

Endothelial Cell Laminin Isoforms, Laminins 8 and 10, Play Decisive Roles in T Cell Recruitment Across the Blood–Brain Barrier in Experimental Autoimmune Encephalomyelitis

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Abstract. An active involvement of blood–brain barrier endothelial cell basement membranes in development of inflammatory lesions in the central nervous system (CNS) has not been considered to date. Here we investigated the molecular composition and possible function of the extracellular matrix encountered by extravasating T lymphocytes during experimental autoimmune encephalomyelitis (EAE).

Endothelial basement membranes contained laminin 8 ($\alpha 4\beta 1\gamma 1$) and/or 10 ($\alpha 5\beta 1\gamma 1$) and their expression was influenced by proinflammatory cytokines or angiostatic agents. T cells emigrating into the CNS during EAE encountered two biochemically distinct basement membranes, the endothelial (containing laminins 8 and 10) and the parenchymal (containing laminins 1 and 2) basement membranes. However, inflammatory cuffs occurred exclusively around endothelial basement mem-

branes containing laminin 8, whereas in the presence of laminin 10 no infiltration was detectable. In vitro assays using encephalitogenic T cell lines revealed adhesion to laminins 8 and 10, whereas binding to laminins 1 and 2 could not be induced. Downregulation of integrin $\alpha 6$ on cerebral endothelium at sites of T cell infiltration, plus a high turnover of laminin 8 at these sites, suggested two possible roles for laminin 8 in the endothelial basement membrane: one at the level of the endothelial cells resulting in reduced adhesion and, thereby, increased penetrability of the monolayer; and secondly at the level of the T cells providing direct signals to the trans-migrating cells.

Key words: laminin • experimental autoimmune encephalomyelitis • endothelium • basement membranes • inflammation

Introduction

One of the major functional components of all basement membranes is the laminin family of glycoproteins which influence motility, proliferation, and differentiation of many cell types (Timpl and Brown, 1994; Ekblom and Timpl, 1996). Laminins are heterotrimers composed of an α , β , and γ chain. To date, 5 α , 3 β , and 3 γ chains have been reported which can combine to form up to 12 different laminin isoforms (Timpl et al., 1979; Hunter et al., 1989; Paulsson and Saladin, 1989; Ehrig et al., 1990; Miner et al., 1995; Frieser et al., 1997; Koch et al., 1999). However, in vitro cellular interactions are well defined only for laminin 1 (Aumailley and Smyth, 1998) and, to a lesser extent, for laminins 2 and 5, which have restricted distributions in vivo. Only two isoforms, laminins 8 (composed of

laminin $\alpha 4$, $\beta 1$, and $\gamma 1$) and 10 (composed of laminin $\alpha 5$, $\beta 1$, and $\gamma 1$) are found in endothelial basement membranes of most tissues (Sorokin et al., 1994, 1997a; Frieser et al., 1997). Further, we have shown that laminin $\alpha 4$ expression by endothelial cells in vitro is strongly upregulated by proinflammatory cytokines such as interleukin (IL)¹-1 (Frieser et al., 1997), suggesting a role in inflammatory events. Blood vessels in the central nervous system (CNS) have also been reported to express laminins 1 and 2 (Jucker et al., 1996; Powell and Kleinman, 1997; Tian et al., 1997), which are not detected in blood vessel basement membranes elsewhere and which may be related to their highly specialized structure. The differential expression of

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¹Abbreviations used in this paper: BBB, blood–brain barrier; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillar acidic protein; IL, interleukin; MMP, matrix metalloproteinase; PECAM, platelet endothelial cell adhesion molecule; PLP, protein–lipid–protein; TNF, tumor necrosis factor.

laminin isoforms under different endothelial cell activation states has prompted us to investigate the role of laminins in inflammatory events. We have used a murine experimental autoimmune encephalomyelitis (EAE), an inflammation model with a well defined immunopathology and clinically relevant similarity to the human disease, multiple sclerosis.

EAE can be induced in susceptible mouse strains by immunization with myelin proteins, myelin protein fragments, or by adoptive transfer of myelin reactive CD4⁺ T cell blasts (Engelhardt, 1997). A critical step in this inflammation is the extravasation of leukocytes from the blood stream into the CNS parenchyma, which involves autoaggressive T cell adhesion to and migration through the endothelial cell monolayer of the postcapillary venules. However, after passage across the endothelial cell monolayer, leukocytes still face the endothelial cell basement membrane and the subjacent glia limitans consisting of astrocyte endfeet and associated basement membrane (Wolburg and Risau, 1995).

Ultrastructurally, at least two basement membranes can be identified in association with larger blood vessels in the brain, an endothelial and an astroglial basement membrane (Wolburg and Risau, 1995). In addition, the epithelium of the meninges coinvasinates with blood vessels from the surface of the brain and contributes to the astroglial basement membrane (Alcolado et al., 1988; Wolburg and Risau, 1990; Zhang et al., 1990; Wolburg and Risau, 1995). Collectively, the astroglial basement membrane and the meningeal epithelium basement membrane are known as the parenchymal basement membrane, as they delineate the border to the brain parenchyma. The endothelial cell and parenchymal basement membranes define the inner and outer limits of the perivascular space where leukocytes accumulate during acute EAE before infiltrating the brain parenchyma (Cross et al., 1993; Engelhardt et al., 1997). However, studies to date have not distinguished between penetration of the endothelial cell basement membrane and the process of parenchymal invasion across the glia limitans. There is evidence that suggests these two steps are distinct and independent of one another: in EAE induced in macrophage-depleted mice (Tran et al., 1998) or in tumor necrosis factor (TNF)-deficient mice (Körner et al., 1997), the inflammatory infiltrate becomes entrapped in the perivascular space and parenchymal infiltration is prevented, indicating that progression through the astrocyte basement membrane is functionally distinct from endothelial cell basement membrane transmigration.

In this study, we define the extracellular matrix barriers encountered by extravasating T cells and the steps involved in their transmigration using two different murine EAE models. Further, the use of encephalitogenic T cell lines in *in vitro* adhesion assays has permitted definition of cell-matrix interactions permissive or restrictive for T cell extravasation. Our data demonstrate that endothelial cell basement membranes containing laminin 8 are permissive for T cell transmigration, whereas those containing laminin 10 are restrictive for T cell transmigration. Penetration of the parenchymal basement membrane, characterized by the expression of laminins 1 and 2, which are not adhesive for T cells, occurs only after disruption of this outer bar-

rier, probably via proteolysis and involving matrix metalloproteinases (MMPs; Graesser et al., 2000).

Materials and Methods

Animals

Female SJL/N and C57Bl/6 mice between 3 and 4 wk of age were obtained from Bomholdgard Breeding.

Induction of EAE and PLP T Cell Lines

EAE was induced in SJL/N mice by immunization with syngeneic spinal cord homogenate in CFA (Engelhardt et al., 1997) or C57Bl/6 mice using myelin oligodendrocyte glycoprotein peptide (Gardinier et al., 1992) as described previously. Animals were observed daily and neurological deficits were scored as follows: 1+, flaccid tail; 2+, hind limb weakness; 3+, severe hind limb weakness; 4+, hind quarter paralysis; and 5+, forelimb weakness. At least three mice at each stage were examined.

Protein-lipid-protein (PLP)-specific T cell lines were established as described in detail previously (Engelhardt et al., 1997, 1998).

Primary Antibodies

Primary antibodies to extracellular matrix and cell surface adhesion molecules used in immunofluorescence and cell adhesion studies are listed in Table I.

Rat anti-mouse platelet endothelial cell adhesion molecule (PECAM)-1 (MEC13.3; Vecchi et al., 1994) was used as an endothelial cell marker (BD PharMingen); rat anti-mouse CD45 (leukocyte common antigen; Ly-5) was used to detect leukocytes (30-F11; BD PharMingen); Cy3 conjugate anti-mouse glial fibrillar acidic protein (GFAP; Sigma-Aldrich) was used as a marker for astrocyte endfeet; and Cy3-coupled anti-mouse smooth muscle actin (Sigma-Aldrich), which in the CNS can be used to distinguish arteries and arterioles from veins, capillaries, and postcapillary venules (Nehls and Drenckhahn, 1991; Nehls et al., 1992; Boado and Partridge, 1994).

Immunofluorescence

Tissues were prepared and immunofluorescently stained as described previously (Sorokin et al., 1992). Bound antibodies were visualized using FITC- or Texas red-conjugated goat anti-rat, goat anti-guinea pig, or goat anti-rabbit IgG second antibodies (Dianova). In the case of anti- β -dystroglycan stainings, unfixed sections were used. Stained sections were examined using confocal laser scanning microscopy (model 1000; Bio-Rad Laboratories).

Isolation of Laminins 8 and 10

Mouse laminin 8 (composed of $\alpha 4\beta 1\gamma 1$ chains) was isolated from the conditioned media of 3T3 fibroblasts and the MC3T3-G2/PA6 preadipocyte cell line using a combination of an ion exchange chromatography (POROS 20 HQ column; Applied Biosystems) and immunoaffinity chromatography (CNBr-Sepharose) with a rat anti-mouse laminin $\gamma 1$ chain monoclonal antibody raised in our laboratory (3E10). In brief, conditioned medium was diluted 1:3 in 50 mM Tris-HCl buffer, pH 8, and concentrated on an ion exchange column, POROS 20 HQ column. After dialysis against PBS, the protein solution was subjected to immunoaffinity chromatography with the rat monoclonal anti-laminin $\gamma 1$ antibody (3E10). The affinity column was prepared by coupling 2 mg purified antibody to CNBr-Sepharose. Bound protein was eluted batchwise from the affinity material with 0.1 M triethylamine, pH 11.5, neutralized, and dialysed against 20 mM Tris-HCl, pH 8. Before final dialysis against PBS, gelatin-Sepharose was used to absorb fibronectin and a small POROS 20 HQ column was used for sample concentration.

Laminin 10 was isolated from human placenta by affinity chromatography using mouse anti-human $\gamma 1$ (D18) (Sanes et al., 1990) and $\alpha 5$ (4C7) monoclonal antibodies (Leivo and Engvall, 1988) as described previously (Lindblom et al., 1994; Sixt et al., 2001). Mouse anti-human laminin $\beta 2$ (C4) antibody was used to immunoabsorb laminin $\beta 2$ -containing complexes where necessary. Before immunopurification, 50–100 gm human placenta was homogenized twice on ice in 300 ml 50 mM Tris-HCl, pH 7.5, plus 150 mM NaCl and protease inhibitors, and finally in 50 mM Tris-HCl,

Table I. Specificities and Sources of Antibodies Used

Specificity	Species	Antibody	Source/reference
Laminin α 1	Rat	198	Sorokin et al., 1992
Laminin α 1	Rabbit	317	Durbeej et al., 1996
Laminin α 2	Rat	4H8-2	Schuler and Sorokin, 1995
Laminin α 4	Rat	341	Ringelmann et al., 2000
Laminin α 4	Rabbit	377	Sorokin et al., 2000
Laminin α 3	Rabbit	Lam α 3	Galliano et al., 1995
Laminin α 5	Rat	4G6	Sorokin et al., 1997a
Laminin α 5	Rabbit	405	Sorokin et al., 2000
Laminin β 1	Rat	3E10	Sixt et al., 2001
Laminin γ 1	Rabbit	N283	Mayer et al., 1993
Laminin β 2	Guinea pig	GP-5	Patton et al., 1997
Pan-laminin 1	Rabbit	308	Sorokin et al., 1990; Ringelmann et al., 2000
Integrin α 6	Rat	GoH3	Sonnenberg et al., 1988
Integrin α 3	Rabbit	340	Valles et al., 1996
Integrin α 3	Rabbit	C-18	Santa Cruz Biotechnology, Inc.
Integrin α 7	Rabbit	242	Echtermeyer et al., 1996; Mayer et al., 1997
Integrin β 1	Hamster	Ha2/5	Lenter and Vestweber, 1994; BD PharMingen
Integrin β 4	Rat	346-11A	Lenter and Vestweber, 1994; BD PharMingen
β -Dystroglycan	Mouse	NCL-43DAG	Novo Castra

pH 7.5, plus 300 mM NaCl and 20 mM EDTA. After at least 3 h of extraction at 4°C with gentle rolling, the placenta extract was clarified by centrifugation and dialysed against PBS. Further purification steps were performed essentially as described for laminin 8.

The chain composition of the laminin 8 and 10 preparations was assessed by immunoblotting and ELISA using laminin chain-specific antibodies (Table I), whereas the purity of the preparations was assessed by silver-stained SDS-PAGE under reducing conditions. The laminin 8 preparation was found to contain only laminin α 4, β 1, and γ 1 chains (Sixt et al., 2001), whereas the laminin isolated from human placenta contained predominantly laminin α 5, β 1, and γ 1 chains (laminin 10), plus a weak laminin β 2 chain band in silver staining (laminin 11) as shown in Sixt et al. (2001). Laminin 11 represented <5% of the human laminin preparation.

Endothelial Cell Lines

Mouse endothelial cell lines derived from brain capillaries (bEND3) and a skin hemangioma (sEND1) were as described previously (Frieser et al., 1997; Sorokin et al., 1994, 1997a). Both cells were grown in DME supplemented with 10% fetal calf serum at 37°C and 7.5% CO₂ in a water-saturated environment.

Northern Blot Analysis

mRNA extraction and Northern blot analyses were performed as described previously (Sorokin et al., 1997a,b). RNA was isolated from unactivated endothelial cell lines (bEND3 and sEND1), and cell lines activated for 4 h at 37°C with 75 U/ml TNF- α (BD PharMingen) or 10 ng/ml IL-1 β (Biomol), or treated for 3 d with 4 μ g/ml hydroxymethylprogesterone (Sigma-Aldrich) and freshly dissected control and EAE brains (clinical scores, +4/+5). Hybridizations were performed with the following ³²P-labeled specific cDNA probes: laminin α 1 covering nucleotides 7786–9286 (Schuler and Sorokin, 1995); laminin α 2 covering nucleotides 6420–6895 (Schuler and Sorokin, 1995); laminin α 4 covering nucleotides 4720–5311 (Frieser et al., 1997); laminin α 5 covering nucleotides 3962–4623 (Sorokin et al., 1997b); or with a glyceraldehyde-3-phosphate dehydrogenase (GADPH)-specific probe (Sorokin et al., 1997b).

In Situ Hybridization

In situ hybridization of E16 and E18 mouse embryos and newborn and adult brains was performed as described previously (Sorokin et al., 1997a), using the cDNA probes for laminin α 1, α 2, and α 4 chains described above for Northern blot analysis, plus an endothelial cell-specific probe, flk-1 (Breier et al., 1992).

Adhesion Assays

In vitro cell attachment assays were performed as described previously (Goodman et al., 1987; Schuler and Sorokin, 1995), using laminins 8 and

10 prepared as described above, laminin 1 isolated from the Engelbreth-Holm-Swarm (EHS) mouse tumor (Paulsson et al., 1987), and laminin 2 isolated from mouse hearts (Paulsson and Saladin, 1989) as substrates. PLP encephalitogenic T cell lines (1 d after restimulation) were washed with PBS and 5 \times 10⁴ cells were added per well in 100 μ l adhesion buffer (RPMI, 0.5% BSA, 10 mM Hepes, pH 7.5). The number of adherent cells after 30 min of incubation at 37°C without shear stress was determined by colorimetric analysis of lysosomal hexosaminidase (OD₄₀₅; Landegren, 1984). Since leukocytes are activated by chemokines and cytokines are released at sites of inflammation in vivo, attachment assays were performed with nonactivated and PMA-activated (100 ng/ml) cells.

To assess which cellular receptors mediated interactions with extracellular matrix molecules, inhibition studies were carried out using specific antibodies to integrin subunits (see Table I). Cells were preincubated with given concentrations of antibodies against integrin subunits or control antibodies for 30 min before addition to protein-coated microtiter plates. Experiments were carried out with different laminin isoforms plated at a concentration of 20 μ g/ml. The experimental procedure was otherwise as described in detail elsewhere (Frieser et al., 1996).

The percentage of cells which bound specifically to the coating substrate was determined as follows: $([OD_{405}$ of total bound cells – OD_{405} of BSA bound cells]/ OD_{405} of 50,000 applied) \times 100 = percentage of specific binding.

Results

Basement Membranes Associated with Brain Blood Vessels

In the noninflamed brain the cellular and extracellular matrix layers of the blood vessels appear as a single structure at the light microscope level and are distinguishable only by electron microscopy. However, during early stages of inflammation or in animals with low clinical EAE scores (1+ to 2+), leukocytes accumulate in the perivascular space between the endothelial and the astrocyte endfeet basement membranes, rendering them distinguishable at the light microscope level and allowing the clear identification of the localization of laminin chains (Fig. 1, A–C). In EAE, local inflammations develop around blood vessels, hence only a fraction of blood vessels develop a perivascular cuff, allowing analysis of vessels with and without infiltrates in the same section (Fig. 1, B and C). Double staining with a pan-laminin 1 antibody, which recognizes laminin α 1, β 1, and γ 1 chains equally well and

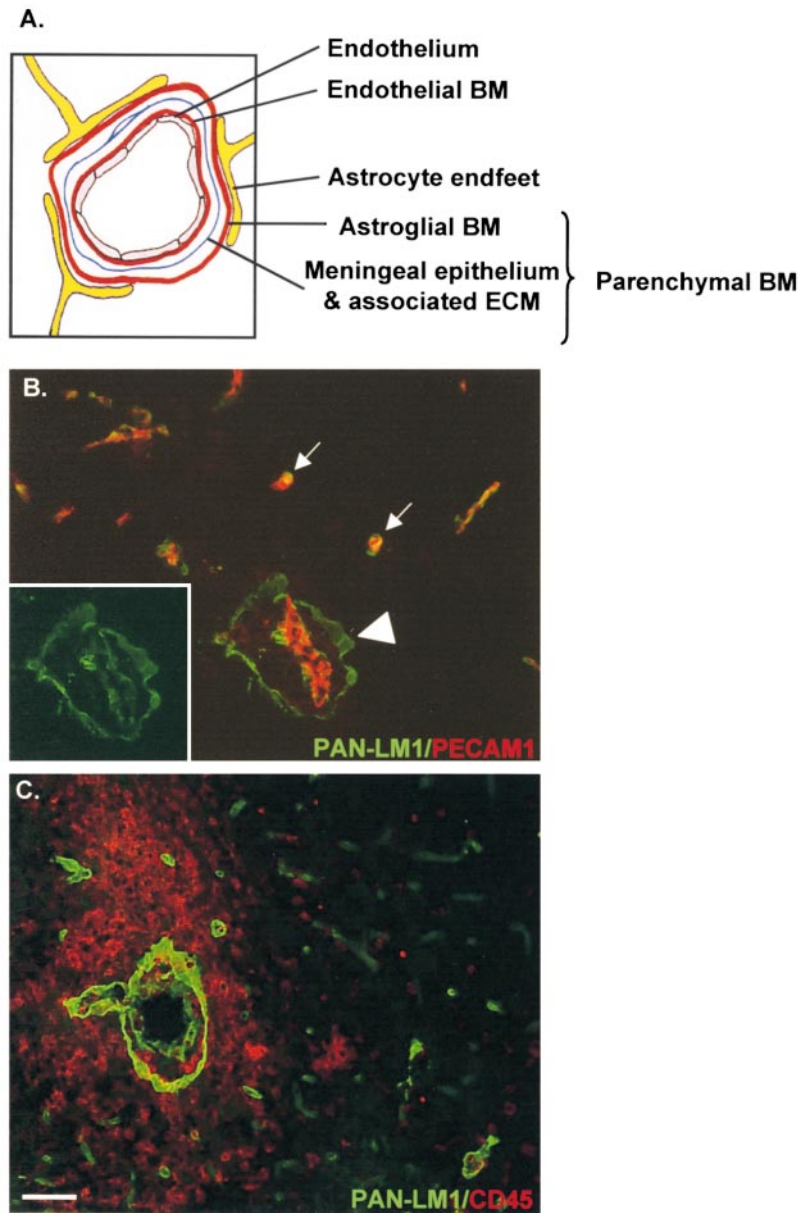


Figure 1. Cell layers and basement membranes which occur in association with blood vessels in the CNS. (A) Schematic representation of blood vessels showing the inner endothelial cell layer and its basement membrane (BM), bordered by epithelial meningeal cells and associated extracellular matrix and outer astroglial basement membrane and astrocyte endfeet. Collectively, the meningeal component and astroglial basement membrane are termed the parenchymal basement membrane. (B) Double staining of EAE brains (clinical scores +1 to +2) for PECAM-1 (red) and pan-laminin 1 antibody (PAN-LM1, green) which recognizes laminin $\alpha 1$, $\beta 1$, and $\gamma 1$ chains and thereby many laminin isoforms. Inset shows pan-laminin 1 staining alone. At sites of mononuclear infiltration the internal endothelial cell layer, bordered by its basement membrane, is distinct from the outer parenchymal basement membrane (arrowhead), whereas where no infiltration has occurred these two basement membranes are indistinguishable (arrows). (C) Double staining of EAE brains (clinical scores +3 to +4) with the leukocyte marker, CD45 and pan-laminin 1 antibody, demonstrating infiltrating mononuclear cells accumulating in the perivascular space and penetrating the outer parenchymal basement membrane. Bar, 50 μm .

hence any laminin containing at least one of these chains and anti-PECAM-1 (Fig. 1 B) or the leukocyte marker, anti-CD45 (Fig. 1 C), reveals the PECAM-1-positive endothelial cell monolayer and the separate inner endothelial and outer parenchymal basement membranes at sites of infiltration (Fig. 1 B, inset), but not where no infiltration has occurred (Fig. 1, B and C).

Stainings with specific monoclonal and/or polyclonal antibodies for the five known laminin α chains ($\alpha 1$ – $\alpha 5$) showed that all α chains except laminin $\alpha 3$ are expressed in association with blood vessels. However, the different laminin α chains were not colocalized in the same basement membrane and they had different cellular origins (Figs. 2 and 3). Fig. 2 shows consecutive sections of an inflamed brain double stained for pan-laminin 1 and CD45 to define sites of mononuclear infiltration (Fig. 2 A), double stained for individual laminin α chains (Fig. 2, B–F), or stained for α -smooth muscle actin alone (Fig. 2 G). Only

laminin $\alpha 4$ and $\alpha 5$ occurred in the inner endothelial cell basement membrane (Fig. 2, B and C), whereas laminin $\alpha 1$ and $\alpha 2$ were restricted to the parenchymal basement membrane (Fig. 2, D–F). The inset in Fig. 2 B shows a double staining for PECAM-1 and laminin $\alpha 1$, revealing clear separation of immunoreactivity, which was also observed for laminin $\alpha 2$ /PECAM-1 double stainings. One of the most striking findings was the absence of laminin $\alpha 5$ in the endothelial cell basement membrane where mononuclear infiltration had occurred (compare Fig. 2, A and C). Spinal cords and brains of mice afflicted with EAE with clinical scores of 1+ to 5+ showed essentially the same pattern of results. Recruitment of inflammatory cells, as defined by CD45 reactivity, was consistently localized around blood vessels where only laminin $\alpha 4$ could be detected in the endothelial cell basement membrane (compare Fig. 2, A and B). No infiltrate was observed around blood vessels where both laminin $\alpha 4$ and $\alpha 5$ were detected

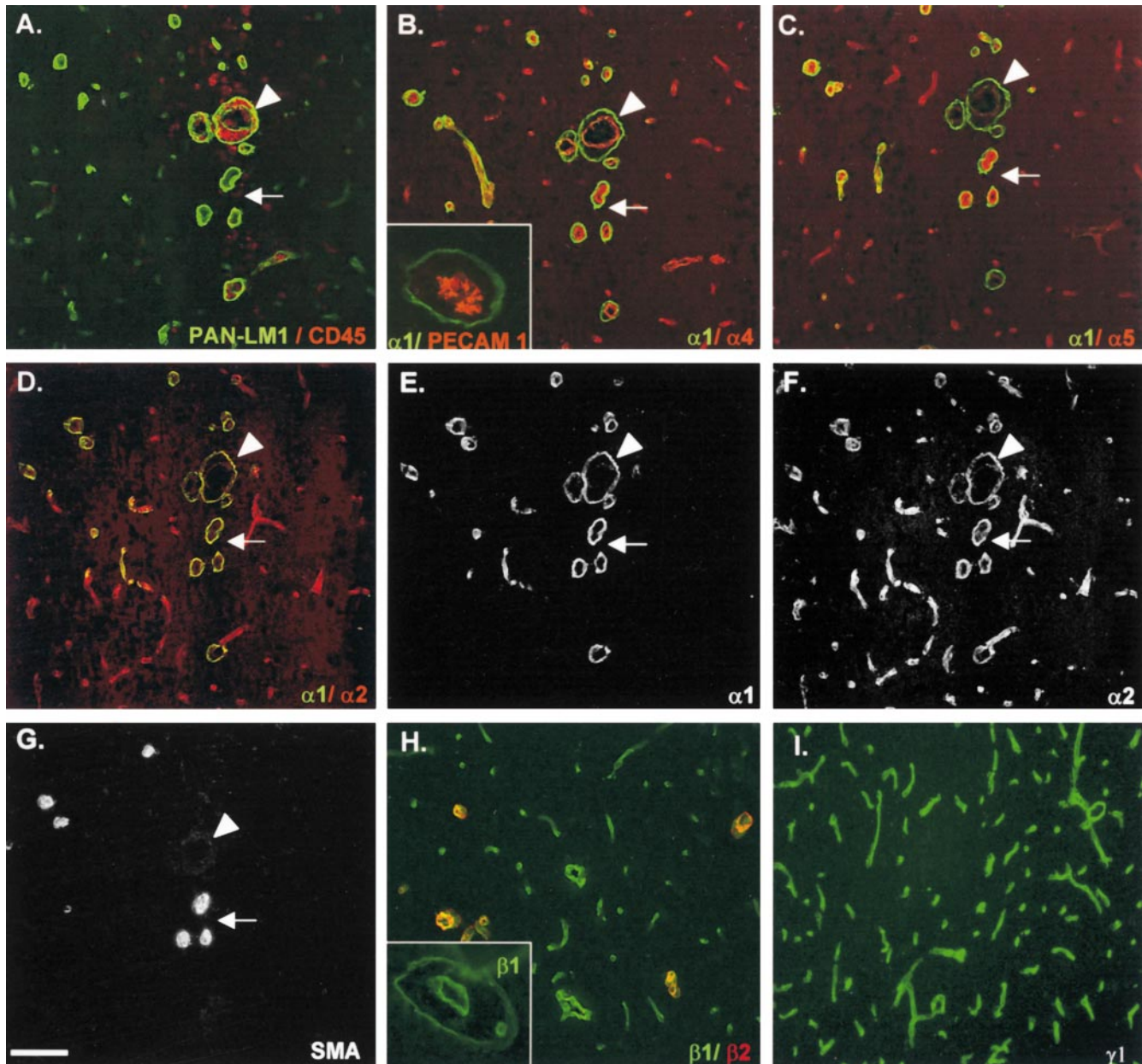


Figure 2. Confocal microscopy analysis of consecutive sections of EAE brains (clinical score +1 to +2) double stained for pan-laminin 1 (PAN-LM1) and CD45 (A), laminins $\alpha 1$ and $\alpha 4$ (B), laminins $\alpha 1$ and $\alpha 5$ (C), laminins $\alpha 1$ and $\alpha 2$ (D–F), or α -smooth muscle actin (SMA) alone (G). Inset in B shows double staining for PECAM-1 and laminin $\alpha 1$ at sites of mononuclear infiltration. Superimposed images are shown in A–D; individual images for laminins $\alpha 1$ and $\alpha 2$ are shown in E and F, respectively. Arrowheads in all sections mark the same blood vessel where mononuclear infiltration has occurred, revealing the presence of laminin $\alpha 4$ alone in the endothelial cell basement membrane. The arrows mark vessels where no infiltration has occurred, revealing the presence of laminin $\alpha 4$ and $\alpha 5$ in the endothelial cell basement membrane and α -smooth muscle actin reactivity (G). Note the presence of laminin $\alpha 1$ and $\alpha 2$ in parenchymal basement membrane only (B–F) and the restricted distribution of laminin $\alpha 1$ (D and E). Double staining for laminin $\beta 1$ and $\beta 2$ (H) and for laminin $\gamma 1$ alone (I) revealed the presence of laminin $\beta 1$ and $\gamma 1$ chains in association with all blood vessels, whereas laminin $\beta 2$ is restricted to larger vessels. Inset in H shows laminin $\beta 1$ staining at a site of mononuclear infiltration, localizing this chain to endothelial and parenchymal basement membranes. The same pattern of results was observed for laminin $\gamma 1$. Bar, 80 μm .

(compare Fig. 2, A–C). Further, mononuclear infiltration occurred at sites where the parenchymal basement membrane contained both laminin $\alpha 1$ and $\alpha 2$ (Fig. 2, D–F), but where no smooth muscle actin reactivity was detectable (Fig. 2 G).

Laminin $\alpha 2$ immunoreactivity was more widespread than that of laminin $\alpha 1$ and was detected in association

with all blood vessels within the brain (Fig. 2, D–F). This is well illustrated in Fig. 3, A–C, which shows laminin $\alpha 1/\alpha 2$ double staining of a single microvessel, revealing the more extensive staining for laminin $\alpha 2$ and the restriction of laminin $\alpha 1$ to a portion of the vessel. The close association of the laminin $\alpha 2$ and GFAP (Fig. 3, D–F) suggests that laminin $\alpha 2$ is expressed by astrocytes and that the protein

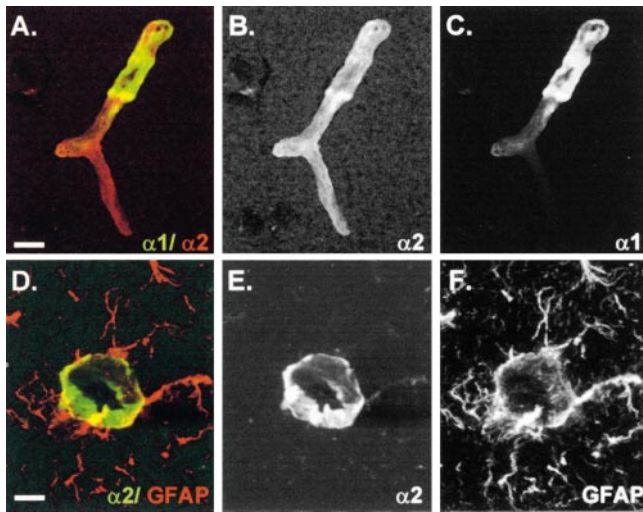


Figure 3. Confocal microscopy analysis of individual blood vessels in the CNS double stained for laminin $\alpha 1$ and $\alpha 2$ (A–C) or laminin $\alpha 2$ and GFAP (D–F). Superimposed images are shown in A and D; individual images are shown in B, C, E, and F. Note the restricted distribution of laminin $\alpha 1$ which disappears at the precapillary level (A–C). Laminin $\alpha 2$ localizes at the GFAP-positive astrocyte endfeet (D and E). Bars: (A–C) 8 μm ; (D–F) 12 μm .

is deposited at their endfeet. Laminin $\alpha 1$ chain immunoreactivity was restricted to vessels with diameters $>10 \mu\text{m}$ throughout the CNS (Fig. 2, D and E) and was contiguous with pia mater epithelium covering the brain, disappearing abruptly in the precapillary region of arteries and veins (Fig. 3, A and C). No laminin $\alpha 1$ staining was detected in association with capillaries (Fig. 2 E).

To define the laminin isoforms localized in endothelial and parenchymal basement membranes, stainings were also performed for laminin $\beta 1$, $\beta 2$, and $\gamma 1$ chains, revealing the presence of laminin $\beta 1$ (Fig. 2 H) and $\gamma 1$ (Fig. 2 I) in endothelial and parenchymal basement membranes (Fig. 2 H, inset), whereas laminin $\beta 2$ was restricted to the smooth muscle layer of larger blood vessels (Fig. 2 H). This suggests that laminin 8 ($\alpha 4\beta 1\gamma 1$) and 10 ($\alpha 5\beta 1\gamma 1$) are the main laminin isoforms in the endothelial cell basement membrane, whereas laminin 1 ($\alpha 1\beta 1\gamma 1$) and 2 ($\alpha 2\beta 1\gamma 1$) occur in the parenchymal basement membrane.

Cellular Origins of Laminin Chains

In situ hybridization experiments were performed to define the cellular origin of the laminin α chains. The low turnover of most laminin α chains in adult tissues (data not shown) made it necessary to compare the expression patterns for the individual laminin α chains in embryonic and/or newborn brains (Fig. 4). In E16, E18, and, more weakly, in newborn mouse brains, laminin $\alpha 1$ mRNA was not expressed by endothelial cells, but rather by the leptomeningeal cells of the pia mater that encase the brain and are infolded from the brain surface (Fig. 4, A and B), correlating well with the protein distribution data. In situ hybridization of E16 and E18 mouse brains revealed a strong laminin $\alpha 2$ mRNA signal in the meninges (data for E16 is shown in Fig. 4 C), which is most likely to be a glial product

(Sixt, M., and L. Sorokin, manuscript in preparation). Laminin $\alpha 4$ mRNA was expressed in all endothelial cells of embryonic, newborn, and adult vessels (data for E16 brain are shown in Fig. 4, E and F), suggesting a high turnover of this molecule even in the mature blood vessel basement membrane. Our previous studies have shown that laminin $\alpha 5$ mRNA appears in endothelium of the brain (and other tissues) only 3–4 wk after birth and remains weak in the adult brain endothelium (Sorokin et al., 1997a).

Laminin Receptor Expression in Normal and Inflamed Blood Vessels

Immunoreactivity for the major laminin-binding receptors ($\alpha 6\beta 1$, $\alpha 3\beta 1$, $\alpha 7\beta 1$, $\alpha 6\beta 4$, and dystroglycan; Delwel and Sonnenberg, 1996; Talts et al., 1999; Kikkawa et al., 2000; Kortessmaa et al., 2000) was analyzed in inflamed and non-inflamed blood vessels. Due to the absence of antibodies to α -dystroglycan which function well in immunofluorescence of tissues other than skeletal muscle, it was necessary to utilize a β -dystroglycan-specific antibody for investigations of α -dystroglycan localization. β - and α -dystroglycan are products of the same gene and colocalize in most tissues (Henry and Campbell, 1998). However, there are reports of β -dystroglycan expression in the absence of α -dystroglycan (Saito et al., 1999; Losasso et al., 2000).

Integrin $\alpha 6$ and β -dystroglycan were prominent in CNS blood vessels (Fig. 5, A–C), whereas no staining was observed for integrin $\alpha 3$, $\alpha 7$, and $\beta 4$ subunits (data not shown; Pinkstaff et al., 1999). Confocal microscopy revealed a mutually exclusive expression of integrin $\alpha 6$ and β -dystroglycan on all blood vessels except capillaries (Fig. 5, A–C). It was not possible to perform double staining for integrin $\alpha 6$ and PECAM-1, as both were rat antibodies; however, comparison of the staining patterns for the two molecules revealed identical patterns. This suggests expression of integrin $\alpha 6$ on PECAM-1-positive endothelium in close association with laminin $\alpha 4$ and $\alpha 5$, but not with the laminin $\alpha 1$ and $\alpha 2$ chains of the parenchymal basement membranes (double staining for laminin $\alpha 1$ and integrin $\alpha 6$ is shown in Fig. 5, D–F). This pattern was even more distinct in EAE brains, where the presence of a perivascular cuff allowed clear identification of integrin $\alpha 6$ expression on endothelium in close association with the laminin $\alpha 4$ - and/or $\alpha 5$ -containing basement membrane (data for integrin $\alpha 6$ is shown in Fig. 5 F, inset). In contrast, β -dystroglycan immunostaining was restricted to astrocyte endfeet, in close association with GFAP staining (not shown) and laminin $\alpha 2$ immunoreactivity, and was distinct from that of laminin $\alpha 4$ and $\alpha 5$ (reconstruction of serial optical sections for laminin $\alpha 4/\beta$ -dystroglycan double staining is shown in Fig. 5, G–I).

At early stages of EAE and in control brains, all blood vessels showed an intense and continuous staining for integrin $\alpha 6$ (Fig. 5, D and F), whereas at advanced stages of EAE integrin $\alpha 6$ immunoreactivity on endothelium was significantly less intense and discontinuous (Fig. 5 F, inset). β -Dystroglycan did not appear to be altered in its expression pattern or intensity. However, at sites of leukocyte infiltration this was difficult to assess because of high background staining of this mouse monoclonal antibody in EAE brains (data not shown).

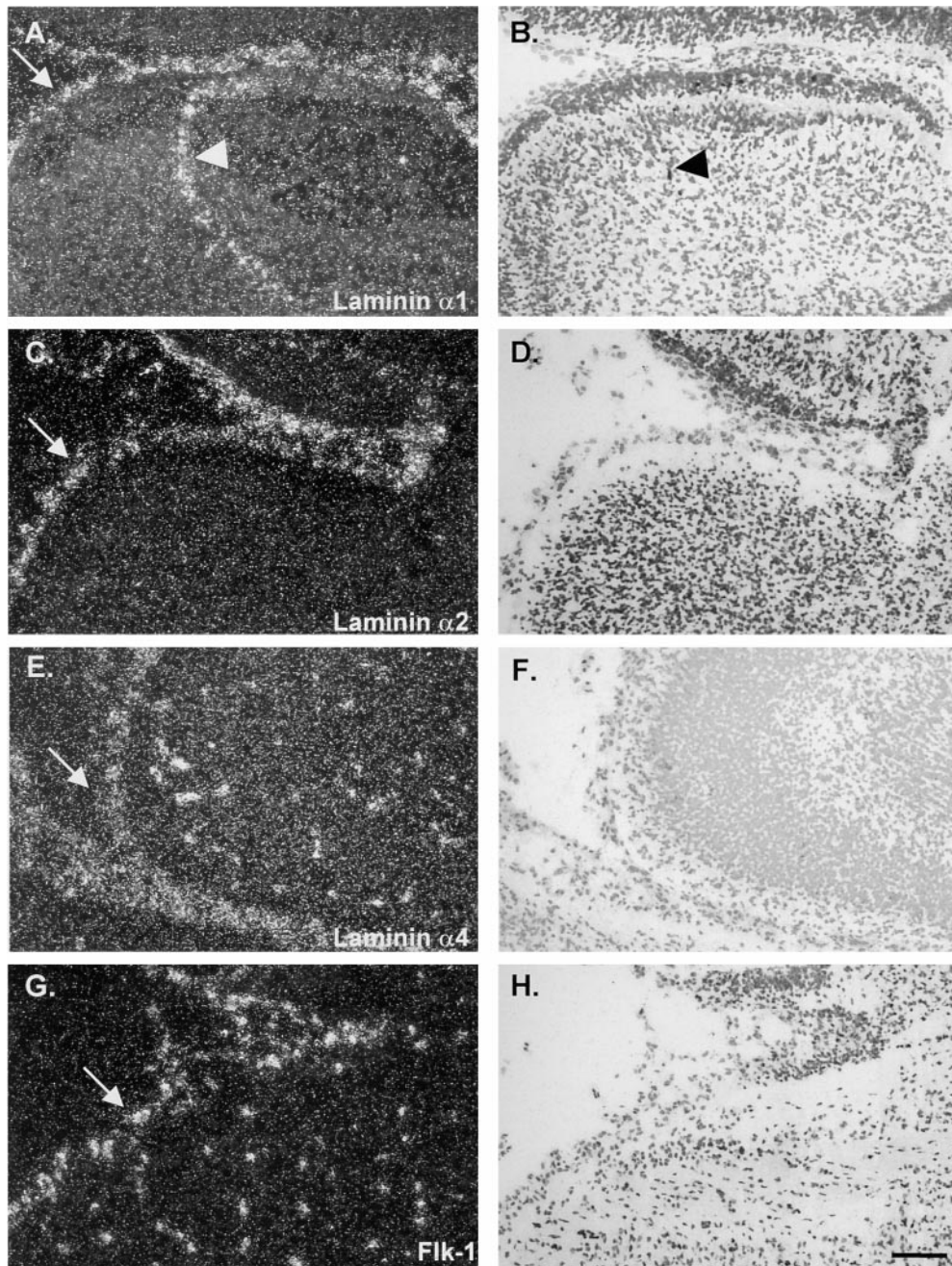


Figure 4. In situ hybridization of consecutive sections of E16 embryonic mouse brains with probes specific for laminin $\alpha 1$ (A and B), $\alpha 2$, (C and D), $\alpha 4$ (E and F), and an endothelial cell-specific probe, flk-1 (G and H). Data shown are for similar areas, encompassing meninges (arrows) and a portion of the cortex of the cerebellum. Similar results were observed for E18 embryos and, in the case of laminin $\alpha 4$, also for newborn and adult tissues. Laminin $\alpha 1$ mRNA was expressed by the leptomeningeal cells of the pia mater that encase the brain and are infolded from the brain surface (arrowheads in A and B); laminin $\alpha 2$ mRNA was restricted to the meninges (C and D), whereas laminin $\alpha 4$ mRNA was detected in endothelial cells within and outside the brain (E and F) in a similar pattern to that observed with the endothelial cell-specific probe, flk-1 (G and H). Bar, 25 μm .

Integrin $\beta 1$ immunoreactivity was more widespread than that of integrin $\alpha 6$ as it occurred on both endothelium and astrocyte endfeet (compare Fig. 5, F and K), and was strong on endothelium and weaker on astrocyte endfeet (Fig. 5, J–L). In EAE brains, venules with perivascular inflammatory cells staining for integrin $\beta 1$ were not altered (Fig. 5 K, inset).

Northern Blot Analysis—Whole Brains

To investigate the possibility that laminin $\alpha 5$ mRNA is downregulated in EAE brains, Northern blot analysis was performed on whole brains of control and EAE mice (clinical score 4+ / 5+) using laminin $\alpha 1$ -, $\alpha 2$ -, $\alpha 4$ -, and $\alpha 5$ -specific probes. Laminin $\alpha 1$ and $\alpha 2$ mRNA were not detectable in either control or EAE brains, whereas the

12-kb laminin $\alpha 5$ mRNA was detectable after 6 d of exposure and a distinct 6.5-kb laminin $\alpha 4$ mRNA signal was seen after 24 h of exposure (Fig. 6 A). No difference was apparent between control and EAE brains.

Northern Blot Analyses of Cultured Endothelial Cells

To test whether laminin isoform expression in microvascular endothelium is regulated by proinflammatory cytokines present at sites of mononuclear infiltration (for review see Sedgwick et al., 2000), mouse brain capillary-derived (bEND3) and vein-derived (sEND1) endothelial cell lines were investigated by Northern blot analysis.

Consistent with the *in vivo* results, both endothelial cell lines expressed laminin $\alpha 4$ and $\alpha 5$ mRNA (Fig. 6, B and C). Laminin $\alpha 4$ mRNA was upregulated in sEND1 after

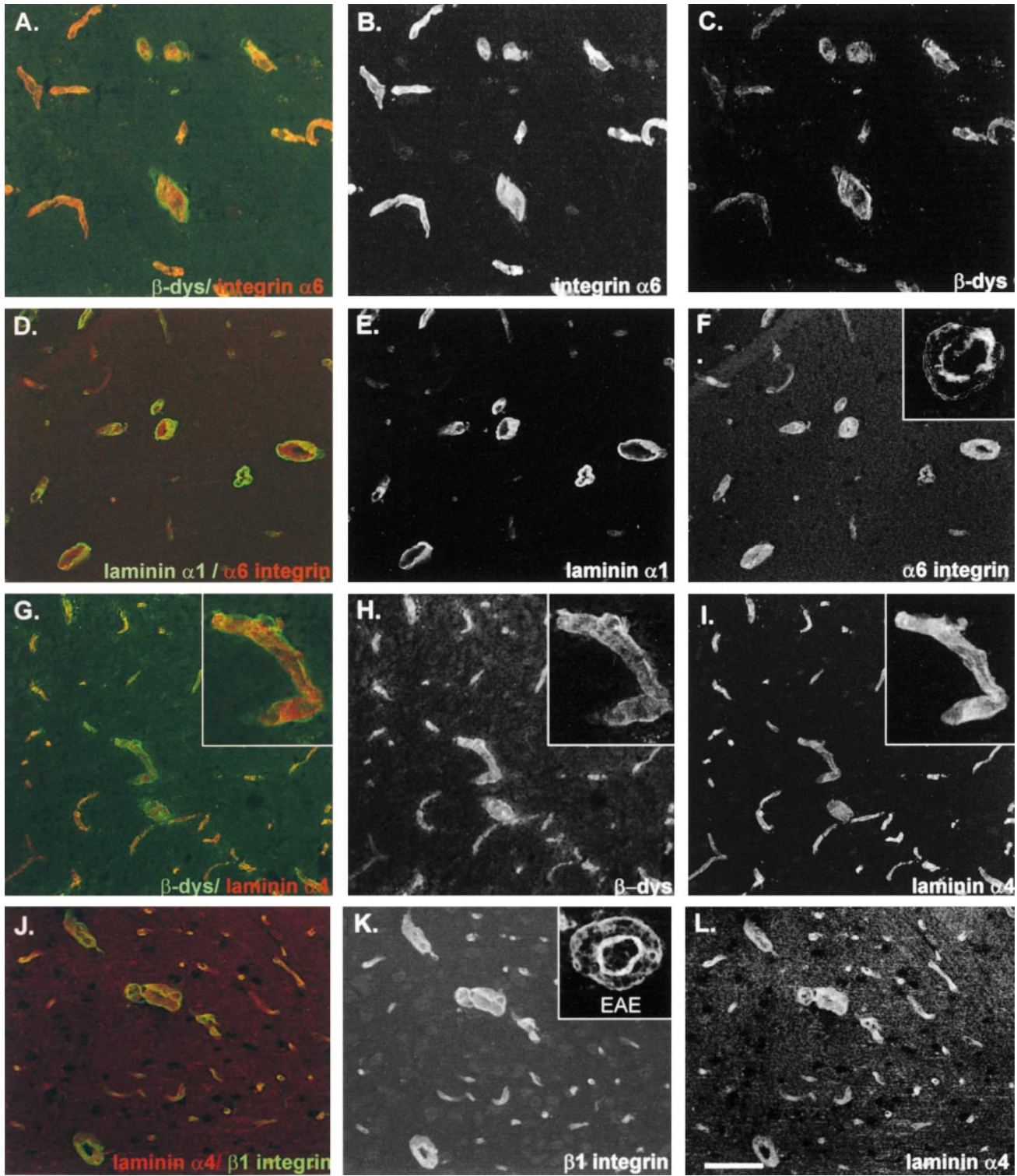


Figure 5. Confocal microscopy analysis for the expression of integrin $\alpha 6$ and β -dystroglycan in mouse brain blood vessels. A–C show double staining for integrin $\alpha 6$ and β -dystroglycan (β -dys) showing mutually exclusive distributions. D–F show double staining for integrin $\alpha 6$ and the parenchymal basement membrane component, laminin $\alpha 1$, localizing integrin $\alpha 6$ on the inner endothelial cell layer; inset in F shows integrin $\alpha 6$ expression at sites of mononuclear infiltration revealing discontinuous staining. G–I show double staining for β -dystroglycan and the endothelial cell basement membrane-specific laminin $\alpha 4$, demonstrating β -dystroglycan expression on astrocyte endfeet; the insets in G–I show a higher magnification of a single vessel. J–L show double staining for integrin $\beta 1$ and laminin $\alpha 4$ showing overlapping distribution patterns; the inset in K shows integrin $\beta 1$ expression at sites of mononuclear infiltration, demonstrating concentration on the inner endothelial cell layer, but also on infiltration cells, with weaker expression on the astrocyte endfeet. Bars: (A–C) 80 μ m; (D–L) 100 μ m.

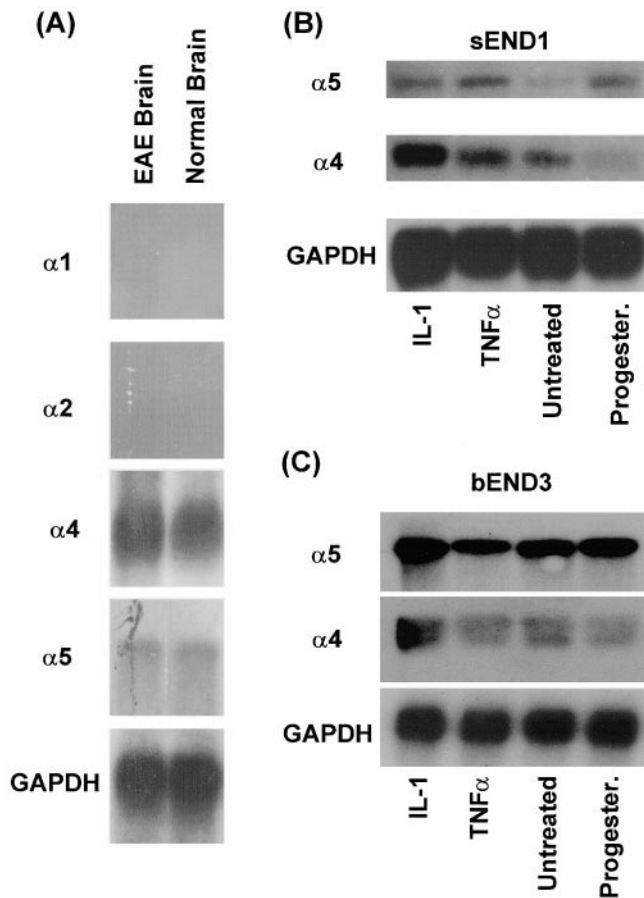


Figure 6. Northern blot analysis for the expression of laminin $\alpha 1$, $\alpha 2$, $\alpha 4$, and $\alpha 5$ mRNA in control and EAE (clinical score 4+/5+) mouse brains (A), and for laminins $\alpha 4$ and $\alpha 5$ mRNA in sEND1 (B) and bEND3 (C) mouse endothelial cell lines. Endothelial cell lines were either untreated, activated with IL-1 or transforming growth factor α (TNF α), or treated with hydroxymethylprogesterone (Progester.). Data in A for laminin $\alpha 1$ and $\alpha 2$ is after 2 wk of exposure, for laminin $\alpha 5$ after 6 d of exposure, and for laminin $\alpha 4$ after 24 h of exposure.

activation with TNF- α and IL-1 (Fig. 6 B), whereas treatment with the angiostatic agent, hydroxymethylprogesterone, decreased expression (Fig. 6 B). Interestingly, laminin $\alpha 5$ mRNA was also upregulated by TNF- α and to lesser extent by IL-1, whereas the 12-kb laminin $\alpha 5$ mRNA increased compared with the controls after hydroxymethylprogesterone treatment (Fig. 6 B). bEND3 responded less intensely than sEND1 to either TNF or IL-1 activation, consistent with its capillary origin (Fig. 6 C).

We have shown in a previous study that bEND3 and sEND1 do not express laminin $\alpha 1$ mRNA (Sorokin et al., 1994); experiments performed here with specific probes for laminin $\alpha 2$ and $\alpha 3$ mRNA also revealed no signal, even after 6 d of exposure (data not shown).

Adhesion of Encephalitogenic T Cell Lines to Purified Laminins

To investigate whether encephalitogenic T cells were capable of interacting with endothelial cell laminins, SJL/N mouse-derived T cell lines specific for the encephalite-

nic peptide (amino acids 139–153) of PLP were tested for adhesion to mouse laminins 1, 2, and 8, and human laminin 10. T cells adhered weakly to the laminin $\alpha 5$ chain containing laminin 10 (11% of total cells added) and exhibited minimal binding to the laminin $\alpha 4$ chain containing laminin 8 (5%; Fig. 7 A). The parenchymal basement membrane laminins 1 and 2 were not adhesive, even at high coating concentrations (Fig. 7 A).

Upon stimulation of T cells with PMA, encephalitogenic T cells bound principally to laminin 10 (60%) and showed increased binding to laminin 8 (20%; Fig. 7 A). However, no specific binding to laminins 1 or 2 could be induced, even under conditions that maximally activate integrins (10 mM Mn²⁺). The differential adhesion of the T cells to the different laminin isoforms was not due to either the quality of the laminin preparations or their ability to bind to the culture plates, as C2C12 myoblasts were found to bind significantly (50–100% cell adhesion) to all four laminin isoforms (Karosi, S., O. Wendler, and L. Sorokin, manuscript in preparation). To ensure that the different purified laminin isoforms bound with the same efficiency to the plastic plates used in cell adhesion assays, the laminins were labeled with ¹²⁵I and coated under the same conditions as applied for cell binding assays. It was shown that all four laminin isoforms used in this study bound to the plastic surfaces in a comparable and concentration-dependent manner (data not shown).

Adhesion of encephalitogenic T cells to laminins 8 and 10 was inhibited in a concentration-dependent manner with the function-blocking antibodies to integrin $\alpha 6$ chain (GoH3) and the mouse integrin $\beta 1$ chain (Ha2/5; data for laminin 10 is shown in Fig. 7 B), indicating $\alpha 6\beta 1$ -mediated binding to both laminin 8 and 10.

Discussion

We have shown previously that different mouse endothelial cell lines express predominantly either laminin $\alpha 4$ or $\alpha 5$ and that this expression pattern can be altered by the growth or activation state of the cells (Sorokin et al., 1994, 1997a; Frieser et al., 1997), suggesting that the laminin isoforms containing these chains have different functions in endothelial cell basement membranes. The data presented here support this hypothesis. Further, we have shown for the first time that mononuclear cells infiltrating the CNS during the course of EAE encounter two biochemically and functionally distinct basement membranes, the endothelial and parenchymal basement membranes. To date, most studies of EAE have concentrated on leukocyte-endothelial cell interactions, revealing $\alpha 4\beta 1$ and vascular cell adhesion molecule 1 as significant players (Engelhardt et al., 1998; Laschinger and Engelhardt, 2000), or the subsequent penetration of the CNS, with little consideration that after penetrating the endothelial cell monolayer infiltrating cells still face two formidable and distinct extracellular barriers. The localization of a perivascular cuff of infiltrating mononuclear cells surrounding blood vessels at early stages of EAE allowed clear identification of the endothelial cell and parenchymal basement membranes, demonstrating that endothelial cell basement membranes contain laminin $\alpha 4$ and/or $\alpha 5$ chains which are produced by endothelial cells, whereas parenchymal basement mem-

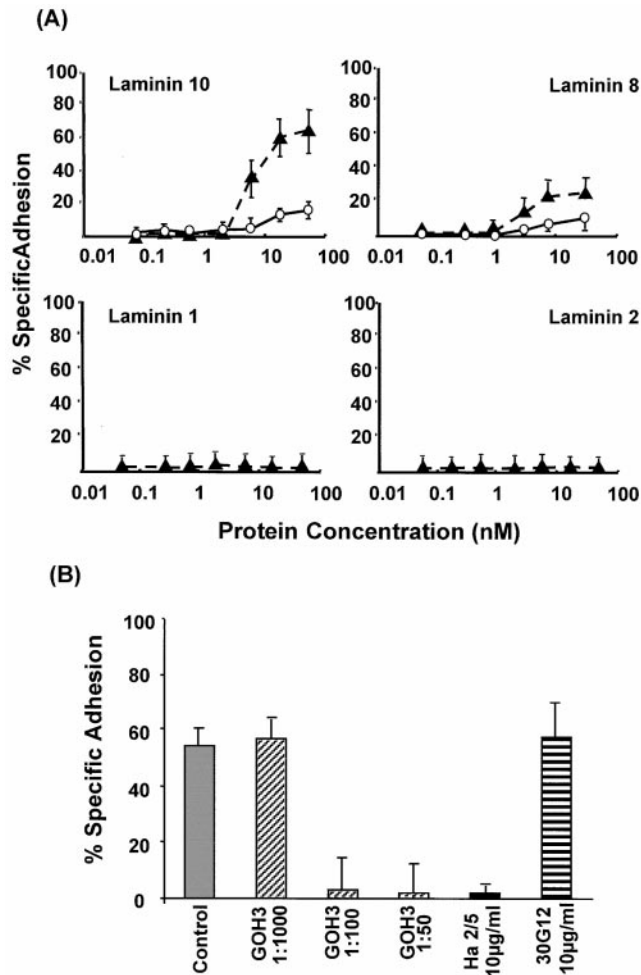


Figure 7. (A) Specific binding of nonactivated (○) and PMA-activated (▲) PLP-specific, encephalitogenic T cell lines to increasing concentrations of the endothelial cell basement membrane laminins 10 and 8, and the parenchymal basement membrane laminins 1 and 2. Saturable T cell binding occurred only to laminins 10 and 8; binding to laminins 1 and 2 could not be induced, even under maximal integrin activation conditions (10 mM Mn^{2+}). (B) Binding to 30 nM laminin 10 was completely inhibited by antiintegrin $\alpha 6$ (GoH3) or antiintegrin $\beta 1$ (Ha2/5), whereas anti-CD45 antibody (30G12) had no effect. The same pattern of results was observed for laminin 8. Values represent means of at least six experiments \pm SD.

branes contain laminin $\alpha 1$ and $\alpha 2$ produced by leptomeningeal cells and astrocytes, respectively (see Fig. 8 for summary of data). Interestingly, mononuclear recruitment in the perivascular space correlated with the laminin composition of the endothelial cell basement membrane: recruitment of T cells occurred exclusively around endothelial cell basement membranes containing laminin $\alpha 4$, but not laminin $\alpha 5$, suggesting that laminins containing the laminin $\alpha 4$ chain are permissive for T cell infiltration, whereas those containing the laminin $\alpha 5$ chains are restrictive or inhibitory (Fig. 8). In vitro studies demonstrated that this is probably due to high affinity integrin $\alpha 6\beta 1$ -mediated binding of encephalitogenic T cells to laminin 10, which has been shown in other cells not to be conducive to migration, whereas the labile, low affinity binding

to laminin 8 is more likely to support cell migration (Goodman et al., 1989; von der Mark et al., 1999; Pedraza et al., 2000). Whether this is the case with the encephalitogenic T cell lines is currently under investigation.

Integrin $\alpha 6\beta 1$ has been reported to be a specific receptor for laminin 1 on several cell types, including T cells (Shimizu et al., 1990; van de Wiel-van Kemenade et al., 1992), and in some cells also mediates binding to laminin 2 (Chang et al., 1995). However, despite constitutive surface expression of this integrin, the encephalitogenic T cell lines could not be induced to bind the parenchymal basement membrane laminins 1 and 2. Even maximal cell (Mn^{2+}) and integrin (Mn^{2+}) activation conditions were ineffective in inducing binding to either of these laminins, demonstrating that the presence of the $\alpha 6\beta 1$ receptor is insufficient for ligand binding. The data also suggest that T cell transmigration of the parenchymal basement membrane is fundamentally different from transmigration of the endothelial cell basement membrane. This has been suggested by the results of several other studies which have shown that additional activating agents or comigrating bystander cells are necessary for the final entry of T cells into the brain parenchyma. In macrophage-depleted (Tran et al., 1998) and $TNF-\alpha^{-/-}$ (Körner et al., 1997; Riminton et al., 1998) mice, infiltrating T cells cross the endothelium and its basement membrane, but remain in the perivascular space even during peak stages of EAE. Furthermore, GFAP knockout mice, which show a disrupted association of the astrocyte endfeet with blood vessels, exhibit an early spreading of the infiltrating cells into the CNS parenchyma during EAE (Liedtke et al., 1998). Our data suggest that the parenchymal barrier breaks down in lesions only at advanced stages of inflammation in a process which is likely to be dependent on the proteolytic activity of MMPs. MMPs have been extensively studied in EAE (Chandler et al., 1997; Madri and Graesser, 2000), demonstrating a significant role for MMPMT-1/MMP2 (Graesser et al., 1998, 2000) and MMP9 (Dubois et al., 1999; Lichtinghagen et al., 1999; Hartung and Kieseier, 2000) in T cell entry into and residency in the parenchyma of the CNS, as well as in demyelination. Although protease inhibitors have been shown to reduce the severity or delay the onset of EAE, to date no protease inhibitor has been shown to completely ablate T cell migration into the perivascular space or the brain parenchyma (Clements et al., 1997; Graesser et al., 2000). Until now it was not clear that emigrating T cells face more than just the subendothelial cell basement membrane. Therefore, the results of the MMP inhibitor experiments were interpreted as reduced transmigration across the endothelial cell basement membrane. However, they can equally well be explained by inhibition of transmigration across the parenchymal basement membrane. It now remains to be determined whether the main targets of MMP activity are components of the endothelial cell or the parenchymal basement membrane.

One of the major laminin receptors, integrin $\alpha 6\beta 1$, was localized predominantly on the endothelial cells, where it is likely to mediate interactions with the endothelial cell laminins 8 and 10, whereas astrocyte endfeet appear to utilize a different receptor for interactions with the parenchymal laminins. β -Dystroglycan occurred predominantly on astrocyte endfeet where no $\alpha 6$ integrin was detected, sug-

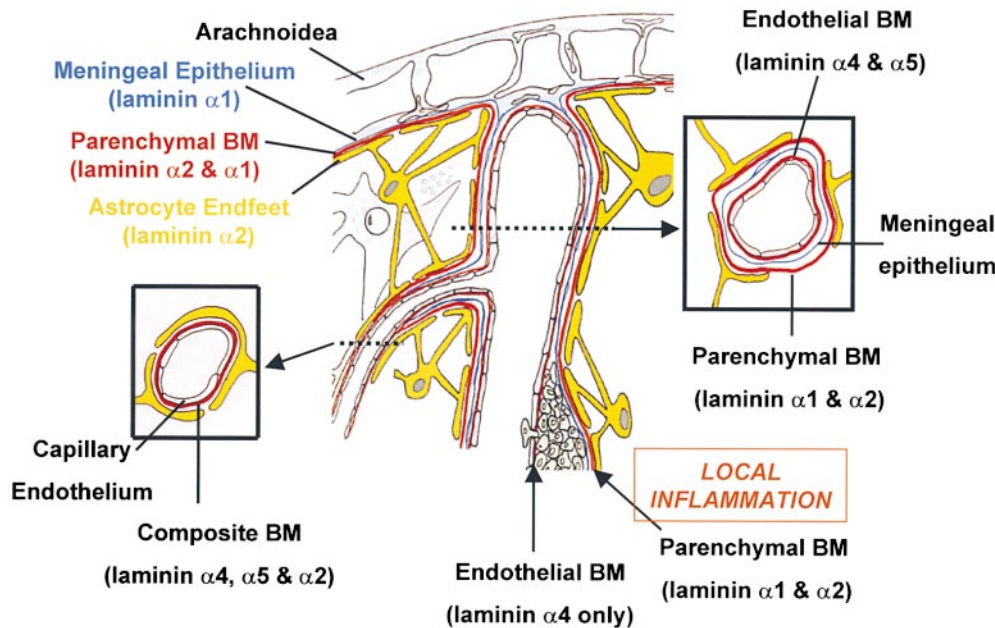


Figure 8. Illustration of cell layers, basement membranes (BM), and their laminin composition of CNS blood vessels with and without an inflammatory cuff. Larger blood vessels consist of an inner endothelial cell layer with a basement membrane (containing laminins $\alpha 4$ and $\alpha 5$), bordered by the meningeal epithelium and its basement membrane (containing laminin $\alpha 1$) and an outer astroglial basement membrane (containing laminin $\alpha 2$) and astrocyte endfeet. The meningeal and astroglial basement membranes are collectively termed the parenchymal basement membrane as they delineate the border to the brain parenchyma. Only at sites of local inflammation

are the endothelial and parenchymal basement membranes distinguishable and define the inner and outer limits of the perivascular space where leukocytes accumulate before infiltrating the brain parenchyma. Examination of such sites demonstrates that mononuclear infiltration occurs across endothelial basement membranes containing only the laminin $\alpha 4$ and bordered by a parenchymal basement membrane containing laminin $\alpha 1$ and $\alpha 2$. The basement membrane of microvessels where no epithelial meningeal contribution occurs appear to have a composite basement membrane containing the endothelial cell laminins, laminin $\alpha 4$ and $\alpha 5$, and laminin $\alpha 2$ produced by the astrocytes and deposited at their endfeet.

gesting that α -dystroglycan may be the laminin receptor on the astrocyte endfeet mediating binding to the parenchymal laminins 1 and 2. We have tested two α -dystroglycan antibodies, IIH6 and VIA4-1, both of which showed extremely weak staining of the CNS blood vessels (data not shown). Hence, it cannot be excluded from the data represented here that β -dystroglycan alone occurs on the astrocyte endfeet as has been shown in other tissues (Saito et al., 1999; Losasso et al., 2000). We and others have shown that during the course of EAE, integrin $\alpha 6 \beta 1$ is downregulated on the endothelial cells at sites of infiltration (Defilippi et al., 1992; Sobel et al., 1998). This, in combination with our observed high turnover of laminin $\alpha 4$ in endothelial cell basement membranes in the brain and the selective upregulation of laminin $\alpha 4$ expression by cytokines such as TNF- α , which have been shown to play a role in EAE, may lead to a loosening of the endothelial cell-basement membrane interaction, resulting in the reported “rounding up” of endothelial cells observed at sites of T cell infiltration in EAE (Wolburg et al., 1999) and further facilitating the infiltration process.

The clear identification of the endothelial cell and parenchymal basement membranes possible in the present study and characterization of their laminin isoform content clarifies confusion in the literature concerning laminin distribution in blood vessels in the CNS. Both laminin $\alpha 1$ and $\alpha 2$ have been reported to be expressed by brain blood vessels in noninflamed brains (Sanes et al., 1990; Jucker et al., 1996; Virtanen et al., 2000). However, in the absence of an inflammation, as shown here, the endothelial and parenchymal basement membranes lie in such close proximity that at the light microscopy level they may be mistaken

for a single structure, leading to the misconception that laminins $\alpha 1$ and $\alpha 2$ are present in endothelial cell basement membranes. Further discrepancies may also result from the fact that the laminin $\alpha 1$ monoclonal antibodies used in earlier studies were subsequently shown to recognize the laminin $\alpha 5$ chain (Virtanen et al., 1995; Jucker et al., 1996; Tiger et al., 1997).

The process of extravasation in the CNS is frequently described as passage through the blood-brain barrier (BBB; Noseworthy, 1999), a structure characterized by interendothelial cell tight junctions and induced and maintained by the close association between microvascular endothelial cells and astrocyte endfeet processes (Janzer and Raff, 1987; Wolburg and Risau, 1995). However, our data clearly showed that T cell transmigration never occurred at microvascular sites, but rather at postcapillary venules where endothelial cells were separated from the astrocyte endfeet by two basement membranes and at least one cell layer (leptomeningeal cells). Therefore, this site does not fulfill the above described characteristics of the BBB. The association of epithelial leptomeningeal cells with veins and postcapillary venules has not been reported previously, but may be related to the nature of the studies performed to date: ultrastructural studies on human tissues have shown that cerebral arteries and arterioles are ensheathed by leptomeningeal cells (Alcolado et al., 1988; Zhang et al., 1990), but such studies cannot easily distinguish between vessel types. Only through the use of laminin chain-specific antibodies and our knowledge of their cellular origins, in combination with examination of inflamed brain tissue, was it possible to specifically define the cell layers and basement membranes associated with

sites of cellular infiltration. It now remains to be determined how the leptomeningeal sheet appears at the ultrastructural level in postcapillary venules and veins in the mouse brain, and the nature of the BBB at such sites.

In conclusion, our data enforce the concept that T cell emigration of the endothelial cell basement membrane is distinct from transmigration of the parenchymal basement membrane. For the first time, two biochemically distinct basement membranes encountered by emigrating T cells in EAE have been defined, and not only laminin $\alpha 4$ in the endothelial cell basement membrane and integrin $\alpha 6$ on emigrating T cells, but also the endothelium of inflamed vessels, have been identified as key players in transmigration of the endothelial basement membrane.

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