Surface Expression of α4 Integrin by CD4 T Cells Is Required for Their Entry into Brain Parenchyma

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Summary

Cloned CD4 T cell lines that recognize the Ac1–16 peptide of myelin basic protein bound to I-A^u were isolated and used to analyze the immunopathogenesis of experimental autoimmune encephalomyelitis (EAE). T helper type 1 (Th1) clones induced disease, while Th2 clones did not. Using variants of a single cloned Th1 line, the surface expression of α4 integrins (very late antigen 4 [VLA-4]) was identified as a major pathogenic factor. Encephalitogenic clones and nonencephalitogenic variants differ by 10-fold in their level of surface expression of α4 integrin and in their ability to bind to endothelial cells and recombinant vascular cell adhesion molecule 1 (VCAM-1). The α4 integrin–high, disease-inducing cloned Th1 T cells enter brain parenchyma in abundance, while α4 integrin–low, nonencephalitogenic Th1 cells do not. Moreover, antibodies to α4 integrin, its ligand VCAM-1, and intercellular adhesion molecule 1 all influence the pathogenicity of this encephalitogenic clone in vivo. The importance of the expression of VLA-4 for encephalitogenicity is not unique to cloned T cell lines, as similar results were obtained using myelin basic protein–primed lymph node T cells. α4 integrin levels did not affect antigen responsiveness or production of the Th1 cytokines interleukin 2, interferon γ, and lymphotoxin/tumor necrosis factor β; and antibodies against α4 integrin did not block antigen recognition in vitro. Thus, we conclude that surface expression of α4 integrin is important in CD4 T cell entry into brain parenchyma. A general conclusion of these studies is that α4 integrins may be crucial in allowing activated effector T cells to leave blood and enter the brain and other tissues to clear infections.

Cell-mediated immune responses involve the clonal selection of antigen-specific CD4 T cells in draining lymph nodes. Once these cells have proliferated and differentiated to effector function, they emigrate via the efferent lymphatics and enter the blood stream. For such cells to mediate their effector function, they must leave blood vessels and recognize antigen in the tissues. During infection, this process contributes to host defense; in autoimmunity, it leads to tissue damage. The process of T cell migration from blood to tissue raises many questions. How do T cells enter tissues that normally do not contain significant numbers of such cells, such as the central nervous system (CNS);[1] what molecules are involved in the emigration of T cells from the blood; and can this process be manipulated to block local inflammation?

In infection, local signals activate endothelial cells to interact with leukocytes. Activated endothelium expresses endothelial leukocyte, vascular cell, and intercellular adhesion molecules (ELAM-1, VCAM-1, and ICAM-1), all involved in leukocyte/endothelial cell binding (1–9). Thus, local infection signals for leukocyte emigration, and local lymphocyte activation can sustain the process by producing inflammatory cytokines (5, 6, 10–17). However, when T cells specific for autoantigens are infused into normal mice, they can bind endothelium and enter tissues in the absence of local inflammatory-inducing signals. This suggests that activated effector T cells may enter all tissues in search of antigen, and it seems certain that such behavior is critical in host defense against infectious agents that do not elicit potent inflammatory responses; it could also contribute to autoimmune disease. Indeed, Mackay et al. (18) have shown that activated T cells preferentially emigrate from the blood into tissues, while resting cells exit in lymph node high endothelial venules. Thus, it is crucial to understand the mechanisms by which activated T cells leave the blood and enter tissues. To study this problem, we have examined the behavior of autoreactive

[1] *Abbreviations used in this paper:* CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; ELAM-1, endothelial leukocyte adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; LT, lymphotoxin; MBP, myelin basic protein; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

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cloned T cells that either do or do not invade the brain and asked what molecules are crucial for the invasive function.

The intact CNS of mammals, considered to be an immunologically privileged site, does not normally contain significant numbers of lymphocytes. Although the blood-brain barrier effectively excludes inert cells such as erythrocytes and resting T lymphocytes, recent experiments have shown that activated T cells can easily cross the blood-brain barrier (19–21). However, the exact mechanism by which these lymphocytes cross into the CNS remains unclear, and the molecules involved have not been defined.

We have used the disease model experimental autoimmune encephalomyelitis (EAE) to study the question of transit of T lymphocytes from the blood into the CNS. EAE is characterized by acute onset of paralysis, perivascular and parenchymal infiltration of the brain and spinal cord by mononuclear cells, and inflammation in the lesions similar to a delayed-type hypersensitivity reaction. EAE can be induced actively by priming an animal to CNS proteins like myelin basic protein (MBP), or adoptively by injection of activated lymphocytes that are specific for these CNS antigens (22). EAE is clearly mediated by $CD4^+$ T lymphocytes. This was originally shown in adoptive transfer studies with lymph node cells (23), and more recently by isolation of encephalitogenic T cell clones (24, 25). In the PL/J mouse strain, the acetylated NH_2-terminal nonapeptide MBP Ac1–9 is the major encephalitogenic peptide (26). Among T cell clones specific for the MBP peptide Ac1–9, α and β TCR gene usage is very restricted (27, 28). However, two T cell clones that share peptide specificity and have identical TCR chains can differ in their ability to transfer disease (28). Therefore, the TCR on a $CD4^+$ T cell does not by itself determine encephalitogenicity. Furthermore, the secretion of TNF-α also plays a crucial role in the pathogenesis of EAE (29–31). It is not clear what aspect or aspects of EAE are influenced by cytokine expression.

In this paper we address the question of T cell entry into the CNS by comparing an encephalitogenic (EAE-inducing), MBP-specific, $CD4^+$ T cell clone with its variants that have lost encephalitogenic activity. The variant clones have decreased cell surface expression of α4 integrin and show very minimal lymphocytic infiltration of brain upon transfer. In addition, injection of antibodies to α4 integrin and its ligand VCAM-1 can inhibit pathogenicity of the encephalitogenic clone in vivo. Thus, these studies strongly implicate a direct role of α4 integrins in the entry of activated T cells into normal CNS tissue.

Materials and Methods

Mice. PL/J, (SJL/J × PL/J)F_1, and BALB/c (*nu/nu*) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in our facility. They were housed in the animal facilities of the Howard Hughes Medical Institute, Section of Immunobiology, Yale University School of Medicine.

mAbs. MK/1 (anti-VCAM-1) (32) and PS/2 (anti-α4 integrin) (33) were provided by Dr. Paul Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK). LPAM-1 (anti-α4 integrin (34, 35) was provided by Dr. Irving Weissman (Stanford, CA). C508.19G is a mAb that recognizes a clonotypic determinant of the TCR on MBP-specific clone 19. It was generated according to a standard protocol for hybridoma production (36). Other antibodies used were YCD3-1 (anti-CD3) (37), F23.2 (anti-Vβ8.2) (38), GK1.5 (anti-CD4) (39), TIB 124-M1/89.18.7.HK (anti-CD45) (40), TIB 122-M1/9.3.HL.2 (anti-CD45) (40, 41), 7D4 (anti-IL-2R) (42), M17/5.2 (anti-LFA1) (43), YN/1 (anti-ICAM-1) (44), IM7.8.2 PGP1 (anti-CD44) (45), RM2-1 (anti-CD-2) (46), Y3P (anti-$I\text{-}A^u$) (47), and Lenny (polyclonal rabbit serum to β1 integrin), provided to us by Drs. Clayton Buck and Steven Albelda (Wistar Institute, Philadelphia, PA).

Preparation of MBP and MBP Peptide Ac1-16. Peptides were synthesized on a solid phase peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) and purified by HPLC (48). All peptide preparations ran as a single peak on HPLC and had the predicted amino acid content and molecular weight by mass spectroscopy. Mouse and guinea pig MBP were purified from spinal cord tissue as previously described (49, 50).

T Cell Lines and Clones. CD4 T cell clones were derived as previously described (25, 51). In brief, PL/J or (SJL/J × PL/J)F_1 mice were immunized with 50 μg whole guinea pig MBP in CFA in the hind foot pads and the base of the tail. 10 d later, local lymph nodes were removed and stimulated with syngeneic spleen cells plus 50 μg/ml mouse MBP. T cell lines were obtained by stimulation of these cultures ($\sim 2 \times 10^6$ T cells) every 14 d with 6×10^6 irradiated (2,700 rad) syngeneic spleen cells per T-75 flask, plus MBP or MBP peptide Ac1-16 (5 μg/ml), and 5 U/ml rIL-2. T cell clones were obtained by soft agar cloning of the lymph node cultures 3 d after stimulation in vitro, and recloned by limiting dilution.

Proliferation and Lymphokine Studies. 10^4 of various T cell clones were cultured with 5×10^5 irradiated syngeneic spleen cells as feeders, with and without MBP or MBP peptide Ac1-16, in 0.2-ml culture medium in 96-well flat-bottomed microtiter plates. After 48–72 h, 1 μCi/well [^{3}H]thymidine was added and cells were harvested 15 h later. The mean counts per minute of [^{3}H]thymidine incorporation were calculated for triplicate measurements. The standard deviations were within 10% of the mean value. The experiments were repeated at least twice with similar results.

Inhibition of proliferation with different antibodies was accomplished by adding varying amounts of antibody into the microtiter wells. The plates were harvested, counted, and analyzed as described above.

Lymphokine assays were done by standard protocols. IL-4 production was determined using CT4S cells and 11B11, antibody to IL-4 (52). TNF α/β units were determined by cytotoxicity against WEHI 164 cells (53). Units of INF-γ were determined by a viral plaque reduction assay using Sindbis virus on WISH cells (54).

FACS® Analysis and Sorting. T cells were stained by indirect immunofluorescence. $0.5–1 \times 10^6$ cells were incubated with the primary antibodies for 30 min at 4°C. The primary antibody was washed away, and the cells were incubated with appropriate secondary antibodies conjugated to fluorescein (goat anti-rat or anti-mouse Ig; Hyclone Laboratories, Logan, UT). Cells were washed and fixed with 1% paraformaldehyde. Immunofluorescent analysis was performed on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA) equipped with logarithmic amplifiers. Peak fluorescence intensity is the channel number out of 1,000 with the most fluorescent events.

Staining for cell sorting experiments was done according to a standard protocol using the LPAM-1 antibody (anti-α4 integrin) diluted in cell culture medium plus FCS. The secondary antibody was a goat anti-rat IgG (HyClone Laboratories) conjugated to

fluorescein, which was dialyzed to remove azide. Cells were sorted according to fluorescence intensity. Cells collected were the top and bottom 10% of the fluorescence intensity curve. Sorted cells were then simulated with APC, antigen, and IL-2 several times to obtain enough cells for in vivo transfer. The phenotype of these sorted lines was stable in culture as assessed by functional binding studies and FACS® analysis carried out on the day of cell transfer.

Binding to Recombinant Soluble (rs)VCAM-1. rsVCAM-1 at 10 μg/ml was coated onto appropriate 96-well plates in $NaHCO_3$ buffer, pH 9.2. Plates were blocked with 1% BSA in PBS. T cells (2.5×10^5) that had been pulsed with [^{3}H]thymidine were added to the coated wells in triplicate. If blocking antibodies were used, they were added to the T cells or the coated wells 30 min before addition of the T cells to the wells. Cells were incubated on the coated plates for 10 min at room temperature, washed with culture medium to remove unbound cells, lysed, and harvested for counting (55).

EAE Induction, Scoring, and Antibody Blocking. For testing the ability of T cell clones and lines to mediate EAE in vivo, varying numbers of T cells were injected intravenously into (PL/J × SJL)F_1 mice irradiated with 350 rad. Mice were observed daily by an unbiased observer beginning on day 5 for signs of EAE. The animals were graded according to the following scale: 1, limp tail; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, hind and front limb paralysis; 5, moribund. Animals were cared for in accordance with Yale University guidelines. The score for each group at each day is the mean disease score of at least five animals. The total disease index is calculated as the sum of the mean disease score at each day for a given group over the period of the experiment.

For in vivo disease experiments using mAbs to block disease, $2–3 \times 10^7$ T cells were incubated with ascites fluid containing antibodies for 30 min before injection. Ascites fluid was standardized for protein concentration by spectrophotometry (λ, 280 nm). Each antibody dose contained 12 mg of protein. Antibodies were also standardized for mean fluorescence intensity staining on the T cells. Animals that were given multiple doses of antibody were injected intravenously with antibody every 3 d.

Immunohistology. Animals were perfused with PBS and then periodate-lysine-paraformaldehyde (PLP) through the left ventricle of the heart (56). Fixed brains and spinal cords were removed, allowed to soak in PLP fixative for an additional 2 h, and then infused with increasing concentrations of sucrose in phosphate buffer (final sucrose concentration, 18%). Tissue was snap frozen in tissuetek compound in 2-methyl butane. Tissue was cut into 7-μm sections. Tissue was stained with methylene blue.

Brain and spinal cord sections were stained according to standard protocol using goat serum and Triton X-100 as preincubation blockers (56). Antibodies were diluted in the goat serum and Triton X-100 solution, and added to the sections to incubate for 2 h. Sections were washed and incubated with secondary antibodies conjugated to horseradish peroxidase. Sections were washed and developed with diaminobenzidine (DAB). The counter stain is methylene blue.

Results

Cloning and Characterization of MBP-specific T Cells. T cells responding to the encephalitogenic peptide Ac1-16 of MBP were isolated from PL/J mice. Of five clones tested for encephalitogenicity, two of the clones were Th1 cells, established by the fact that they secrete large amounts of IFN-γ and TNF-α and/or -β but no IL-4, while the other three clones were Th2 cells, secreting IL-4 but no IL-2 or IFN-γ. The Th1 clones induced the disease EAE when injected intravenously into irradiated female (PL/J × SJL)F_1 mice, while the three Th2 clones did not (data not shown). One Th1 clone, clone 19, will be analyzed in detail in this report. It is a $CD4^+$ T cell specific for I-A^u plus MBP Ac1-16 that expresses Vβ8.2, and secretes large amounts of IFN-γ and TNF-α and/or -β upon activation. Clone 19 induces EAE in a reproducible and titratable fashion.

Clone 19 Variants That Lack Encephalitogenic Activity Have Decreased Cell Surface α4 Integrin. Over time in tissue culture, certain lines of clone 19 lost encephalitogenic activity (called 19B [benign]). To determine the mechanism of lowered encephalitogenic potential, these variants were compared with encephalitogenic lines of clone 19 (called 19P [pathogenic]). Both clones retained their ability to proliferate when stimulated with APC plus MBP Ac1-16, their original phenotype ($CD4^+$, TCR $V\beta8.2^+$) (Fig. 1), and produced equivalent levels of IFN-γ and TNF-α and/or -β upon activation (data not shown). Clones 19P and 19B were then stained with antibodies to a wide range of surface molecules. As seen in Fig. 1 and Table 1, the level of cell surface expression of these molecules was virtually identical for the two clones with the exception of α4 integrin. The disease-inducing clone 19P had approximately three times higher levels of α4 integrin than the 19B subline that lacks encephalitogenic potential.

Selection for the α4 Integrin High Phenotype of Clone 19 Coselects Pathogenic Potential. Clone 19B was sorted by FACS®

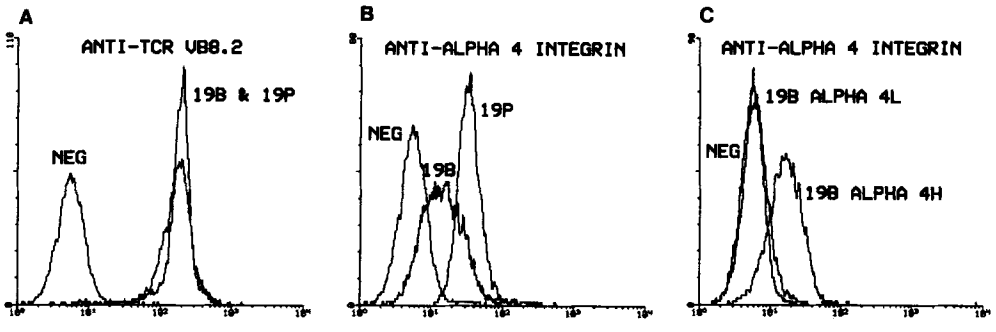


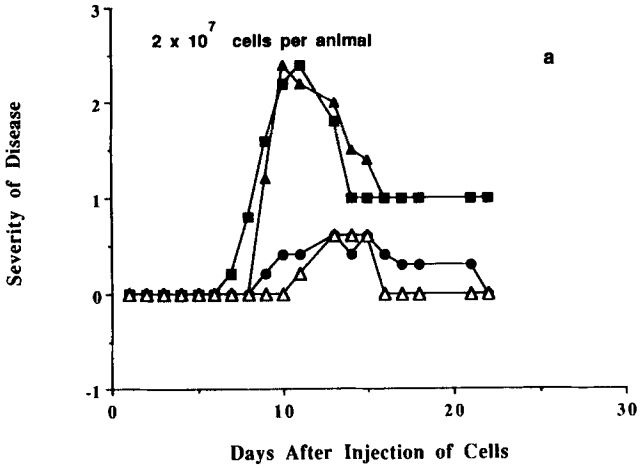
Figure 1. Clone 19 variants that lack encephalitogenic activity have decreased cell surface α4 integrin, but equivalent levels of various other molecules. The clones were stained on the day of transfer in in vivo studies. FACS® profiles of variants of clone 19: (*A*) 19B and 19P stained with antibodies against TCR Vβ8.2 (F23.2); (*B*) 19B and 19P stained with antibodies against α4 integrin (LPAM); (*C*) 19B α4H and 19B α4L stained with antibodies against α4 integrin (LPAM). These profiles are from representative experiments. The staining was repeated numerous times.

Table 1. *Cell Surface Molecule Density on Clone 19 Sublines*

Antibody specificity	Peak channel fluorescence staining on subline			
	19P	19B	19Bα4H	19Bα4L
–	5	5	6	5
Anti-CD3	–*	–*	142	140
Anti-Vβ8.2	–*	–*	108	103
Anti-TCR (clonotype)	50	41	43	41
Anti-CD4	673	528	621	519
Anti-CD45	–*	–*	632	704
Anti-IL-2 rec.	–*	–*	60	50
Anti-LFA-1	204	262	205	276
Anti-ICAM-1	12	13	15	12
Anti-CD44	154	237	227	221
Anti-CD2	ND	ND	97	95
Anti-VLA-4 (LPAM)	14	8	17	5
Anti-VLA-4 (PS/2)	ND	ND	26	8
Anti-β1 integrin	24	34	30	31

Clone 19 variants that lack encephalitogenic activity have decreased cell surface expression of α4 integrin, but equivalent levels of various other molecules. Clones were stained with the indicated antibody and analyzed by FACS®. Peak channel fluorescence is the channel number out of 1,000 with the most fluorescent events. These results are from a representative experiment. The staining was repeated many times.

* FACS®profiles were identical and could be superimposed, however, peak channel fluorescence was not recorded.



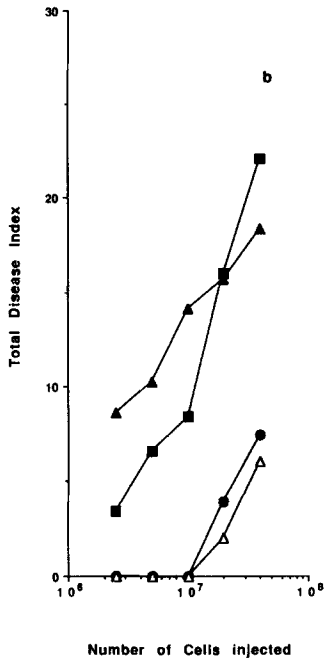


Figure 2. The α4 integrin high sublines of clone 19 are much better at inducing disease than are those that are α4 integrin low. (*a*) T cells were stimulated with MBP Ac1-16, syngeneic feeders, and IL-2 3 d before transfer. Five animals in each group were given 2 × 10^7 T cells intravenously. Animals were scored according to the standard scale for EAE by a blind observer. (*b*) A total disease index was calculated for each subline of clone 19 at each dose of cells by adding the mean disease score at each day over a given period of time. This allows the potency of each line to be directly compared. (●) Clone 19B; (▲) clone 19P; (■) clone 19B α4H; (△) clone 19B α4L.

to obtain two populations of cells, 19B α4 integrin high and 19B α4 integrin low. This yielded four distinct populations of clone 19: the nonpathogenic clone 19, which had almost completely lost the ability to induce disease (19B); the α4 integrin high (19Bα4H)- and low (19Bα4L)-sorted populations of this nonpathogenic clone 19B; and the pathogenic subline 19P.

The four populations of clone 19 were expanded to sufficient numbers and tested for their ability to induce EAE (Fig. 2). As seen in Fig. 1, the phenotype of the α4 integrin high and low populations is stable in vitro. Fig. 2 *a* shows that at 2 × 10^7 cells per animal, the α4 integrin high sublines were much more effective at inducing disease than those that were α4 integrin low. Not only was the onset of disease earlier in mice that were given the α4 integrin high lines, but the severity of disease was also greatly increased in these recipients. A total disease index was calculated for each subline by adding the mean disease score at each day over the course of the experiment. Fig. 2 *b* shows the total disease index for each subline as a function of cell dose. Where the dose-response curves overlap, the two α4 integrin high sublines are 8–16 times more active per cell than are the two α4 integrin low sublines.

Only Pathogenic Sublines of Clone 19 Bind Strongly to Endothelial Cells and VCAM-1. For T cells to produce disease, they must leave the blood and enter brain parenchyma. Since the pathogenic subline of clone 19 expressed greater levels of α4 integrin than the benign subline, we asked whether there was also a difference between these cell lines in binding to microvascular endothelial cells from rat epididymal fat pads. Many more cells of the 19P subline bound to the endothelial cell line than did cells of the 19B subline. Furthermore, antibodies to α4 integrin reduced the binding of the 19P subline to approximately the levels of binding of the 19B subline. By contrast, antibodies to ICAM-1 and to LFA-1 significantly decreased the binding of both sublines of clone 19 to the endothelial cell line (data not shown).

As VCAM-1 is the endothelial cell ligand for α4:β1 integrin (very late antigen 4 [VLA-4]), we also tested the binding of these different populations of clone 19 to recombinant soluble VCAM-1. As shown in Fig. 3, a much larger proportion of cells from the two pathogenic populations of clone 19 (19P and 19Bα4H) bound to the rsVCAM-1 molecule than

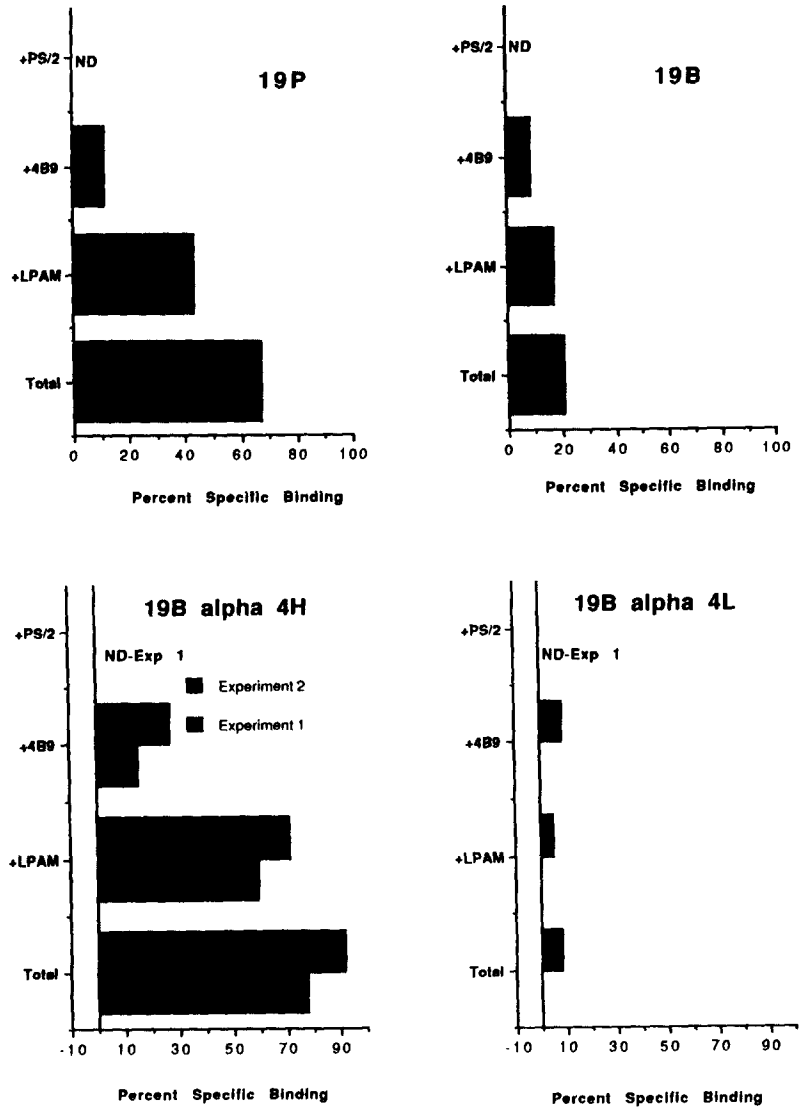


Figure 3. The α4 integrin high sublines of clone 19 (19P and 19B α4H) bind to rsVCAM-1 more effectively than the α4 integrin low sublines (19B and 19B α4L). rsVCAM-1 was coated onto 96-well plates for 24 h and the plates were washed and then blocked with heat-inactivated BSA. The various sublines of clone 19 were pulsed with ^3H for 24 h, washed, counted, and incubated at 37°C for 30 min with and without antibodies to α4 integrin. Some wells previously coated with rsVCAM-1 were incubated for 30 min with antibodies against VCAM-1 (4B9). The cells were added to the coated wells in triplicate with and without antibody, and incubated for 10 min. The plates were then washed and the adherent cells were lysed and harvested. Percent specific binding is calculated as: 100× (counts in the experimental wells on rsVCAM-1 − the background counts of cells bound to wells coated with BSA)/total counts.

did cells of the two populations of clone 19 that expressed lower levels of α4 integrin (19B and 19Bα4L). Moreover, this binding could be partially (LPAM) or completely (PS-2) blocked by anti-α4 integrin and strongly inhibited by anti-VCAM-1 (4B9) mAb.

Pathogenic Sublines of Clone 19 Enter Brain Parenchyma, while Benign Sublines Enter Ependymal Tissue. To determine whether sublines of clone 19 differ in invasiveness of brain parenchyma, perfusion-fixed brain tissue was taken from representative animals in the groups given the different populations of clone 19. The brains from the diseased animals given clone 19P were extensively infiltrated with $CD4^+$ lymphocytes both in perivascular cuffs as well as in the brain parenchyma (Fig. 4, *A* and *B*). By contrast, brains from the nondiseased animals given the same number of clone 19B showed almost no infiltration in the perivascular area or in the brain parenchyma, but did show slight infiltration within the choroid plexus and the meninges (Fig. 4, *C* and *D*; it should be noted that the area photographed represents the area with the most lymphocytic infiltrate). The entire parenchyma and perivascular areas were basically devoid of lymphocytes. Similar results were obtained using spinal cord sections from diseased and nondiseased animals, and from mice given clones 19Bα4H or 19Bα4L. Brain sections were also stained with antibodies to Vβ8.2. The great majority of the lymphocytes in the brains of the diseased animals, as well as in the meninges of the nondiseased animals, express CD4 and Vβ8.2, suggesting that the cells infiltrating brain in these experiments are either the cloned T cells that were injected or their direct progeny (Fig. 4, *E* and *F*).

The Pathogenicity of Clone 19 Can Be Inhibited by mAbs Specific for α4 Integrin, VCAM-1, or ICAM-1. To determine whether α4 integrin binding to VCAM-1 is crucial for disease induction in vivo, we asked whether antibodies to α4 integrin and to its ligand VCAM-1 could influence the course of EAE. Various antibodies were mixed with the pathogenic clone 19 and injected into irradiated $(PL/J \times SJL)F_1$ female mice. As a control, in vitro activation of clones 19Bα4H and 19Bα4L

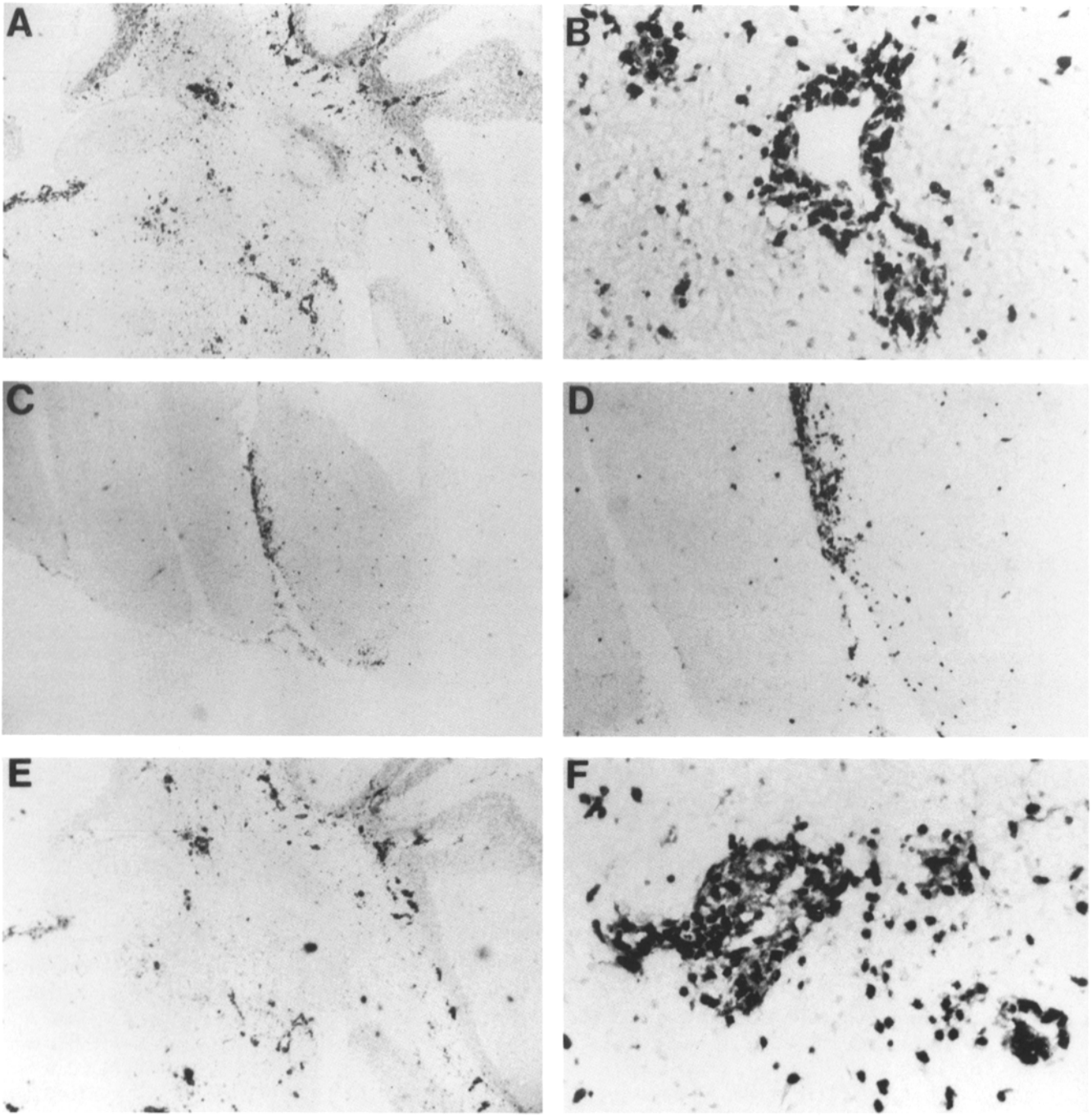


Figure 4. Perfusion-fixed brain tissue from diseased animals given α4 integrin high clone 19P (*A*, *B*, *E* and *F*) and from nondiseased animals given α4 integrin low clone 19B (*C* and *D*). It should be noted that the areas photographed of the nondiseased brains represent the areas with the greatest number of lymphocytes. The entire parenchyma and perivascular areas were basically devoid of lymphocytes. Low-power (*A*) and high-power (*B*) views of brains from diseased animals stained with antibodies to CD4. Low-power (*C*) and high-power (*D*) views of brains from nondiseased animals stained with antibodies to CD4. Low-power (*E*) and high-power (*F*) views of brains from diseased animals stained with antibodies to Vβ8.2 (F23.2). Note that virtually all the cells express CD4 and Vβ8.2.

was examined in the presence and absence of antibodies against α4 integrin (LPAM) and CD45 (TIB 124). As can be seen in Fig. 5, the proliferation of both 19B α4H and 19B α4L was unaffected by antibodies to α4 integrin and CD45, while proliferation was blocked in the presence of antibodies against I-A^u (Y3P) and the TCR clonotype (19G).

In vivo blocking studies demonstrated that antibodies to α4 integrin, VCAM-1, and ICAM-1 can influence both the

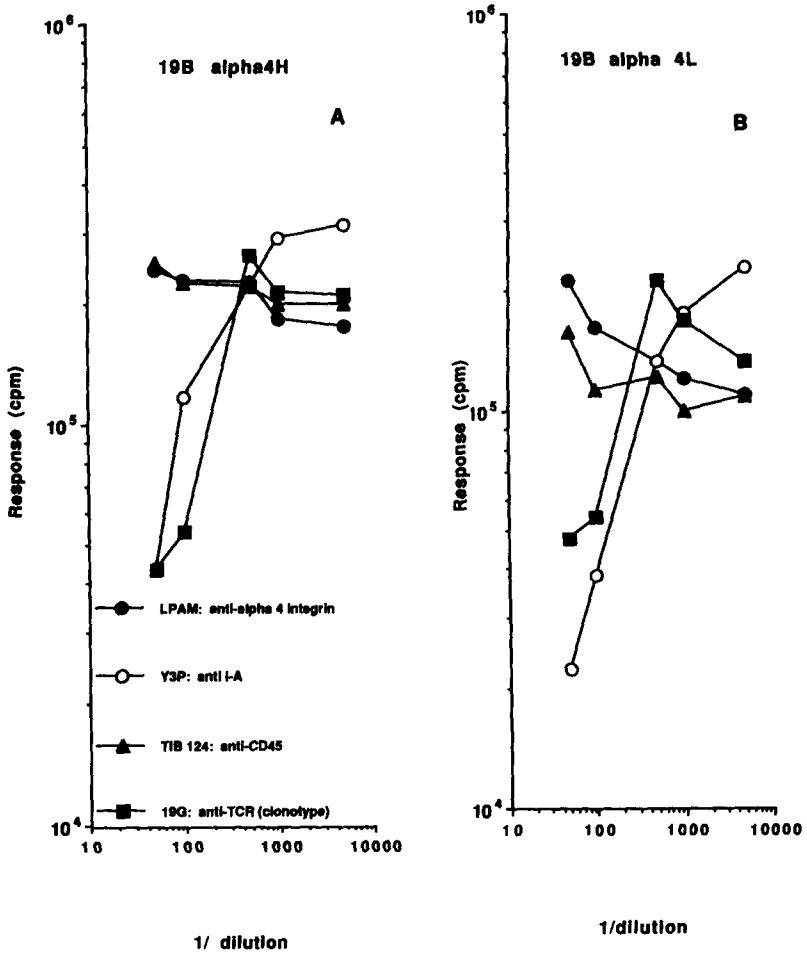


Figure 5. The disease-inducing subline 19B α4H (*A*) and the non–disease-inducing subline 19B α4L (*B*) proliferate equivalently in response to MBP Ac1-16 plus syngeneic feeders. Antibodies against α4 integrin (LPAM [●]) and the isotype-matched anti-CD-45 control antibody (TIB124 [▲]) used in the in vivo blocking of disease have no effect on this proliferative response, while mAb Y3P directed against I-A^u (○) and mAb 19G (■) against a clonotypic determinant of the TCR were able to block proliferation. 2 × 10^4 cells were plated in triplicate in 96-well plates along with 10^5 syngeneic spleen cells as feeders and MBP peptide Ac1-16 at 10 μg/ml. The various antibodies were titrated as shown starting at 20 μg/ml. Wells were pulsed with ^{3}H on day 3, and harvested and counted on day 4.

time of onset and the severity of disease (Fig. 6). Treatment with antibody to α4 integrin delayed the onset of disease by 2–3 d but only slightly decreased the mean peak severity of disease as compared with the animals that received no antibody or an isotype-matched control antibody (anti-CD44). The isotype control antibody to CD44 did not block disease, and might even have accelerated the day of onset (Fig. 6 *A*). Antibody to VCAM-1 had the same effect as antibody to α4 integrin. Anti-VCAM-1 given once at the time of injection of the T cell clone delayed onset of disease by 2 d; continued injection of anti-VCAM-1 on days 3 and 6 further delayed onset of disease (Fig. 6 *B*). However, peak disease grade was essentially the same in all groups. These results suggest that the antibodies to α4 integrin and VCAM-1 are blocking the entrance of the cells into the CNS; once the antibody disappears, the cells can enter the CNS and cause disease. It is unlikely that the antibody delays onset disease by depletion of cells, since decreasing the number of injected T cells decreases the severity of disease before it delays onset (data not shown), and since isotype-matched antibodies that bind to the cells at higher density do not have this effect. Furthermore, brain sections from animals given T cells together with antibody to α4 integrin of VCAM-1, and sampled before onset of disease at a time when the control animals showed clinical signs of EAE, showed the same staining pattern as animals that received clone 19B (data not shown). Antibody to ICAM-1 markedly delayed onset of disease as compared with animals that received either no antibody or the isotype-matched control antibody to CD45 (Fig. 6 *C*), and may decrease disease severity. When injection of antibody to α4 integrin (LPAM-1 and PS/2) was continued every 3 d until after onset of disease, not only was the onset of disease delayed, but in this case the severity of disease was also significantly decreased (data not shown). This decrease was not seen when the isotype-matched control antibody was injected every 3 d.

Response to MBP Peptide Ac1-16 Segregates with Expression of α4 Integrin on Lymph Node Cells from Animals Primed with MBP. All of the prior studies were carried out with a long-term in vitro cloned T cell line. For comparison, recently isolated polyclonal T cells specific for MBP were stained with antibody to α4 integrin (LPAM-1) and sorted according to fluorescence intensity. Two populations that differ in α4 integrin expression were plated with syngeneic feeder cells and varying amounts of MBP peptide Ac1-16. As can be seen from Fig. 7, almost all of the reactivity to MBP peptide Ac1-16 segregated with the α4 integrin high population of cells. Thus, α4 integrin expression correlates with priming, and all re-

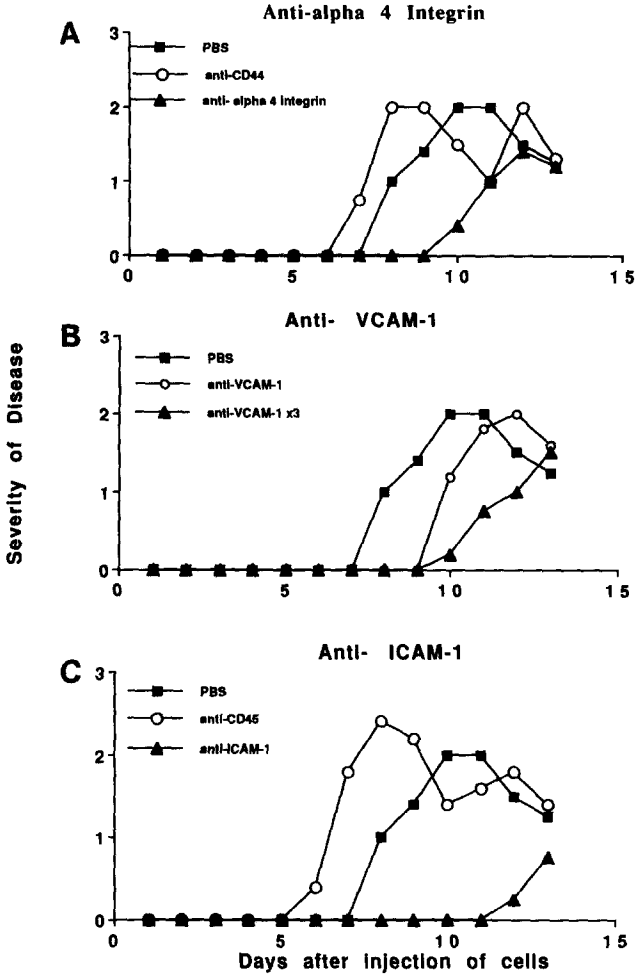


Figure 6. The pathogenicity of clone 19 can be inhibited by mAbs specific for α4 integrin, VCAM-1, and ICAM-1. 2 × 10^7 cells were incubated either in PBS or the specified antibody and injected into irradiated female (PL/J × SJL)F_1 mice. (*A*) Clone 19P treated with PBS (■), anti-α4 integrin (LPAM-1, IgG2b [▲]), and the isotype-matched control anti-CD44 (PGP-1, IgG2b [O]). (*B*) Clone 19 transferred with PBS (■), anti-VCAM-1 given only at the time of injection of cells (O), or also on days 3 and 6 (VCAM-1 ×3 [▲]). (*C*) Mice given clone 19 in PBS (■), with anti-ICAM-1 (YN1, IgG2a [▲]), and the isotype-matched control, anti-CD45 (TIB 122, IgG2a [O]).

cently primed cells that can proliferate to antigen appear to express high α4 integrin levels in this system.

The Pathogenicity of the MBP-primed Lymph Node Cells Can also Be Inhibited by mAbs Specific for α4 Integrin. To test the role of α4 integrin in EAE produced by primary lymph node cells, the effect of anti-α4 integrin mAb on disease induced by adoptive transfer of lymph node cells was examined. 2 ×10^7 MBP-primed lymph node cells were injected into female irradiated (PL/J × SJL)F_1 mice with and without antibody to α4 integrin (LPAM-1). One group received LPAM-1 only at the time of injection of lymph node cells, while another group received LPAM-1 at the time of injection of cells as well as on days 3 and 6 after injection of cells. As can be seen in Fig. 8, antibody to α4 integrin given once significantly delayed the onset of EAE, while in the group that received

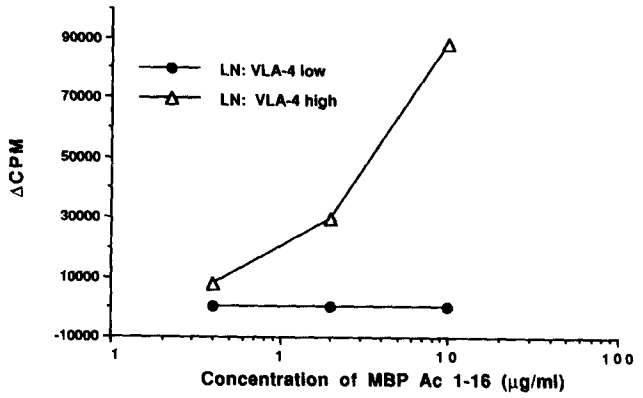


Figure 7. Proliferation of MBP-primed lymph node cells, sorted according to α4 integrin expression, in response to APC plus MBP peptide Ac1-16. MBP-primed lymph node cells were sorted by FACS® into α4 integrin high (Δ) and low (●) populations. Cells were plated in triplicate with APC and MBP peptide Ac1-16 at varying concentrations. Plates were pulsed with [^{3}H]thymidine after 72 h and harvested 15 h later. Data are expressed as cpm by subtraction of the background cpm in the absence of MBP Ac1-16; these were 49 cpm for LN-VLA-4 low and 6,271 cpm for LN-VLA-4 high. Draining inguinal, popliteal, and para-aortic lymph nodes were isolated from (PL/J × SJL)F_1 female mice 10 d after subcutaneous injection of 50 μg guinea pig MBP emulsified in CFA. Lymph nodes were separated into single cell suspension and cultured for 8 d in medium with syngeneic feeder cells, FCS, 25 μg/ml guinea pig MBP, and 10 μg/ml MBP peptide Ac1-16. 5 U/ml rIL-2 was added on day 3.

continued injection of LPAM-1, disease was further delayed and the severity of disease was also diminished.

Discussion

The basic question we have sought to address in the present experiments is what determines the ability of a T cell to enter

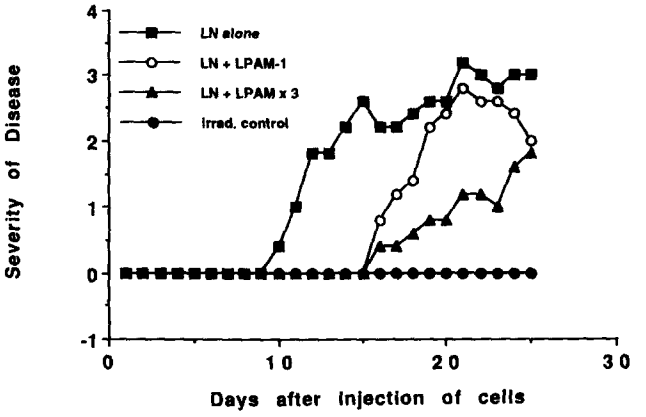


Figure 8. The pathogenicity MBP-primed lymph node cells can also be inhibited by mAbs specific for α4 integrin. 2 × 10^7 MBP-primed lymph node cells were incubated with either PBS (■) or antibody to α4 integrin (LPAM-1) and then injected into irradiated (PL/J × SJL)F_1 female mice. One group was given cells and LPAM-1 (O), one group was given cells and LPAM-1 followed by two more doses of LPAM-1 on days 3 and 6 (▲), while a control group receiving no cells (●) did not develop disease.

brain parenchyma. It has been previously shown that T cells specific for an encephalitogenic peptide of MBP must secrete lymphotoxin (LT) to cause disease (29–31). Our initial results support these prior conclusions since in our studies $CD4^+$ Th-1 cells, which are specific for an encephalitogenic MBP peptide and secrete large amounts of IFN-γ and TNF-α and/or -β, are encephalitogenic, while $CD4^+$ Th-2 clones, which are specific for the same encephalitogenic peptide but secrete large amounts of IL-4 and no IFN-γ or LT, are unable to induce disease.

Our studies add an additional requirement to those cited above; to cause disease, a CD4 cell must be able to leave the blood and enter the brain parenchyma. We demonstrate that this requires cell surface VLA-4 binding to endothelial VCAM-1, and probably LFA-1 binding to endothelial ICAM-1 as well. Several lines of evidence point to the idea that loss of encephalitogenic potential correlates with a decreased level of cell surface VLA-4, and a corresponding loss of this ability of the lymphocytes to enter the brain. The above results found with a T cell clone appear to apply to uncloned T lymphocytes as well. In addition, as VLA-4 is an activation antigen found on memory cells, specificity for MBP peptide Ac1-16 cosegregates with expression of α4 integrin on lymph node cells from animals primed with MBP. On this basis, naive T cells should not be able to enter brain, perhaps explaining the absence of tolerance to the neural antigen MBP.

If α4 integrin expression is required for disease, what is its role in pathogenesis? It seems unlikely that VLA-4 is required for T cell activation in EAE, since both the encephalitogenic clone 19P and its nonencephalitogenic variant clone 19B respond equivalently to MBP peptide Ac1-16 in terms of proliferation and lymphokine production, and antibodies to α4 integrin do not block this response in vitro. A more likely role for α4 integrin in the pathogenesis of EAE would be in allowing lymphocytes access to the CNS. This is supported by the demonstration that pathogenic sublines of clone 19 bind strongly to endothelial cells and VCAM-1 and enter into brain parenchyma, while nonpathogenic sublines of clone 19 bind weakly to endothelial cells and VCAM-1 and are found in brain only in ependymal tissue. This idea is further supported by the fact that antibodies to α4 integrin, VCAM-1, and ICAM-1 appear to inhibit pathogenicity by excluding lymphocytes from the CNS. However, when antibody levels decline, the animals become sick.

It has been proposed that lymphocyte entry into the CNS is initiated when an activated CNS antigen-specific T cell binds to endothelium by LFA-1-ICAM-1 interaction (57, 58). Our data are compatible with this, as anti-ICAM-1 inhibits disease effectively. We propose that once the T cell binds to endothelium, whether it be by an LFA-1–ICAM-1 interaction or by selectin binding, the activated cell induces upregulation of VCAM-1 on the endothelium by virtue of its production of IFN-γ and both soluble and membrane-bound LT and TNF. In this regard, we have observed staining of endothelium with anti-VCAM-1 where perivascular cuffs are present (data not shown). The activated T lymphocyte must have surface expression of VLA-4 to bind to this newly induced VCAM-1 and enter into the CNS. This hypothesis would predict that a T lymphocyte that can enter into the CNS must be able to secrete appropriate lymphokines for upregulation of adhesion molecules and must express VLA-4 and LFA-1. This is consistent with the failure of naive T, Th2, rested Th1, or activated Th1 cells with decreased production of LT/TNF or IFN-γ to enter the CNS on their own. Most importantly for this study, cells with decreased levels of VLA-4 could not cross the brain endothelium. It is likely that only a small number of lymphocytes need to cross the endothelial barrier initially, and that the majority of lymphocytes present in the inflammatory lesions are the progeny of those few cells that originally entered the tissue or are recruited by local inflammation triggered by the early immigrants.

A more general conclusion suggested by these studies, but not directly addressed in them, is that VLA-4 is involved in immune surveillance of many tissues including the CNS. We suggest that the α4 integrins play a crucial role in the emigration of recently activated lymphocytes from blood into tissues. If specific antigen is present in the tissues, then the lymphocyte would remain in the tissue and in responding to antigens would activate local endothelium to recruit further effector cells. This mechanism would allow effector lymphocytes to reach sites of infection involving pathogens that do not normally elicit inflammation, such as certain viruses. This conclusion attributes to α4 integrins a role in the differential migration of activated or memory T cells described by McKay et al. (18).

A recent paper suggests that both VLA-4 and CD44 confer the ability of a $CD8^+$ clone specific for a peptide from a rodent malaria species to be protective against malarial infection (59). We did not detect a role of CD44 in the pathogenesis of EAE, since both the encephalitogenic and nonencephalitogenic sublines of a T cell clone expressed equivalent levels of CD44. More important, in vivo studies using antibodies to CD44 had no effect on the pathogenesis of EAE. Another recent study has also suggested a role for VLA-4 in rat EAE (60). Unlike our studies, these earlier studies did not examine VLA-4 expression as a critical component of encephalogenic potential, and did not follow disease beyond 7 d. Thus, it is unclear whether disease in that model was delayed (as in our studies) or totally prevented by anti-VLA-4. Moreover, that study concluded that VLA-4 may be crucial for nonspecific leukocyte recruitment, whereas our studies point strongly to a role for VLA-4 in the entry of specific T cells into brain and did not address its role in recruitment. In addition, other recent studies have suggested a role for VLA-4 in the passage of T cells from blood into sites of inflammation (61–63). These studies are consistent with our results, but did not address the issue of how activated T cells enter tissues in the absence of an inflammatory stimulus, the main focus of our experiments. Taken together, the results of all these studies support the hypothesis that VLA-4 is a crucial mediator of effector T cell emigration from blood into infected tissues. Finally, as we have also observed modulation of VLA-4 expression in vivo when cloned T cells migrate from perivascular cuffs into brain parenchyma, the regulated expression of this and other integrins may have importance in effector T cell function.

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