CNS lack surface expression of α4β7 (3, 4).

Using four different mAbs directed against VCAM-1, we provide solid evidence that the α4-integrin ligand VCAM-1 is involved in EAE pathogenesis. Our study provides the first evidence that the pathogenic role for α4 and VCAM-1 observed in the tEAE model can be confirmed in the aEAE model, where treatment with anti-α4 and anti–VCAM-1 significantly delayed the onset of the disease.

The therapeutic effect of the anti-α4 mAbs was more successful than was that of the different anti–VCAM-1 mAbs, implying that other α4 ligands such as fibronectin (29, 30) or its splice variant CS-1 might play a role in inflammatory cell recruitment into the CNS during EAE (32). It has been suggested that a CS-1 containing surface protein on astrocytes, which supports α4β1-integrin–mediated adhesion of lymphoblasts in vitro, might provide an additional mechanism for lymphocyte recruitment into the CNS parenchyma during EAE (32).

Yet another explanation would be that α4-integrin is involved in migration-independent pathogenic processes in EAE. This was suggested by our observation that the three different anti–α4-integrin mAbs R1.2, PS/2, and 5-3, which have been shown to recognize different functional epitopes of the α4-integrin subunit (26, 33), exhibited therapeutic efficacies that were indistinguishable from each other. This is surprising since the mAb R1.2 in contrast to PS/2 has been proven to be a poor inhibitor of α4-mediated lymphocyte homing in vivo (18 and Engelhardt, B., unpublished). R1.2 has also been used to successfully interfere with other immune-mediated inflammatory diseases such as spontaneous autoimmune diabetes (34) or T cell–mediated contact hypersensitivity (35) in the mouse. We found that when compared with the other anti–α4-integrin mAbs, R1.2 is most effective in diminishing the antigen-induced proliferation of PLP-specific T cell lines in vitro, independent of their encephalitogenic capacity. Since anti–VCAM-1 mAbs showed no inhibitory effect on PLP-induced T cell proliferation, it is unlikely that R1.2 or PS/2 interfered with α4-mediated T cell binding to VCAM-1 on the surface of APCs, which has been shown to be involved in successful activation of $CD4^+$ T cells (36). Also, R1.2 and PS/2 did not provide a general proliferation-inhibitory signal (37) as they did not interfere with IL-2–dependent proliferation of PLP-specific T cell lines. Recently, binding of the CS1 alternatively spliced domain of fibronectin has been shown to specifically mediate CD3-dependent activation of $CD4^+$ T cells via α4-integrin (38, 39). It remains to be shown whether PS/2 and R1.2 interfere with such an activation signal on PLP-specific T cell lines.

Taken together, our data demonstrate that whereas α4β7-integrin is not required for the development of EAE, α4 and VCAM-1 are critically involved in the pathogenesis of the disease. VCAM-1 seems to be mostly involved in the recruitment of inflammatory cells across the BBB, but α4-integrin appears to play multiple roles in the pathogenesis of EAE.

Acknowledgments

We thank M. Bruckner for expert technical assistance and R. Lobb for recombinant VCAM-1. Gerald Ponath's contributions to some of this work during his stay in our laboratory are gratefully acknowledged. U. Deutsch, T. Yednock, E.C. Butcher, and Y. Reiss are gratefully acknowledged for helpful discussions on the manuscript. Our sincere thanks go to W. Risau for his continuous support of our work.

M. Schulz and this work have been funded by DFG-grant En214/3-2. U. Samulowitz has been funded by the BMBF grant of the IKF-Münster.

References

1. Butcher, E.C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell.* 67:1033–1036.

2. Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell.* 76:301–314.

3. Engelhardt, B., M.-T. Martin-Simonet, L.S. Rott, E.C. Butcher, and S. Michie. 1998. T lymphocytes in the inflamed CNS are phenotypically distinct from T lymphocytes in other inflamed tissues. *J. Neuroimmunol.* 84:92–104.

4. Engelhardt, B., F.C. Conley, P.J. Kilshaw, and E.C. Butcher. 1995. Lymphocytes infiltrating the CNS during inflammation display a distinctive phenotype and bind to VCAM-1 but not to MAdCAM-1. *Int. Immunol.* 7:481–491.

5. Engelhardt, B., D. Vestweber, R. Hallmann, and M. Schulz. 1997. E- and P-selectin are not involved in the recruitment of inflammatory cells across the blood-brain barrier in experimental autoimmune encephalomyelitis. *Blood.* 90: 4459–4472.

6. Cannella, B., A.H. Cross, and C.S. Raine. 1991. Relapsing autoimmune demyelination: a role for vascular addressins. *J. Neuroimmunol.* 35:295–300.

7. Steffen, B.J., E.C. Butcher, and B. Engelhardt. 1994. Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune encephalomyelitis in the central nervous system in the SJL/J mouse. *Am. J. Pathol.* 145:189–201.

8. Engelhardt, B., F.K. Conley, and E.C. Butcher. 1994. Cell adhesion molecules on vessels during inflammation in the mouse central nervous system. *J. Neuroimmunol.* 51:199–208.

9. Cannella, B., A.H. Cross, and C.S. Raine. 1990. Upregulation and coexpression of adhesion molecules correlate with relapsing autoimmune demyelination in the central nervous system. *J. Exp. Med.* 172:1521–1524.

10. Stamper, H.B.J., and J.J. Woodruff. 1976. Lymphocyte homing into lymph nodes: in vitro demonstration of the selective affinity of recirculating lymphocytes for high endothelial venules. *J. Exp. Med.* 144:828.

11. Yednock, T.A., C. Cannon, L.C. Fritz, F. Sanchez Madrid, L. Steinman, and N. Karin. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature.* 356:63–66.

12. Archelos, J.J., S. Jung, M. Maurer, M. Schmied, H. Lassmann, T. Tamatani, M. Miyasaka, K.V. Toyka, and H.P. Hartung. 1993. Inhibition of experimental autoimmune encephalomyelitis by an antibody to the intercellular adhesion molecule ICAM-1. *Ann. Neurol.* 34:145–154.

13. Willenborg, D.O., R.D. Simmons, T. Tamatani, and M. Miyasaka. 1993. ICAM-1-dependent pathway is not critically involved in the inflammatory process of autoimmune encephalomyelitis or in cytokine-induced inflammation of the central nervous system. *J. Neuroimmunol.* 45:147–154.

14. Kent, S.J., S.J. Karlik, C. Cannon, D.K. Hines, T.A. Yednock, L.C. Fritz, and H.C. Horner. 1995. A monoclonal antibody to alpha 4 integrin suppresses and reverses active experimental allergic encephalomyelitis. *J. Neuroimmunol.* 58:1–10.

15. Keszthelyi, E., S. Karlik, S. Hyduk, G.P.A. Rice, G. Gordon, T. Yednock, and H. Horner. 1996. Evidence for a prolonged role of α4 integrin throughout active experimental allergic encephalomyelitis. *Neurology.* 47:1053–1059.

16. Kuchroo, V.K., C.A. Martin, J.M. Greer, S.-T. Ju, R.A. Sobel, and M.E. Dorf. 1993. Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis. *J. Immunol.* 151:4371–4382.

17. Baron, J.L., J.A. Madri, N.H. Ruddle, G. Hashim, and C.A. Janeway, Jr. 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med.* 177:57–68.

18. Hamann, A., D.P. Andrew, W.D. Jablonski, B. Holzmann, and E.C. Butcher. 1994. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J. Immunol.* 152:3282–3293.

19. Andrew, D.P., C. Berlin, S. Honda, T. Yoshino, A. Hamann, B. Holzmann, P.J. Kilshaw, and E.C. Butcher. 1994. Distinct but overlapping epitopes are involved in alpha 4 beta 7-mediated adhesion to vascular cell adhesion molecule-1, mucosal addressin-1, fibronectin, and lymphocyte aggregation. *J. Immunol.* 153:3847–3861.

20. Ge, A.Z., and E.C. Butcher. 1994. Cloning and expression of a cDNA encoding mouse endoglin, an endothelial cell TGF-beta ligand. *Gene.* 138:201–206.

21. Holzmann, B., B.W. McIntyre, and I.L. Weissman. 1989. Identification of a murine Peyer's patch-specific lymphocyte homing receptor as an integrin molecule with an alpha chain homologous to human VLA-4 alpha. *Cell.* 56:37–46.

22. Jalkanen, S., R.F. Bargatze, L.R. Herron, and E.C. Butcher. 1986. A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. *Eur. J. Immunol.* 16:1195–1202.

23. Lenter, M., H. Uhlig, A. Hamann, P. Jeno, B. Imhof, and D. Vestweber. 1993. A monoclonal antibody against an activation epitope on mouse integrin chain beta 1 blocks adhesion of lymphocytes to the endothelial integrin alpha 6 beta 1. *Proc. Natl. Acad. Sci. USA.* 90:9051–9055.

24. Miyake, K., I.L. Weissman, J.S. Greenberger, and P.W. Kincade. 1991. Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J. Exp. Med.* 173:599–607.

25. Miyake, K., K. Medina, K. Ishihara, M. Kimoto, R. Auerbach, and P.W. Kincade. 1991. A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. *J. Cell Biol.* 114: 557–565.

26. Altevogt, P., M. Hubbe, M. Ruppert, J. Lohr, P.v. Hoegen, M. Sammar, D.P. Andrew, L.M. McEvoy, M.J. Humphries, and E.C. Butcher. 1995. The α4-integrin chain is a ligand for α4β7 and α4β1. *J. Exp. Med.* 182:345–355.

27. Röhnelt, R., G. Hoch, Y. Reiss, and B. Engelhardt. 1997. Immunosurveillance modelled in vitro: naive and memory T cells spontaneously migrate across unstimulated microvascular endothelium. *Int. Immunol.* 9:435–450.

28. Reiss, Y., G. Hoch, U. Deutsch, and B. Engelhardt. 1998. T cell interaction with ICAM-1 deficient endothelium *in vitro*: requisite role for ICAM-1 and ICAM-2 in transendothelial migration of T cells. *Eur. J. Immunol.* 28:3086–3099.

29. Ruegg, C., A.A. Postigo, E.E. Sikorski, E.C. Butcher, R. Pytela, and D.J. Erle. 1992. Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J. Cell Biol.* 117:179–189.

30. Chan, B.M., M.J. Elices, E. Murphy, and M.E. Hemler. 1992. Adhesion to vascular cell adhesion molecule 1 and fibronectin: comparison of α4β1 (VLA-4) and α4β7 on the human B cell line JY. *J. Biol. Chem.* 267:8366–8370.

31. Michie, S.A., H.-K. Sytwu, H.O. McDevitt, and X.-D. Yang. 1998. The roles of α4-integrins in the development of insulin-dependent diabetes mellitus. *In* Leukocyte Integrins in the Immune System and Malignant Disease. Vol. 231. B. Holzmann and H. Wagner, editors. Springer-Verlag, Berlin, Germany. 66–83.

32. van der Laan, L.J., C.J. de Groot, M.J. Elices, and C.D. Dijkstra. 1997. Extracellular matrix proteins expressed by human adult astrocytes in vivo and in vitro: an astrocyte surface protein containing the CS1 domain contributes to binding of lymphoblasts. *J. Neurosci. Res.* 50:539–548.

33. Kamata, T., W. Puzon, and Y. Takada. 1995. Identification of putative ligand-binding sites of the integrin α4β1 (VLA-4, CD49d/CD29). *Biochem. J.* 305:945–951.

34. Yang, X.-D., S.A. Michie, R. Tisch, N. Karin, L. Steinman, and H.O. McDevitt. 1994. A predominant role of integrin α4 in the spontaneous development of autoimmune diabetes in nonobese diabetic mice. *Proc. Natl. Acad. Sci. USA.* 91:12604–12608.

35. Ferguson, T.A., and T.S. Kupper. 1993. Antigen-independent processes in antigen-specific immunity. A role for alpha 4 integrin. *J. Immunol.* 150:1172–1182.

36. Damle, N.K., and A. Aruffo. 1991. Vascular cell adhesion molecule 1 induces T cell antigen receptor dependent activation of $CD4^+$ T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 88:6403–6407.

37. Hurley, R.W., J.B. McCarthy, E.A. Wayner, and C.M. Verfaille. 1997. Monoclonal antibody crosslinking of the α4 or β1 integrin inhibits committed clonogenic hematopoietic progenitor proliferation. *Exp. Hematol.* 25:321–328.

38. Nojima, Y., M.J. Humphries, A.P. Mould, A. Komoriya, K.M. Yamada, S.F. Schlossman, and C. Morimoto. 1990. VLA-4 mediated CD3-dependent $CD4^+$ T cell activation via the CS1 alternatively spliced domain of fibronectin. *J. Exp. Med.* 172:1185–1192.

39. Sato, T., K. Tachibana, Y. Nojima, N. D'Avirro, and C. Morimoto. 1995. Role of VLA-4 molecule in T cell costimulation. *J. Immunol.* 155:2938–2947.