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# Overlapping Roles of P-Selectin and $\alpha_4$ Integrin to Recruit Leukocytes to the Central Nervous System in Experimental Autoimmune Encephalomyelitis<sup>1</sup>

Steven M. Kerfoot and Paul Kubes<sup>2</sup>

**Experimental autoimmune encephalomyelitis (EAE) is mediated by inflammatory cells recruited from the circulation to the CNS. We used intravital microscopy to investigate the mechanisms of this recruitment. No leukocyte rolling and very little adhesion was observed in healthy control mice. In contrast, both rolling and adhesion was observed in brain postcapillary venules before onset of physical symptoms of EAE. Rolling and adhesion remained elevated for 2 wk and returned to near normal levels by 5 wk postsymptom onset. Consistent with a role for P-selectin in recruitment to the CNS, P-selectin protein was detected in the brains and spinal cords of EAE mice. Expression was highest before symptom onset and decreased over the next 2 wk. The importance of  $\alpha_4$  integrin increased with time as anti- $\alpha_4$  integrin blocked ~20, 50, and 60% of leukocyte rolling 2 days before disease onset, 5 days and 2 wk postonset of symptoms, respectively, and 85% of rolling 5 wk postsymptoms. Addition of anti-P-selectin to  $\alpha_4$  integrin Ab-treated mice blocked all remaining rolling at each time point. Interestingly, however,  $\alpha_4$  integrin-mediated rolling appeared to be entirely dependent on P-selectin as anti-P-selectin alone was able to completely block all leukocyte rolling. In the absence of rolling (with P-selectin Ab), a 70% reduction in adhesion was noted. A very similar reduction was seen when mice were treated with  $\alpha_4$  integrin-blocking Ab. In conclusion, we describe increased leukocyte trafficking in the brains of EAE mice with important overlapping roles for both P-selectin and  $\alpha_4$  integrin in mediating leukocyte-endothelial cell interactions. *The Journal of Immunology*, 2002, 169: 1000–1006.**

**T**he human disease multiple sclerosis (MS)<sup>3</sup> and its animal model, experimental autoimmune encephalomyelitis (EAE), are mediated by inflammatory cells recruited from the circulation into the CNS. Disease is initiated when activated autoreactive T cells cross the blood brain barrier into the healthy CNS. When Ag is encountered, these cells initiate inflammation and the recruitment of inflammatory effector cells to the CNS, resulting in devastating demyelination and axon destruction.

The mechanisms of leukocyte recruitment to the CNS are not well understood. The current paradigm of recruitment has largely been worked out in vitro and in a few easily accessible tissues in vivo (mesentery, cremaster, and skin) in which intravital microscopy can be performed. Circulating leukocytes first tether to and then roll on endothelial cells expressing adhesion molecules. If appropriate signals are encountered, leukocytes then firmly adhere to the vessel wall before transmigrating into the surrounding tissue (1–3). The tethering and rolling steps are mediated by the selectins, as well as  $\alpha_4$  integrins. Firm adhesion occurs when integrins are

induced to bind their endothelial ligands with high affinity. Until very recently, in vivo investigations of EAE have had to rely on histological observations and clinical outcome following blockade of various adhesion molecules to infer the mechanisms of leukocyte recruitment to the CNS. These studies have primarily focused on a role of  $\alpha_4$  integrin. Indeed, treatment with anti- $\alpha_4$  integrin Abs has some protective effects in EAE and can partially reduce the numbers of inflammatory cells in the CNS of diseased animals (4–6). However, because  $\alpha_4$  integrin has additional roles to leukocyte recruitment, including T cell and effector cell activation, the mechanism of action of  $\alpha_4$  integrin blockade in EAE is not yet clear.

A role for selectins in recruitment to the CNS has been discounted, largely due to their limited expression in the CNS microvasculature (7, 8). Despite this, recent evidence suggests that selectins may yet have a role in leukocyte recruitment to the CNS. Intravital microscopy of TNF- or LPS-treated mice revealed that P-selectin blockade reduced leukocyte rolling in brain microvessels (9, 10). Indeed, a recent study by Carrithers et al. (11) implicated P-selectin in the initial recruitment of activated encephalitogenic T cells into the healthy CNS. However, a role for P-selectin in the inflammatory stage of EAE has not been assessed to date.

We (9) and others (10, 12) have recently developed intravital microscopy of CNS microvessels. Using this technique, we for the first time directly investigate the mechanisms of leukocyte recruitment to the CNS in EAE. Early in disease, P-selectin played a principal role in mediating leukocyte rolling in brain microvessels, which corresponded with an induction of P-selectin expression in the CNS. As disease progressed,  $\alpha_4$  integrin gained importance as the molecule to mediate leukocyte rolling. Nevertheless,  $\alpha_4$  integrin was insufficient to support rolling in the absence of P-selectin. Inhibition of either P-selectin or  $\alpha_4$  integrin reduced subsequent leukocyte adhesion in EAE, supporting their potential role in the pathogenesis of this disease.

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<sup>3</sup> Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; FACs, flow-assisted cytometry.

## Materials and Methods

### Mice and reagents

RB40.34 (anti-P-selectin) and R1-2 (anti- $\alpha_4$  integrin) were purchased from BD PharMingen (Mississauga, Ontario, Canada) as were FITC-conjugated anti-murine CD3 $\epsilon$  (clone 145-2C11) and Mac-1 (clone M1/70). RME-1 (anti-E-selectin) was generously supplied by Dr. A. Issekutz (Dalhousie University, Halifax, Nova Scotia, Canada). Myelin oligodendrocyte glycoprotein (MOG ) 35–55 peptide was initially a gift from Dr. C. Bernard (La Trobe University, Melbourne, Australia), and later generated in our own peptide synthesis laboratory (University of Calgary, Calgary, Alberta, Canada). Female C57BL/6 mice were purchased from Charles River Breeding Laboratories (Montreal, Quebec, Canada).

### Induction of EAE

EAE was induced in mice as previously described (13). Briefly, 9–11-wk-old C57BL/6 mice were immunized s.c. with 50  $\mu$ g of a peptide generated from MOG<sub>35–55</sub> in CFA (Sigma-Aldrich, Oakville, Ontario, Canada). Mice were immunized twice, 1 wk apart. Pertussis toxin (200 ng; List Biological Laboratories, Campbell, CA) was injected i.p. on the day of the first immunization and then again 2 days later. Disease was monitored daily and scored as follows: 0, no clinical signs; 1, tail paralysis; 2, tail paralysis and hind-limb weakness; 3, hind limb paralysis; 4, complete hind limb paralysis and front limb weakness.

### Intravital microscopy

Intravital microscopy of the mouse cerebrovasculature was performed as previously described (9). Briefly, the tail vein was cannulated for the administration of additional anesthetic, fluorescent dyes, and other reagents. A craniotomy was performed using a high-speed drill (Fine Science Tools, North Vancouver, British Columbia, Canada) and the dura matter was removed to expose the underlying pial vasculature. Throughout the experiment, the mouse was maintained at 37°C and the exposed brain was kept moist with an artificial cerebrospinal fluid buffer.

To observe leukocyte/endothelial interactions, leukocytes were fluorescently labeled by i.v. administration of rhodamine 6G (0.3 mg/kg body weight) and observed using a microscope (Axioskop,  $\times 10$  eyepiece and  $\times 25$  objective lens; Zeiss, Don Mills, Ontario, Canada) outfitted with a fluorescent light source (epi-illumination at 510–560 nm using a 590-nm emission filter). A low light intensifier charge-coupled device camera (Stanford Photonics, Palo Alto, CA) mounted on the microscope was used to project the image to a monitor. Three different postcapillary venules with a diameter between 30 and 70  $\mu$ m were chosen for observation. All experiments were recorded for later analysis. Rolling leukocytes were defined as those cells moving at a velocity less than that of erythrocytes within a given vessel. Leukocytes were considered adherent if they remained stationary for 30 s or longer.

### Experimental protocol

Leukocyte rolling and adhesion was observed by intravital microscopy at various time points corresponding to different phases of disease: 2–3 days before predicted onset of symptoms (10 days post first immunization with MOG<sub>35–55</sub>) corresponding to developing presymptomatic EAE, 4 days postdevelopment of symptoms corresponding to acute disease, and 2 and 5 wk postsymptoms corresponding to chronic disease. Depending on the experiment, 70  $\mu$ g R1-2, 100  $\mu$ g RME-1, or 20  $\mu$ g RB40.34 in 200  $\mu$ l saline were administered i.v. based on previous work from our laboratory showing that these are optimal concentrations of Ab needed to inhibit rolling in muscle microvessels. For experiments requiring pretreatment with adhesion molecule blocking Ab, Abs were administered at the same concentrations as described above. Five-hour pretreatment was used for R1-2 and 24-h pretreatment was used for RB40.34.

### Flow-assisted cytometry (FACs) analysis of inflammatory cells in the CNS

Infiltrating inflammatory cells in the CNS were analyzed by FACs as previously described (14). Mice were perfused through the heart with PBS to clear circulating blood from the vasculature. Brain and spinal cord tissue of EAE mice were dissociated through a wire mesh. Mononuclear cells were then separated on a Percoll (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada) gradient. Cells were then incubated with FITC-conjugated anti-CD3 or Mac-1 and analyzed with a BD Biosciences FACScan (Mountain View, CA). Untreated mice were used as controls to ensure that mononuclear cell counts were not simply due to vascular leukocyte content.

### Dual-radiolabeled Ab assay for P-selectin expression

P-selectin expression was quantitatively measured in the brain and other tissues of healthy control and EAE mice by the dual-radiolabeled Ab assay as previously described (9, 15). Briefly, a mixture of 10  $\mu$ g <sup>125</sup>I-labeled anti-P-selectin (RB40.34) and a dose of <sup>131</sup>I-labeled nonbinding Ab (A110-1, anti-keyhole limpet hemocyanin) calculated to achieve a total <sup>131</sup>I activity of 4–6  $\times 10^5$  cpm were injected i.v. through the jugular vein. The Abs were allowed to circulate for 5 min. A blood sample was taken from the carotid artery and the mouse was then completely perfused with saline through the jugular vein to remove all circulating blood. Tissues were harvested, weighed, and measured for <sup>125</sup>I and <sup>131</sup>I activity. P-selectin expression was calculated per gram of tissue by subtracting accumulated <sup>131</sup>I activity (labeled nonbinding Ab) from accumulated <sup>125</sup>I activity (labeled anti-P-selectin). The <sup>131</sup>I activity of the nonbinding Ab accounts for circulating Ab or protein that has leaked into tissue nonspecifically. Data are represented as the percentage of the injected dose of Ab per gram of tissue. This approach is sufficiently sensitive to detect small but significant amounts of constitutive P-selectin in wild-type relative to no P-selectin in P-selectin-deficient mice (15).

### Statistics

Data in graphs is shown as mean  $\pm$  SEM unless indicated otherwise. A Student's *t* test with Bonferroni correction was used for multiple comparisons. Statistical significance was set at *p* < 0.05.

## Results

### MOG<sub>35–55</sub>-induced EAE

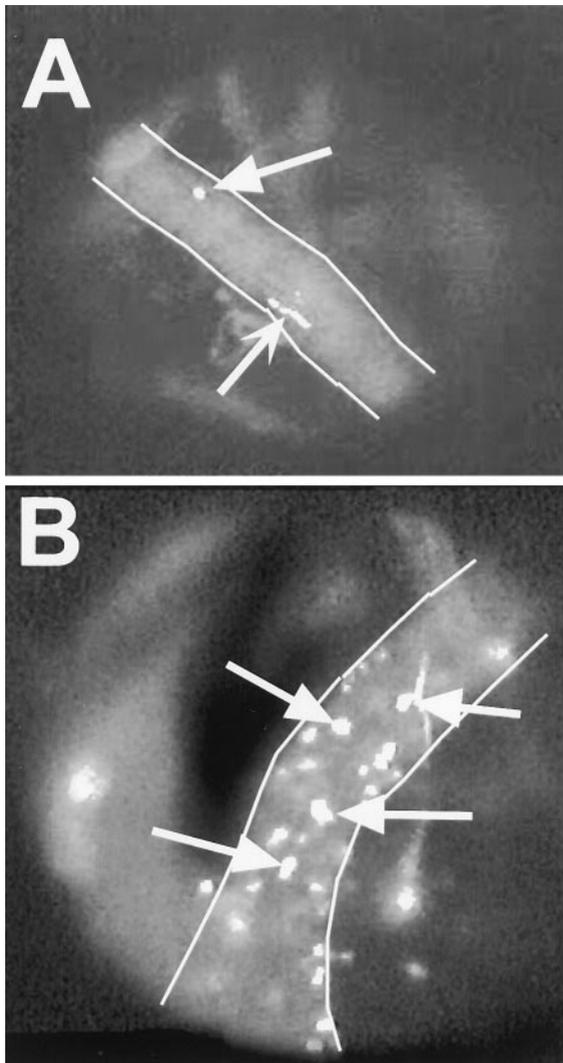
EAE was induced in C57BL/6 mice as described in *Materials and Methods*. This protocol resulted in a chronic, nonremitting disease consistent with previous reports (16). Symptoms arose between 11 and 15 days following the first immunization with MOG<sub>35–55</sub>, and escalated rapidly over 6–7 days at which point they stabilized. Only very slight recovery was observed over the next 4 wk (data not shown).

### Leukocyte rolling and adhesion is increased in the CNS microvasculature of EAE mice

We used intravital microscopy to directly observe leukocyte-endothelial interactions in the cerebrovasculature of EAE mice (Fig. 1). Very little leukocyte recruitment was observed in the brains of healthy control mice. Rolling cells were almost never observed and adherent leukocytes were also rare (Fig. 2). In contrast, profound recruitment was observed in EAE mice. Approximately 30 cells/min were observed rolling in postcapillary venules of presymptomatic mice (Fig. 2A). Rolling remained elevated over the next week, significantly falling to <15 cells/min 2 wk postdevelopment of symptoms and returning to near-normal levels 5 wk postdevelopment of symptoms. Leukocyte adhesion was also significantly induced in presymptomatic mice to 10-fold above control (Fig. 2B). Adhesion peaked in the acute phase of disease and returned to near normal levels 2 wk following symptom onset.

### FACs analysis of infiltrating inflammatory cells

Infiltrating inflammatory cells in the CNS of EAE mice were determined by FACs (Fig. 3). Very few inflammatory cells could be detected in healthy control mice. In contrast, large numbers of lymphocytes and macrophages could be detected in the CNS of mice with EAE. Inflammatory cells were detected in greatest numbers in mice 4 days postdevelopment of symptoms, but were also present in presymptomatic mice and mice 2 wk postdevelopment of symptoms. The ratio between lymphocyte and macrophages (~1:3) did not change over the course of disease. Neither did the ratio between CD3<sup>+</sup> and CD3<sup>-</sup> cells in the lymphocyte population favoring CD3<sup>+</sup> cells to a small degree (~58%).



**FIGURE 1.** Intravital microscopy of the murine microvasculature in a healthy control (A) and a presymptomatic EAE mouse (B). Leukocyte endothelial interactions were very rarely observed in healthy control mice, although adherent cells were observed occasionally in some mice (*top* arrow in A). The *lower* arrow in A points to a free leukocyte in the circulation. In contrast, many rolling and adherent cells were observed in EAE mice (arrows in B).

*P-selectin and  $\alpha_4$  integrin together mediate leukocyte rolling in the cerebrovasculature of EAE mice*

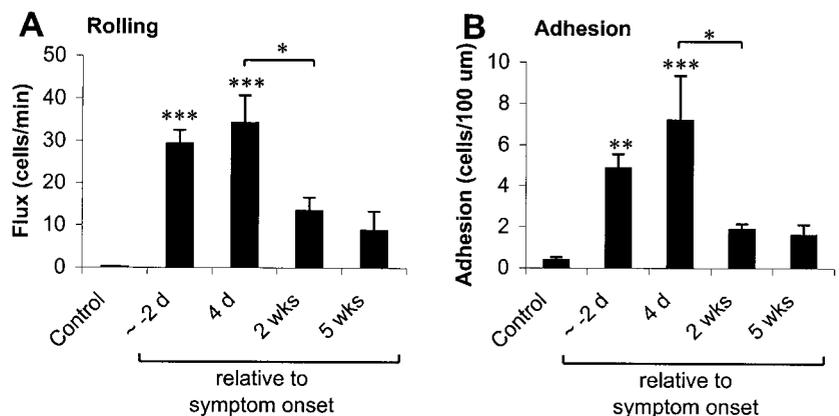
To determine the mechanism by which leukocytes are recruited to the brain in EAE mice, intravital microscopy was used to directly

observe the affects of adhesion molecule blockade on leukocyte recruitment. After baseline rolling was measured in mice at various stages of disease, anti- $\alpha_4$  integrin was administered i.v. and leukocyte rolling was observed 20 min later. We have previously shown that 20 min is required for optimal inhibition of rolling by R1-2 (17) and that isotype-matched Ab has no effect on leukocyte recruitment (18). Fig. 4 summarizes that the role for  $\alpha_4$  integrin-mediated rolling increased over the course of disease. Although  $\alpha_4$  integrin blockade decreased rolling by ~20% in presymptomatic mice (Fig. 4A), this increased to 50% in mice in the acute (Fig. 4B, 4 days postsymptom onset) and 60% in chronic (Fig. 4C, 2 wk postsymptom onset) phases of disease. The rolling cells observed in mice 5 wk postsymptom onset were almost completely (90%) blocked by anti- $\alpha_4$  integrin (Fig. 4D). Following blockade of  $\alpha_4$  integrin, all remaining rolling was further blocked by anti-P-selectin at all stages of disease (Fig. 4). We have previously shown that an isotype-matched Ab has no effect on leukocyte rolling (18). In a separate series of experiments, we administered anti-P-selectin alone and surprisingly found that it was sufficient to completely block all leukocyte rolling (Fig. 5A). This demonstrates that  $\alpha_4$  integrin-mediated rolling is completely dependent on P-selectin. This observation has been made before, that  $\alpha_4$  integrin can support rolling but is unable to tether the cells, an event requiring selectins (17). Anti-E-selectin had no effect on leukocyte rolling (Fig. 5B), demonstrating that the blockade was specific for P-selectin, not E-selectin.

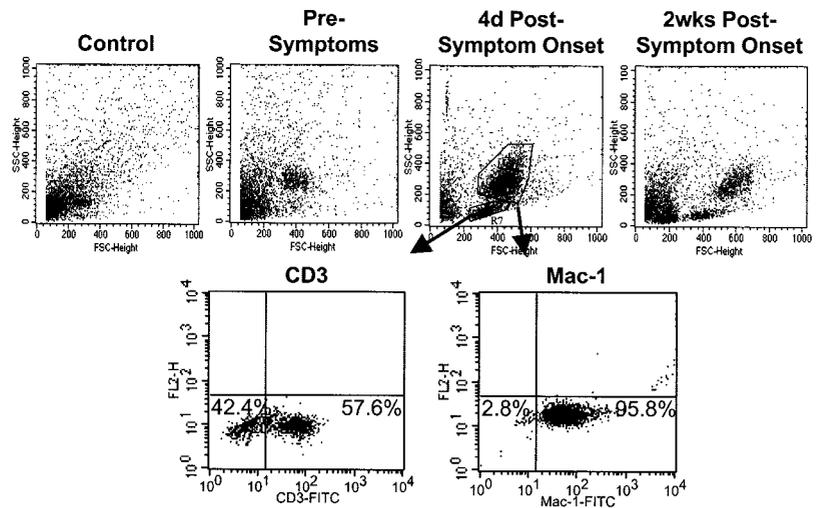
*$\alpha_4$  integrin mediates leukocyte adhesion in the brain microvasculature*

To determine the mechanisms mediating leukocyte adhesion in the cerebrovasculature of EAE mice, mice were pretreated with anti- $\alpha_4$  integrin 5 h before adhesion was observed by intravital microscopy. As anti- $\alpha_4$  integrin was unable to displace already adherent cells (data not shown) pretreatment with the Ab was required to prevent the accumulation of new cells. Mice in the acute phase of disease (4 days postonset of symptoms) were investigated as maximal adhesion was observed at this time point (Fig. 2B). Blockade of  $\alpha_4$  integrin resulted in a 70% reduction in leukocyte adhesion (Fig. 6B), demonstrating that  $\alpha_4$  integrin is an important mediator of adhesion in EAE. A similar reduction in adhesion was observed with 24-h pretreatment with  $\alpha_4$  integrin Ab (data not shown). To determine whether leukocyte adhesion in the cerebrovasculature was dependent on leukocyte rolling, acute-phase mice were pretreated for 24 h with anti-P-selectin. Rolling was completely blocked in these mice over the 24 h (Fig. 6A). Leukocyte adhesion was reduced by 70% (Fig. 6B).

**FIGURE 2.** Leukocyte recruitment to the brain over the course of EAE. Intravital microscopy was performed before and 4 days, 2 wk, and 5 wk following development of physical symptoms. Leukocyte recruitment was also observed in healthy control mice. Leukocyte rolling (A) and adhesion (B) were observed. Results are shown as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  vs control.  $n = 4$  for 4 days and 2 wk postsymptom onset;  $n = 5$  for control, -2 days, and 5 wk postsymptom onset.



**FIGURE 3.** Flow cytometry analysis of inflammatory cells in the CNS of EAE mice over the course of disease. *Top row* are plots based on forward and side scatter. As a representative, fluorescence plots gated on the lymphocyte and monocyte populations (based on forward and side scatter) from mice 4 days postonset of symptoms are shown on the *lower row*. One representative of two separate batches of MOG-treated mice is shown for each time point.



#### *P-selectin is expressed in the CNS of EAE mice*

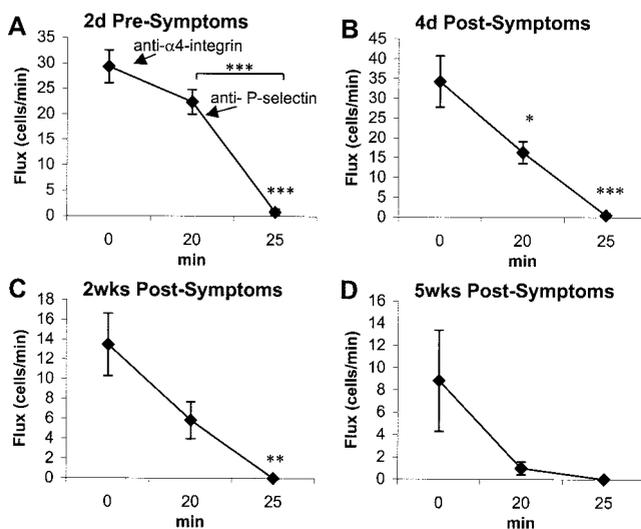
P-selectin protein expression in the brain and spinal cord of EAE mice was investigated using a quantitative radiolabeled Ab assay. Although completely absent in the CNS of healthy control mice, a dramatic induction of P-selectin expression was observed in the brains of EAE mice, with greatest expression in presymptomatic mice (Fig. 7A). Levels fell over the course of disease, but remained significantly elevated compared with control. P-selectin expression was variable in the spinal cords of presymptomatic mice (Fig. 7B), and therefore did not reach significance over healthy controls. However, similar to the brain, significant expression was observed in mice 4 day and 2 wk postdevelopment of symptoms.

Although the largest increases in P-selectin were observed in the CNS, P-selectin expression was also assayed in a wide range of other tissues (Table I). Increased expression was largely restricted

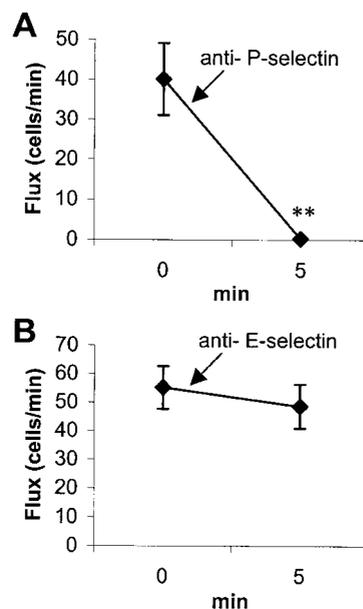
to the CNS, with the exception of presymptomatic mice which had significantly increased levels of P-selectin protein in a number of abdominal organs including the liver, pancreas, spleen, mesentery, and stomach. P-selectin was also up-regulated in the bone marrow of mice 2 wk after development of symptoms. P-selectin expression in the brain was very low compared with other tissues as optimal expression in presymptomatic mice was lower than baseline expression observed in tissues like the skin and muscle of healthy control mice. Despite this, the relative increase in P-selectin expression was greatest in the brain.

#### Discussion

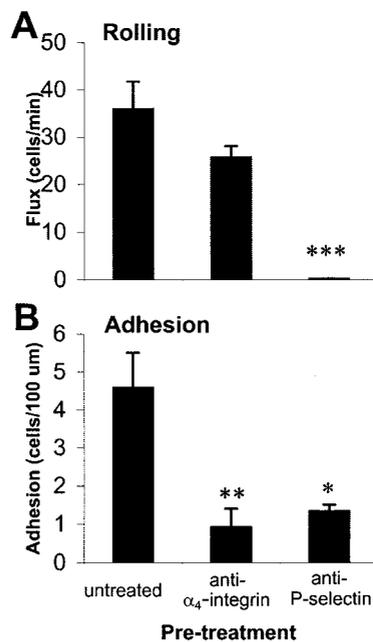
It is well-established that the infiltration of leukocytes into the brains and spinal cords of humans beset with MS or the spinal cords of EAE mice underlies the pathology of this disease. To date, the majority of murine studies have focused on the spinal cord,



**FIGURE 4.** Leukocyte rolling in the brain microvasculature of EAE mice is mediated by P-selectin and  $\alpha_4$  integrin. Baseline rolling was determined by intravital microscopy in EAE mice at various stages of disease. Leukocyte rolling was again measured 20 min following administration of an  $\alpha_4$  integrin-blocking Ab and then 5 min following administration of a P-selectin-blocking Ab. In each panel results are shown as mean  $\pm$  SEM. Please note the difference in scale on the y-axis. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  vs baseline.  $n = 4$  for 4 days and 2 wk postsymptom onset;  $n = 5$  for 2 days presymptom onset and 5 wk postsymptom onset.



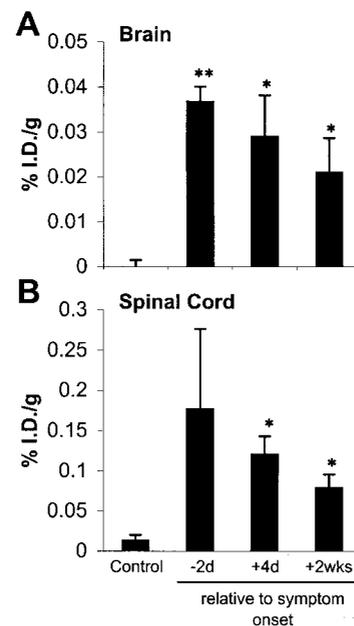
**FIGURE 5.** Leukocyte rolling in the brain microvasculature of EAE mice is entirely dependent on P-selectin. Baseline rolling in mice 4 days postsymptom onset was determined by intravital microscopy before the administration of anti-P-selectin (A) or anti-E-selectin (B). Rolling was observed 5 min later. \*\*,  $p = 0.0023$  vs baseline, determined by a two-tailed  $t$  test.  $n = 5$  for anti-P-selectin-treated mice;  $n = 3$  for anti-E-selectin-treated mice.



**FIGURE 6.** Mice were pretreated with anti-P-selectin for 24 h or anti- $\alpha_4$  integrin for 5 h before leukocyte rolling (A) or adhesion (B) was determined by intravital microscopy. Neither P-selectin nor  $\alpha_4$  integrin blockade completely blocks leukocyte adhesion in the brain microvasculature of EAE mice. Results are shown as mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  vs untreated control.  $n = 8$  for the untreated group;  $n = 6$  for the anti- $\alpha_4$  integrin-treated group;  $n = 5$  for the anti-P-selectin-treated group.

which unlike in humans, appears to be the primary target organ in EAE mice. We visualized the murine brain microvasculature, a primary target in human MS, and observed that whereas there is absolutely no trafficking of leukocytes in healthy mice, there is a profound increase in leukocyte rolling and adhesion in the brain microvasculature of mice with EAE. We observed a very significant increase in P-selectin expression 2–3 days before onset of disease. At this stage, P-selectin mediated the majority of leukocyte rolling, but with time the contribution of  $\alpha_4$  integrin to rolling increased dramatically and was dominant for adhesion. However, the role for P-selectin has clearly been underappreciated as throughout disease P-selectin was required for  $\alpha_4$  integrin to mediate rolling.

Initial work proposed that there was a limited role for selectins in leukocyte recruitment to the CNS, due to limited selectin ex-



**FIGURE 7.** P-selectin expression in the CNS of EAE mice. P-selectin protein expression in the brain (A) and spinal cord (B) microvasculature of healthy control mice and mice at various stages of EAE was measured by accumulation of radiolabeled-specific Ab. Results are shown as the mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  vs control.  $n = 5$  for control and 2 wk postsymptom onset;  $n = 4$  for 2 days presymptom onset;  $n = 6$  for 4 days postsymptom onset.

pression. For example, Engelhardt et al. (7) reported a lack of P-selectin and E-selectin expression in EAE. However, more recent work has proposed a role for the selectins in the inflamed brain microvasculature. Although Barkalow et al. (8) were unable to see rapid P-selectin expression in cultured cerebral endothelial cells, this group reported that these same cells did have the capacity to synthesize P-selectin at a more delayed time point. Intravital microscopy of the brain revealed that TNF induces P-selectin- and E-selectin-dependent neutrophil recruitment in the brain microvasculature (9). A very recent study by Piccio et al. (10) suggested that TNF- or LPS-stimulated brain microvasculature induced autoreactive T cells to roll and adhere. In that study, the authors also used fluorescently labeled anti-P-selectin Abs and demonstrated an increase in P-selectin expression in brain vessels of EAE mice. Using an extremely sensitive and quantitative approach to measuring P-selectin expression, we demonstrated no basal P-selectin

Table I. P-selectin expression over disease course in EAE<sup>a</sup>

Tissue	Control	2 Days Pre	4 Days Post	2 wk Post
Skin	0.106 $\pm$ 0.0270	0.190 $\pm$ 0.0663	0.133 $\pm$ 0.0403	0.144 $\pm$ 0.0403
Muscle	0.052 $\pm$ 0.0187	0.113 $\pm$ 0.0451	0.056 $\pm$ 0.0154	0.026 $\pm$ 0.0324
Lung	0.115 $\pm$ 0.0640	0.083 $\pm$ 0.0477	0.059 $\pm$ 0.0301	0.048 $\pm$ 0.0241
Heart	0.005 $\pm$ 0.0050	0.025 $\pm$ 0.0245	0.015 $\pm$ 0.0094	0.020 $\pm$ 0.0100
Liver	0.037 $\pm$ 0.0267	0.326 $\pm$ 0.1221**	0.002 $\pm$ 0.0018	0.030 $\pm$ 0.0304
Pancreas	0.051 $\pm$ 0.0106	0.148 $\pm$ 0.0306**	0.070 $\pm$ 0.0183	0.074 $\pm$ 0.0139
Spleen	0.191 $\pm$ 0.0731	0.709 $\pm$ 0.0984**	0.454 $\pm$ 0.0642	0.396 $\pm$ 0.1111
Mesentery	0.018 $\pm$ 0.0103	0.180 $\pm$ 0.0262**	0.092 $\pm$ 0.0325	0.096 $\pm$ 0.0210
Stomach	0.072 $\pm$ 0.0151	0.163 $\pm$ 0.0272*	0.096 $\pm$ 0.0127	0.087 $\pm$ 0.0220
Small bowel	0.103 $\pm$ 0.0161	0.142 $\pm$ 0.0334	0.125 $\pm$ 0.0394	0.096 $\pm$ 0.0206
Large bowel	0.036 $\pm$ 0.0087	0.048 $\pm$ 0.0159	0.030 $\pm$ 0.0137	0.065 $\pm$ 0.0223
Bone marrow	0.378 $\pm$ 0.0113	0.368 $\pm$ 0.0257	0.393 $\pm$ 0.0365	0.588 $\pm$ 0.0486**

<sup>a</sup> Control, 2 wk post,  $n = 5$ ; 3 days pre,  $n = 4$ ; 4 days post,  $n = 6$ .

\*,  $p < 0.05$  vs control.

\*\*,  $p < 0.01$  vs control.

expression, but a very significant increase in P-selectin expression before disease onset that remained elevated for at least 2 wk postsymptoms. P-selectin was detected in both the brain and the spinal cord, confirming that its expression was not limited to the brain microvasculature. The potential explanation for the lack of previous detection of cerebral P-selectin by some groups may be related to the fact that our assay revealed that optimal P-selectin expression in the brain was only at levels seen basally in organs like muscle and skin (Table I). Nevertheless, these levels have previously been reported to be sufficient to mediate basal rolling in muscle and skin (19). Most importantly, our study demonstrates a functional role for P-selectin in the leukocyte rolling during EAE, particularly a few days before disease onset where P-selectin mediated the large majority of rolling independent from  $\alpha_4$  integrin. P-selectin maintained a role throughout disease as with disease progression, rolling was mediated by a combination of P-selectin and  $\alpha_4$  integrin.

As the disease progressed, the importance of P-selectin as a rolling molecule was replaced by the  $\alpha_4$  integrin pathway. This integrin is very unusual in its ability to induce tethering, rolling, and adhesion thereby potentially bypassing the need for the selectins. Indeed, Alon et al. (20) and Berlin et al. (21) both demonstrated that immobilizing a ligand for  $\alpha_4$  integrin within a laminar flow chamber was sufficient to induce flowing lymphocytes to tether and roll, and when an appropriate activation signal was provided, then the lymphocytes were also able to firmly adhere. In vivo,  $\alpha_4$  integrin was also shown to support rolling and adhesion in inflamed mesenteric microvessels of adjuvant-treated rats (17), but in this case selectins were also required. By contrast,  $\alpha_4$  integrin did support tethering, rolling, and adhesion in muscle microvessels treated with IL-4 in the absence of all selectins (22). Clearly, the tissue and/or the inflammatory process dictated whether  $\alpha_4$  integrin required selectins. In our study, early in EAE development,  $\alpha_4$  integrin appeared to have only a minor role in the rolling process. By contrast, with progression of disease, blockade of  $\alpha_4$  integrin could reduce both rolling as well as adhesion suggesting a more and more important role for this molecule. However,  $\alpha_4$  integrin-mediated rolling remained additionally dependent on P-selectin, likely for initial leukocyte tethering.

The importance of  $\alpha_4$  integrin is underscored by the reduced symptoms in EAE mice associated with spinal cord disease (4–6, 23). The fact that anti- $\alpha_4$  integrin blocks recruitment to the brain may bode well for future therapeutic intervention in human disease. However, blockade of  $\alpha_4$  integrin is not completely protective from disease, and we show in this study that it cannot prevent all leukocyte adhesion in the brain microvasculature, suggesting that other adhesion molecules may also have a role. Interestingly, by blocking all leukocyte rolling with anti-P-selectin Ab, it was also not possible to completely prevent leukocyte adhesion. Rolling is usually considered to be a prerequisite for adhesion, but recent studies have suggested that in some cases leukocytes may be able to tether and immediately adhere, bypassing the rolling stage. Vajkoczy et al. (12) recently showed that activated T cells can adhere without rolling in spinal cord microvessels via  $\alpha_4$  integrin. This may be the mechanism responsible for residual adhesion we observed in anti-P-selectin-pretreated mice. Alternatively, other selectins (E-selectin or L-selectin) may contribute. We observed no role for E-selectin, but it is not clear whether L-selectin could contribute to tethering. L-selectin-deficient mice are protected from EAE, however, this may have more to do with the inability of L-selectin<sup>-/-</sup> monocytes to migrate through the tissue once across the vascular wall or even to recognize targets rather than a defect in leukocyte recruitment per se (24). Regardless, the inability of anti-P-selectin to block all leukocyte adhesion in the

CNS of EAE mice may explain why Engelhardt et al. (7) did not observe any benefit in EAE mice. Based on our work, we would propose dual inhibition of  $\alpha_4$  integrin and P-selectin may provide optimal benefit in human disease.

In conclusion, our data demonstrate that during the development of EAE in mice, early P-selectin-dependent leukocyte rolling is induced that correlates with P-selectin expression just before symptom onset. However, with the progression of disease, P-selectin expression is somewhat reduced and the importance of the  $\alpha_4$  integrin as a rolling molecule increases such that by 5 wk, this molecule is able to support essentially all rolling and the majority of adhesion. However, this  $\alpha_4$  integrin-mediated rolling remains entirely dependent on P-selectin, demonstrating the importance of P-selectin to leukocyte recruitment to the CNS in EAE. Finally, our data also suggest that even before disease onset when P-selectin dominates as a key rolling molecule,  $\alpha_4$  integrin mediates much of the observed adhesion. Clearly, designing molecules to inhibit  $\alpha_4$  integrin preferably with anti-selectins may significantly impact upon the development of disease in EAE and possibly in MS.

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