Lycopersicon esculentum lectin: an effective and versatile endothelial marker of normal and tumoral blood vessels in the central nervous system

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The binding of *Lycopersicon esculentum* lectin (LEA) to the vascular endothelium was studied in the central nervous system of rat, mouse and guinea pig at different developmental ages, and in a gliosarcoma model. Our observations showed that LEA consistently stained the entire vascular tree in the spinal cord and in the brain of all animal species at all developmental ages investigated. In the tumor model, the staining of the vascular network was very reproducible, enabled an easy identification of vascular profiles and displayed a higher efficiency when compared to two other commonly used vascular marker (EHS laminin and PECAM-1). Moreover, our results showed that LEA staining was comparable in both vibratome and paraffin sections and could be easily combined with other markers in double labeling experiments.

These observations indicate that LEA staining may represent an effective and versatile endothelial marker for the study of the vasculature of the central nervous system in different animal species and experimental conditions.

An optimal visualization of the vascular network in the central nervous system is required to investigate the anatomical features of the blood supply in either normal or pathological conditions, and is essential for morphometrical studies. In recent years, the determination of the microvessel density in different organs has proven to be a valuable tool to provide information on their metabolic activity and on the rate of neovascularization either during embryonic development or in pathological conditions, such as in response to ischemia or trauma and in tumorigenesis. The identification of blood vessels is usually carried out by a variety of affinity cytochemistry techniques, whose results, however, are conditioned by numerous technical restrictions. Although different vessel wall antigens have been successfully used as markers in human nervous tissue samples, their utilization across species may be hampered by the lack of available cross-reacting antibodies. Moreover, the visualization of the vascular bed may be difficult in tumor models, since many antigens that mark vessels in physiological conditions are not consistently expressed in tumoral tissues (Vermeulen *et al.*, 1996); this shift in the antigenic properties reflects the influence of the microenvironment on the phenotype and function of the endothelial cells (Alessandri *et al.*, 1999; Arosarena *et al.*, 1994)

As a matter of fact, a reliable marker should a) allow a comprehensive identification of the vascular tree and be resistant to different conditions of tissue preparation (for instance, fixation and embedding), b) work in a range of species and experimental conditions, c) be compatible with other vascular and parenchymal markers.

In the present report we describe an easy and effective method to label normal and tumoral blood microvessels in the mouse, rat and guinea pig central nervous system that meets satisfactorily the above listed criteria.

Materials and Methods

Animals and tumor model

Experiments in healthy animals were performed on two rats (Sprague-Dawley) at embryonic day 18 (E18), two rats at postnatal day 1 (P1), five adult rats, five adult mice (C57 Bl) and eight adult Hartley guinea pigs (Charles River, Calco, LC, Italy).

The University of Milan and the Istituto Nazionale Neurologico approved care and handling of animals in accordance with the International guidelines on the animal research of the European Community (86/609). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Adult animals and P1 rats were anaesthetized with an intraperitoneal injection of chloral hydrate (4%, 1 mL/100 g body weight) and perfused transcardially with 1% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 (PB), followed by 4% paraformaldehyde in the same buffer. The E18 embryos, obtained from an anaesthetized pregnant rat, were further anaesthetized with ether and perfused transcardially with the fixative, using a glass capillary. Brains and spinal cords were removed, postfixed in 4% paraformaldehyde in PB at 4°C for 12 h and either cut with a Vibratome in 50-μm-thick sections or embedded in paraffin.

Four male Fisher 344 rats (Charles River) weighing about 180 g were used for intracerebral implantation of the 9L gliosarcoma cell line (ATCC). Deeply anaesthetized animals were immobilized in a stereotactic frame (Bianchi et al., 1992), the skin was incized over the bregma and a small hole was drilled into the skull approximately 1 mm anterior, 4 mm lateral to the bregma. Ten μl of 9L cell suspension (50,000 cells in PBS) were then injected at a depth of 4.5 mm.

At the animal death, the brain was extracted and fixed by immersion in paraformaldehyde-lysine-periodate (PLP, McLean and Nakane, 1974) in PB (4 h at 4°C), rinsed in PB for 12 h and embedded in paraffin.

Lectin histochemistry

Biotinylated lectin from Lycopersicon esculentum (bLEA, Sigma, St. Louis, MO), specific for N-acetyl-D-glucosamine and N-acetyl-polylactosamine oligomers (Kawashima et al., 1990; Porter et al., 1990) was used.

Vibratome sections permeabilized with mild ethanol treatment (10%-25%-10%, 10 min each) or paraffin-embedded dewaxed sections were treated sequentially with a) 1% H_2O_2 for 10 min to inactivate endogenous peroxidase, b) 0.01 M phosphate buffered saline (PBS) containing 1% bovine serum albumin (PBSB) for 30 min, and c) bLEA (10 μg/ml) diluted in PBSB for 2 hr.

Lectin binding was revealed with either a) standard ABC-peroxidase method (Hsu et al., 1981) using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine tetrahydrochloride (DAB) as a chromogen for light microscopy inspection, or b) streptavidin-Rhodamine RedX (Jackson Immunoresearch, West Grove, Pennsylvania) for fluorescence microscopy (in this case the preliminar treatment with H_2O_2 was omitted).

The specificity of lectin labeling was assessed by incubating control sections in either: a) PBSB in place of the lectin; b) bLEA preincubated for 30 min with 0.2 M N,N',N''-triacetyl-chitotriose (Sigma; Goldstein and Poretz, 1986).

Immunocytochemistry

Polyclonal rabbit antiserum directed against laminin purified from Engelbreth-Holm-Swarm murine tumor (EHS laminin, Sigma) and polyclonal goat antiserum to platelet-endothelial cell adhesion molecule-1 (PECAM-1, Santa Cruz Biotechnol., Santa Cruz, CA) were used respectively at 1:200 and 1:100 dilution. Free-floating vibratome sections or dewaxed sections were submitted to the same pretreatments listed for lectin histochemistry. After overnight incubation in the primary antibody at 4°C, antigen-antibody reaction was revealed using an appropriate biotinylated secondary antibody (Vector, 1:200 for 75 min) followed by the ABC method. Prior to immunoreaction with anti-laminin serum, sections were submitted to digestion with 0.4% hyaluronidase from bovine testis (Sigma) in PBS, 30 min at 37°C, for antigen unmasking (Folkvord et al., 1989).

For double labeling experiments, sections were sequentially incubated with bLEA and anti-laminin serum, followed by a mixture of Alexa 488-conjugated goat anti-rabbit IgG antibody and streptavidin-Rhodamine RedX (Jackson).

Single and double labeled sections prepared for fluorescence microscopy were examined under a TCS NT confocal laser scanning microscope (Leica Lasertecknik GmbH, Heidelberg, Germany), equipped with a 75-mW Kripton/Argon mixed gas laser, with a 10x objective lens (NA 0.3). Fluorochromes were
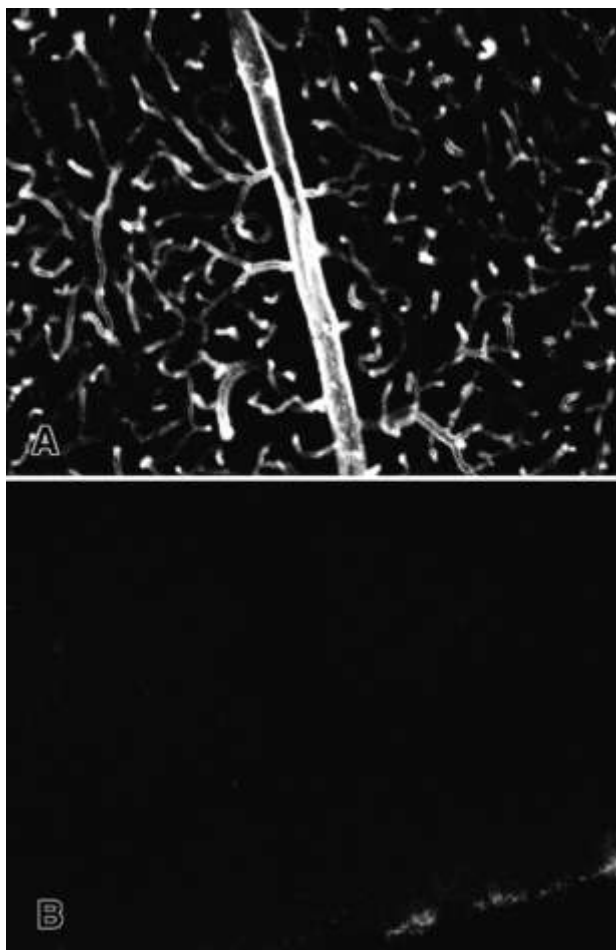
Figure 1. Analysis by confocal microscope of two adjacent vibratome sections of adult guinea pig cerebral cortex labeled respectively with biotinylated LEA (A) and with biotinylated LEA preabsorbed with the inhibiting hapten sugar moiety (B). ×100
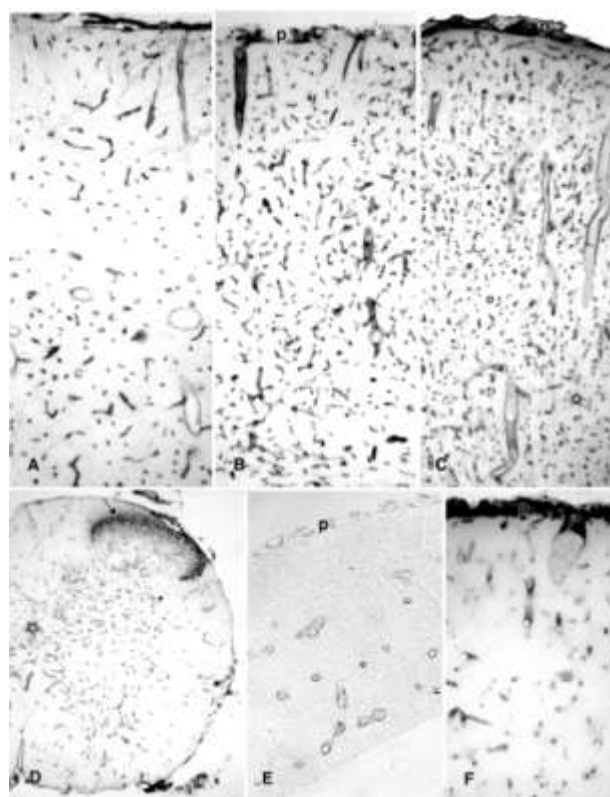

Figure 2. Light microscopy of coronal sections of adult mouse (A), rat (B) and guinea pig (C) parietal cerebral cortex, adult mouse spinal cord (D), rat cerebral cortex at E18 (E) and P1 (F). Binding of biotinylated LEA to the microvasculature was revealed with ABC/DAB method. B-C, F vibratome sections; A, D, E, paraffin sections. The arrow in D points to lamina II of the dorsal horn; p, pia. A, B ×100; C, F ×80; D, ×70; E, ×225.

excited respectively at 488 and 568 nm, visualized with 530/30 nm band-pass and 590 nm long-pass filters, imaged separately and merged with Leica Power Scan software. Images, acquired with an aperture size of 102 μm corresponding to an optical section of approximately 15 μm in thickness, derived from the average of eight frames, were collected digitally as a matrix of 1024 x 1024 pixels and transferred to a graphics program (Adobe Photoshop 6.0). Control experiments showed that the levels of interchannel cross-talk were insignificant.

Results

The pattern of labeling obtained with bLEA was highly reproducible in all animal species, developmental ages and nervous system regions investigated. Moreover the distribution and the intensity of the staining were comparable in vibratome and

paraffin sections.

The specificity of LEA staining was demonstrated by the lack of labeling in control sections incubated in either a) PBSB in place of the lectin or b) bLEA preabsorbed with the appropriate hapten sugar moiety (Figure 1B).

In adult animals, LEA binding (Figures 1A, 2A-D) was localized exclusively to blood vessels in all regions of the central nervous system examined, except for the spinal cord where it specifically labeled also primary afferent fibers in laminae II and III of the dorsal horn (Figure 2D). LEA labeling was continuous along the wall of the vessels which presented a tube-like appearence (Figure 1A, 2, 3A). The examination of sections double-labeled for bLEA and EHS laminin showed that the entire vascular tree was stained in all samples examined, without significant differences in labeling intensity between vessels of different caliber (Figure 3).
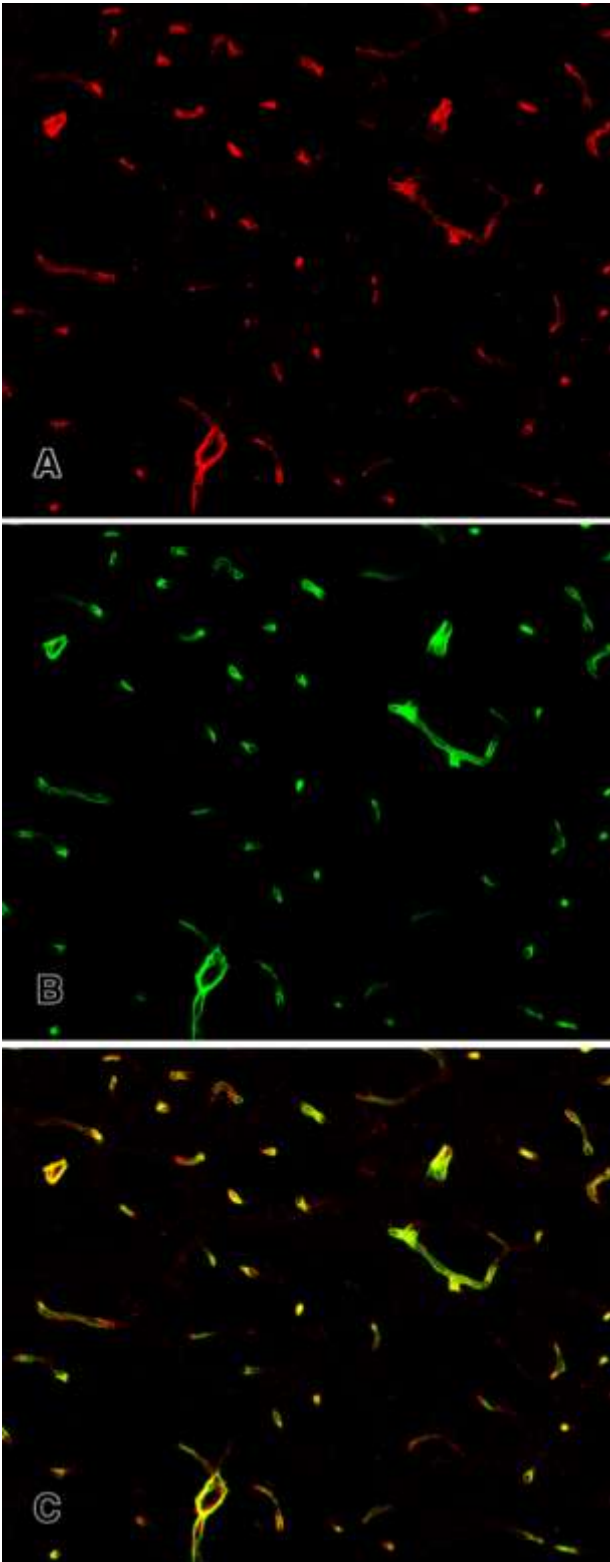
Figure 3. Analysis by confocal microscope of a paraffin section of adult guinea pig thalamus double labeled with biotinylated LEA (A) and serum anti-EHS laminin (B). C: merged images. ×180.

Moreover, at least in the rat, LEA appeared to label consistently the vascular cerebral network both at prenatal (E18) and perinatal developmental stages (Figures 2E,F).

In the tumor model, as in the surrounding normal brain parenchyma, the staining of the vascular network was very reproducible and consistent, with minimal background that enabled an easy identification of vascular profiles (Figures 4A,B).

LEA labeling efficiency of tumoral vessels appeared very high, especially when compared to that of two other frequently used vessel markers. Laminin immunoreactivity was intense in the vessels of the normal cerebral parenchyma (Figure 4D), whereas labeling of the tumoral vessels was very weak and incomplete (Figure 4C). The same irregular staining was observed when tumor sections were immunostained with antibodies to PECAM-1 (not shown) that in addition produced a high background.

Discussion

Most lectins used as vascular markers in different organs and animal species selectively bind to glycoconjugates concentrated in the glycocalyx and in the basal membrane. Endothelial glycocalyx is a complex organization of integral and peripheral glycoproteins, glycolipids and proteoglycans bearing a variety of saccharide residues. These molecules are integrated in a complex filamentous meshwork that subserves multiple functions such as the regulation of microvessel permeability, blood flow, coagulation, fibrinolysis, angiogenesis and the interaction with plasma proteins and blood cells. The carbohydrate residues of the glycocalyx are exposed to vessel luminal surfaces and therefore are easily available to markers, thus making lectin labeling an effective and relatively simple tool to either image the morphology of the vascular tree or investigate the pattern of distribution of sugar residues in physiological and pathological conditions. The success of lectin staining depends on the selection of the appropriate markers, since the pattern of distribution of endothelial sugar moieties is developmentally (Pihush *et al.*, 1994; Quondamatteo *et al.*, 1997; Nico *et al.*, 1998) and spatially regulated (Bankston *et al.*, 1991) and it is, at least to a certain extent, species-specific (Russel and Dalion, 1988). For example, *Ulex europaeus* agglutinin, a lectin specific for non-reducing terminal alfa-fucosyl residues (Hindgaul *et al.*, 1982; Pereira *et al.*, 1978), is an efficient endothelial marker only in human tissues (Holthöfer *et al.*, 1982). Conversely, optimal labeling of dog and chick
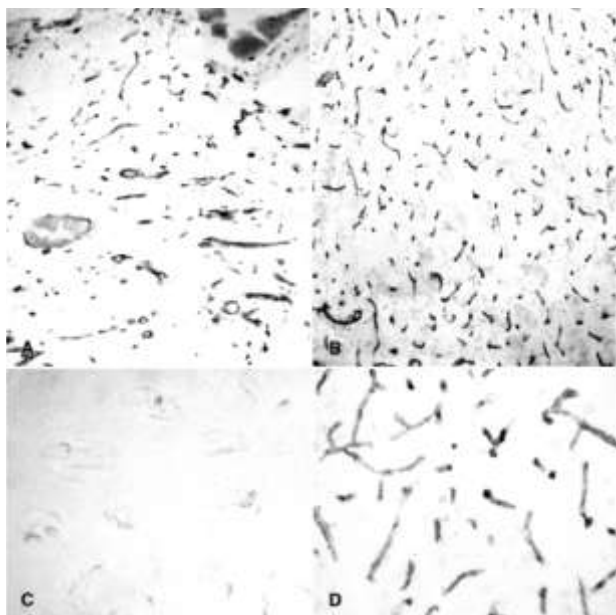
Figure 4. A-D Light microscopy of paraffin sections of 9L gliosarcoma labelled with biotinylated LEA (A, B) and with antiserum to EHS laminin (C, D). ABC/DAB method. Microvasculature labeling is shown in 9L gliosarcoma (A, C) and in the surrounding normal brain tissue (B, D). A, B ×100; C, D ×260.

endothelium has been obtained with *Arachis hypogaea* (Kiatipattanasakul *et al.*, 1998) and *Lens culinaria* lectins (Jilani *et al.*, 2003), respectively.

Lectin histochemistry has been frequently used to visualize the morphology of vascular networks in a variety of organs, either on tissue sections (Porter *et al.*, 1990; Hansen-Smith *et al.*, 1988; Nanka *et al.*, 2001; Beltrão *et al.*, 2003; Jilani *et al.*, 2003; Cebasek *et al.*, 2004) or using intravascular perfusion (Thurston *et al.*, 1998, 1999; Murphy *et al.*, 1999; Debbage *et al.*, 2001; Jilani *et al.*, 2003). However, lectins have been only rarely used to delineate the vascular tree in the central nervous system (Porter *et al.*, 1990), although their affinity to brain endothelium has been extensively characterized (Nag 1985a, b; Vorbrodt, 1986; Vorbrodt *et al.*, 1986; Alroy *et al.*, 1987; Fatehi *et al.*, 1987; Porter *et al.*, 1990; Debbage *et al.*, 1996; Gerhart *et al.*, 1998). In the present paper, we extend previous observations (Porter *et al.*, 1990) showing that LEA staining allows the comprehensive visualization of the vascular network of brain and spinal cord in three mammalian species widely used as models in the studies of the central nervous system. This lectin is specific for (GlcNAc)n (n >3) and poly-N-acetyllactosamine residues (Nachbar *et al.*, 1980; Zeng *et al.*, 1998) and it has been previously shown to label the endothelium in several non nervous organs of different mammal

species (Porter *et al.*, 1990; Bankston *et al.*, 1991; Augustin *et al.* 1995; Thurston *et al.* 1996, 1998; Murphy *et al.*, 1999; Debbage *et al.*, 2001). The presence of binding sites for LEA in the endothelia of all the nervous organs, animal species and developmental stages examined in the present study likely reflects the important recognition functions played by polylactosaminoglycans in cell membranes (Feizi, 1985). Our findings are in line with previous observations (Debbage, 1996) showing that the oligosaccharide sets that characterize the glycosylation pattern of receptors and adhesion molecules and include polylactosamines are highly conserved in brain endothelia.

An optimal visualization of the vascular bed is particularly important to characterize the processes of angiogenesis and vascular remodeling taking place in pathological conditions, like tumors or inflammatory diseases. However, the identification of newly formed blood vessels, especially in tumors, is frequently hampered by the altered expression of the vessel wall antigens commonly used as markers (Vermeulen *et al.*, 1996). In the present investigation, we have compared, using a gliosarcoma model, the staining efficiency of LEA with that of two antibodies recognizing respectively the endothelial adhesion molecule PECAM-1 and laminin, a glycoprotein of basal membranes. Our findings showed that only LEA stained consistently all tumoral vessels and indicate that, at variance with sialylated oligosaccharides (Debbage *et al.*, 1994), the binding sites for this lectin are not lost in tumoral tissue.

LEA staining offers a few distinct advantages over other vascular markers: a) it produces only minimal parenchymal staining, thus allowing an easier identification of the vascular profiles, which is an essential requirement to perform morphometric analysis of the vasculature; b) its binding properties are preserved in paraffin embedded nervous tissue, enabling the analysis of archivial samples; c) it can be combined with other labeling techniques.

In conclusion, the complex of our observations indicate that LEA may represent an effective and versatile endothelial marker for the study of the vasculature of the central nervous system in different animal species and experimental conditions.

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