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Ontogeny of four blood–brain barrier markers: an immunocytochemical comparison of pial and cerebral cortical microvessels

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ABSTRACT

Pial and cortical microvessels possess many blood–brain barrier (BBB) properties in common, including impermeability to electron dense tracers, high transendothelial electrical resistance and specialised endothelial cell ultrastructural features. To compare pial and cortical microvessels further, a developmental, immunocytochemical study was undertaken of 4 BBB markers in the rat: OX-47, EBA, GLUT-1 and s-laminin. The appearance of the markers was monitored from embryonic d 16, to postnatal and adult stages. Each of the 4 markers appeared simultaneously in both pial and cortical vessels. GLUT-1 and OX-47 were present in endothelial cells of the BBB from *E* 16 to the adult. EBA and s-laminin appeared from postnatal d 7 through to the adult. Pial microvessels lack the ensheathment of astrocytes which may be involved in the induction and/or maintenance of BBB markers in the cortex. It is possible that astrocyte-derived factors diffusing from the brain surface are responsible for induction of BBB properties in the pial microvessels.

Key words: Vasculature; endothelium; endothelial cell markers.

INTRODUCTION

Pial microvessels share many of the blood–brain barrier (BBB) properties possessed by capillaries of the cerebral cortex and most other intracerebral regions. For example the transendothelial electrical resistance of pial microvessels is high, a definitive characteristic of BBB microvessels. Average values of 1900 Ω cm^{-2} and 1500 Ω cm^{-2} have been measured in the frog (Crone & Olesen, 1982) and the rat (Butt & Jones, 1992) respectively. Also structural features of pial and cerebral microvessels in the frog have been described as being similar (Bundgaard, 1982). Interendothelial cell junctions were similar both qualitatively and quantitatively in both types of microvessel and the organelle content, in particular the characteristic paucity of plasmalemmal caveolae, was indistinguishable. The permeability of their interendothelial cell junctions to a range of electron dense tracers was also similar. The tracers ferritin, horseradish peroxidase, microperoxidase and ionic lanthanum (ranging in size from MW 900 kDa to ionic lanthanum) all failed to cross the belt-like tight junctions which encircle each cortical and pial endothelial cell (Reese & Karnovsky, 1967; Brightman & Reese, 1969; Westergaard & Brightman, 1973; Westergaard, 1980; Bundgaard, 1982; Butt et al. 1990).

Pial and cerebral microvessels differ in that pial vessels lack the perivascular ensheathment of astrocytes which is found in the brain. There is substantial evidence that astrocytes play a major role both in the induction and maintenance of the BBB characteristics of endothelial cells in the central nervous system (Risau, 1991; Bradbury, 1993; Cancilla et al. 1993). The presence of BBB characteristics in pial microvessels may therefore suggest that diffusible, astrocyte-derived molecules are at least part of the inductive mechanism and that glial–endothelial cell contact is not a prerequisite.

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In order to extend the comparison of pial and cerebral microvessels a developmental, immunocytochemical study was undertaken of 4 BBB markers: OX-47, EBA, GLUT-1 and s-laminin. OX-47, the rat homologue of the chick HT7 antigen (74 kDa), is an integral plasma membrane glycoprotein of unknown function present in BBB endothelia (Risau et al. 1986; Seulberger & Risau, 1993). Endothelial barrier antigen (EBA) is a protein triplet (23.5, 25 and 30 kDa) expressed by rat endothelial cell plasma membranes at the BBB (Sternberger & Sternberger, 1987). It is absent from fenestrated brain microvessels and brain endothelial cells where the BBB has broken down (Sternberger et al. 1989; Rosenstein et al. 1992) though its function is unknown (Sternberger & Sternberger, 1987). GLUT-1 is an isoform of the glucose transporter (52 kDa) which in the brain is expressed selectively by microvascular endothelial cell plasma membranes (Pardridge & Boado, 1993). S-laminin (180 kDa), a homologue of the B1 subunit of laminin, is a component of basal laminae of brain microvessels (Hunter et al. 1992).

MATERIALS AND METHODS

Animals and tissue preparation

Four Wistar rats at each of embryonic (E) day 16 and postnatal (d) days 1, 7, 15, 16, 17, 18, 21, 27 and adult (> d 30) were anaesthetised with Sagatal (1 ml/kg body weight) and either killed immediately by cervical dislocation or perfused transcardially with freshly prepared 4% paraformaldehyde-0.1% glutaraldehyde in phosphate buffer (pH 7.2, 0.1 M, room temperature) for 10 min. For fixed tissue the brain was removed and immersed in the same fixative for 2 h at 4 °C. Slices (5 mm) of brain were processed into paraffin wax using cedarwood oil. Sections (7 μm) were collected on glass slides and hydrated to water. Alternatively, unfixed brains were frozen rapidly in OCT mounting medium in isopentane cooled over liquid nitrogen or in a solid carbon dioxide and 90% ethanol slush. Cryostat sections (7 μm) were cut and collected on gelatin-coated slides.

Antibodies and immunolabelling

The following mouse monoclonal antibodies (mAbs) were obtained: (1) mAbs to s-laminin (C 4, D 7 and D 5) and an mAb against the B2 subunit (D 18) from the Developmental Studies Hybridoma Bank, Baltimore, USA; (2) mAb to EBA from Affiniti Research Products Ltd, Nottingham, UK; (3) mAb to GLUT-1 from Biodesign International, Kennebunk, USA; (4) the mAb MRC OX-47 from Serotec Ltd, Oxford, UK. Antibodies were used at a dilution of 1 in 50, except EBA which was used at dilutions ranging from 1:50 to 1:10000.

Horseradish peroxidase immunolabelling

Endogenous peroxidase was blocked by incubating the cryostat sections with 3% hydrogen peroxide in 90% methanol for 5 min. Dewaxed paraffin sections were incubated with 3% hydrogen peroxide in water for 5 min. The tissue was then incubated with the diluted primary antibody for 30 min. Following washing with phosphate-buffered saline (PBS) for 5 min, the tissue was incubated in biotinylated goat antimouse antibody overnight at 4 °C. After washing in PBS the tissue was then incubated in StreptABComplex (Dako Ltd) for 30 min. A further wash in the PBS was followed by incubation in the metal enhanced 3,3′-diaminobenzidine (DAB) solution (Piece and Warriner). The reaction was followed microscopically and stopped by washing the slides in distilled water with counter staining in haematoxylin before conventional mounting for light microscopy. As a negative control the primary antibody step was omitted. As a positive control adult brain was used for all antibodies employed.

RESULTS

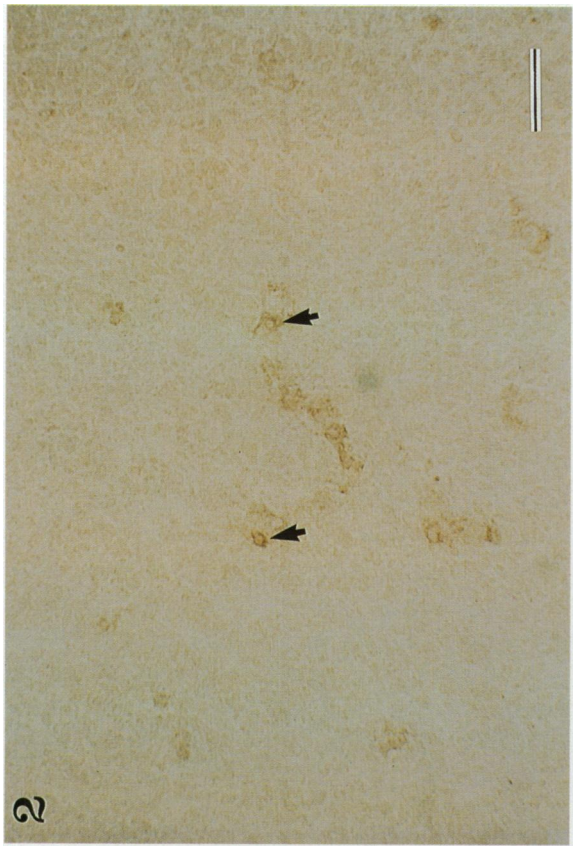
Each of the 4 BBB markers studied appeared at the same developmental stage in both pial and cortical vessels (see Table). No differences were evident subjectively between the 2 vessel types of each of the markers OX-47, GLUT-1 and s-laminin. Only EBA showed any differences between pial and cortical vessels. The 2 vessel types were therefore indistinguishable in their temporal sequence and, with one

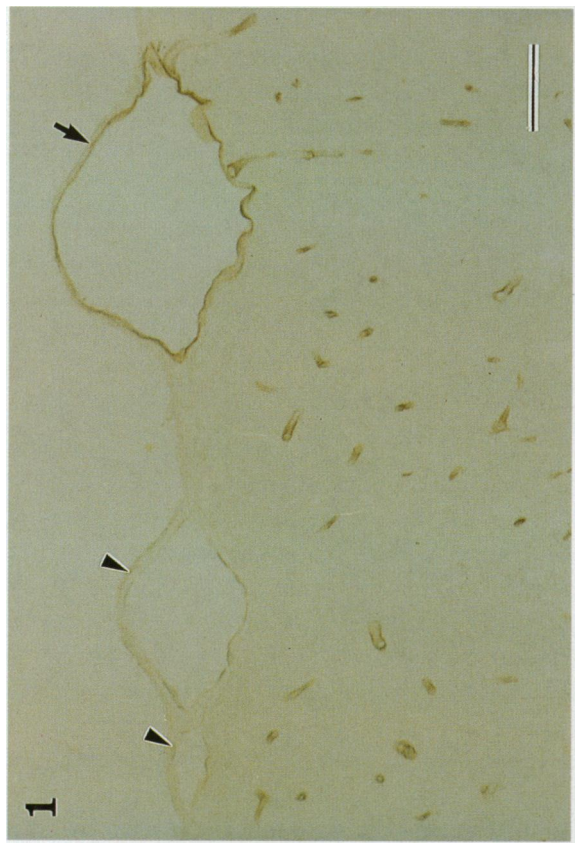
Fig. 1. EBA peroxidase immunolabelling in adult rat brain. The pia mater displays heterogeneity in EBA expression with both positive (arrow) and negative (arrowheads) vessels. In contrast cerebral cortical vessels are all strongly labelled. Bar, 3 μm.

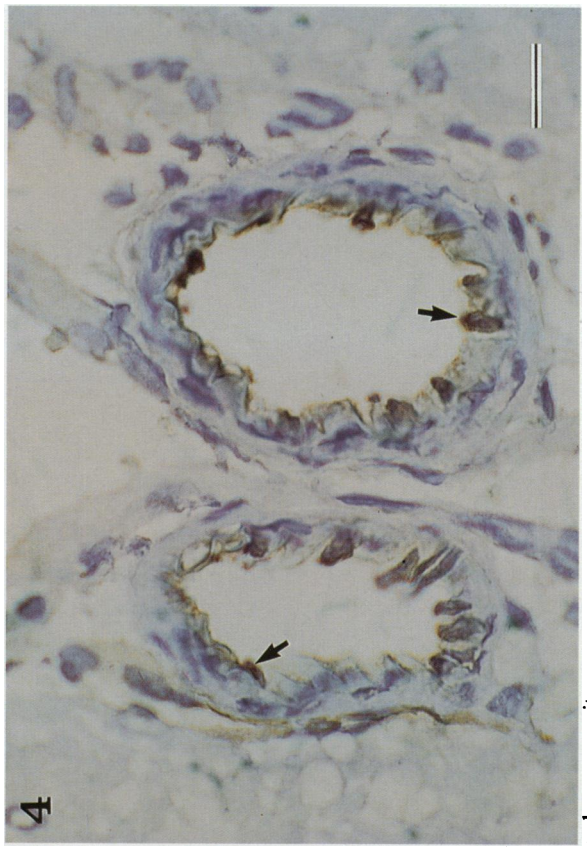
Fig. 2. OX-47 weak peroxidase immunolabelling of cerebral cortical microvessels (arrows) in E 16 rat brain. Bar, 1 μm.

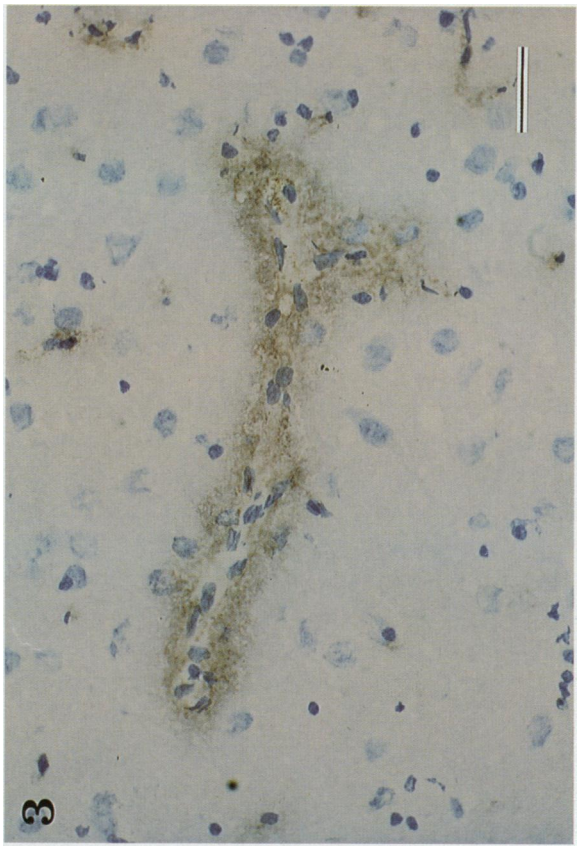
Fig. 3. OX-47 peroxidase immunolabelling of microvessels in adult rat cerebral cortex. Bar, 1 μm.

Fig. 4. OX-47 peroxidase immunolabelling of pial arterioles in adult rat brain. Labelling is restricted to endothelial cells (arrows). Bar, 1 μm.









Figs 1 to 4. For legends see opposite.

Table. *Immunoperoxidase labelling of pial and cortical vessels*

				Antilaminin subunit antibodies			
	EBA	OX-47	GLUT-1	B2 (D18)	S-ln (D7)	S-ln (D5)	S-ln (C4)
Adult	+	+	+	+	+	+	+
d 27	+	+	+	+	+	+	+
d 21	+	+	+	+	+	+	+
d 17	(+)	+	+	+	+	+	+
d 16	(+)	+	+	+	+	+	+
d 15	(+)	+	+	+	+	+	+
d 7	−	+	+	+	(+)	(+)	(+)
d 1	−	+	+	−	−	−	−
E 16	−	(+)	+	−	−	−	−

+ Strongly positive and (+) weak labelling of sections.

exception, level of expression of these particular antigens. All negative control tissue (omission of the primary antibody) was unlabelled. Specific labelling data are given below for each antibody. EBA and GLUT-1 are fixative insensitive so paraffin and cryostat sections were employed. OX-47 and s-laminin are fixation sensitive, therefore only cryostat sections were used.

EBA

EBA labelling was first detected in pial and cortical microvessels at 15 d postpartum (Table). At this stage only a minority of vessels were immunopositive and higher antibody concentrations (1:100) were required for detection compared with adult rats (1:500–1:10000). The number of immunopositive vessels gradually increased from postnatal d 15 to the adult at which point all cerebral cortical endothelial cells showed a strong, uniformly distributed reaction product (Fig. 1). In contrast, adult pial microvessels showed a heterogeneity in EBA expression as previously described (Cassella et al. 1996): vessels could be classified as showing complete, partial or no labelling. In the case of partially labelled vessels the strongest labelling was consistently on the side of the vessel that was closest to the glia limitans and the cut-off point for labelling appeared to be at the level of the paracellular cleft. Only endothelial cells of the cortical and pial vessels were labelled, perivascular cells appeared not to be labelled.

OX-47

Weak labelling of a minority population of pial and cortical vessels was observed at E 16 (Fig. 2; Table). Postnatally there was strong expression in the majority of vessels (Figs 3, 4) although the nature of the labelling of unfixed frozen sections generally precluded precise localisation. However, in pial arterioles (Fig. 4) it was possible to confirm that the labelling was confined to the endothelial cells with only minimal staining of the immediate perivascular space.

GLUT-1

GLUT-1 was strongly expressed from E 16 (Fig. 5) to the adult (Fig. 6) by all pial and cortical vessels (Table). Vessels were uniformly labelled and in thin paraffin sections the reaction product could be precisely localised to endothelial cells. However, it was not possible to differentiate between luminal and abluminal staining. Occasional immunopositive erythrocytes were observed within the vessel lumina.

S-laminin

The 3 antibodies against s-laminin (C 4, D 7 and D 5) gave essentially the same pattern of labelling during development. S-laminin was first detected in the walls of cortical and pial microvessels at d 7 (Table). At this stage the D 18 antibody (directed against the laminin B2 chain) showed a more intense labelling (Table). At later stages of development there was no apparent difference between antibodies. From postnatal d 15 onwards s-laminin expression resembled the pattern in the adult. It was difficult to determine if the labelling was of the endothelial cells or basement membrane in smaller microvessels (Fig. 7). The distribution of the reaction product was most clearly

seen in pial arterioles (Fig. 8), labelling the abluminal front surrounding the perivascular smooth muscle. Vascular smooth muscle was unlabelled.

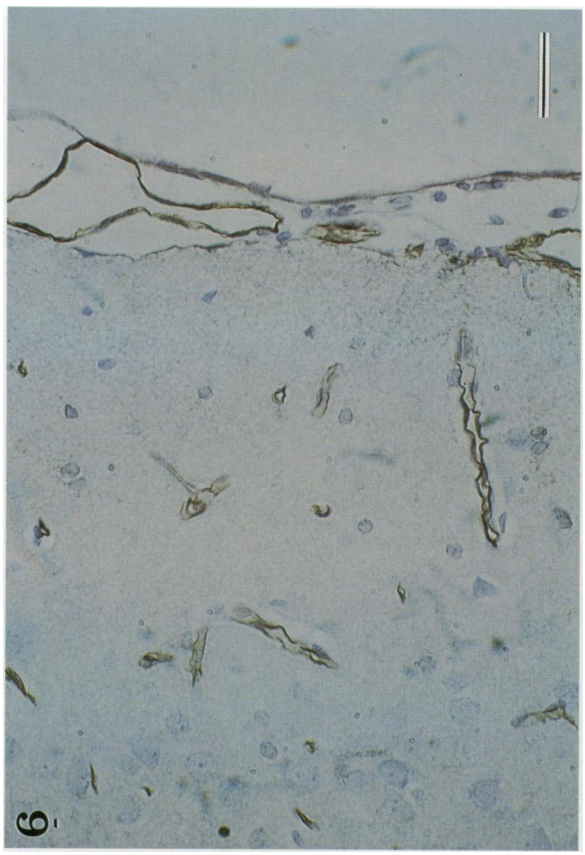
DISCUSSION

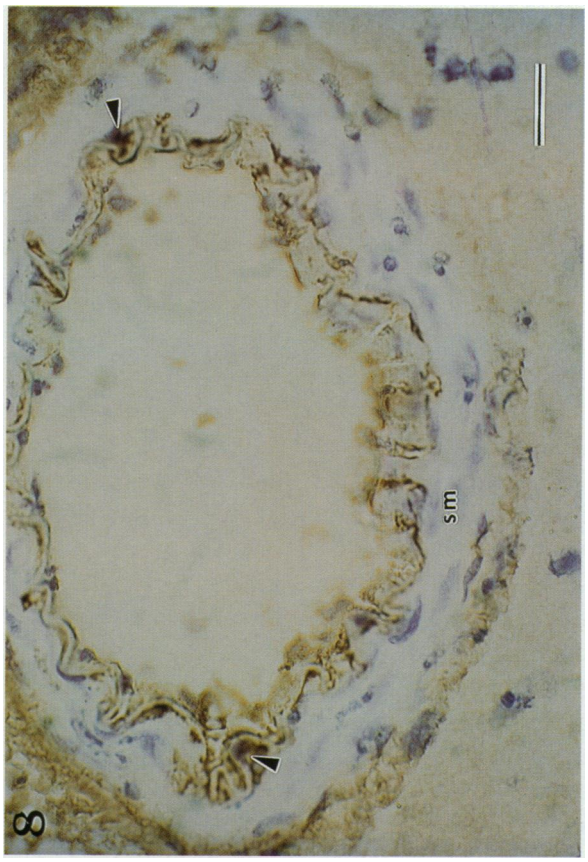
In this developmental study each of the 4 BBB markers, OX-47, EBA, GLUT-1 and s-laminin, appeared simultaneously in cortical and pial microvessels. While an antigen may first appear during embryonic life (e.g. GLUT-1) or postnatal life (e.g. EBA), the onset was the same in both microvessel types. Though the developmental appearance of EBA, GLUT-1 and s-laminin at the BBB have been described previously, there appears to be no previous comparative study of cortical and pial microvessels for endothelial cell antigen markers.

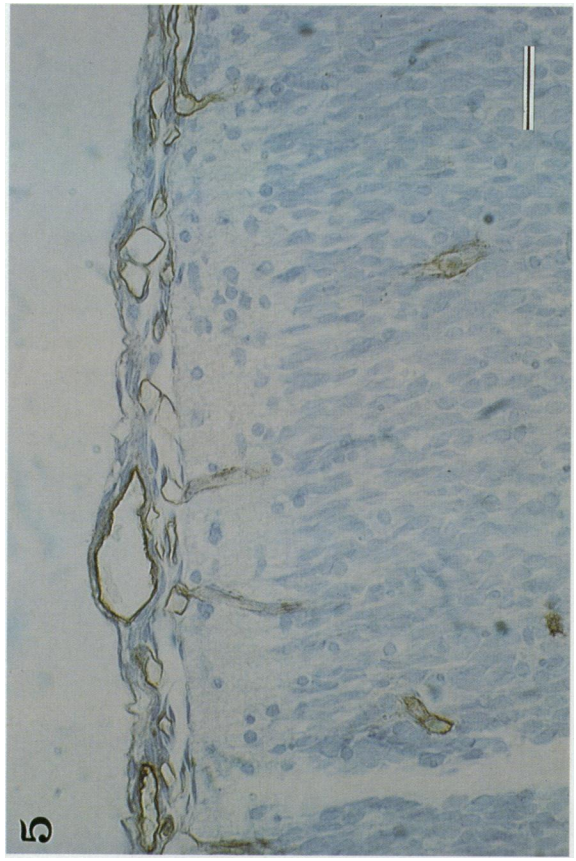
The experimental evidence, though controversial (Harik et al. 1993), indicates that at the earliest embryonic stages examined the rat BBB is impermeable to horseradish peroxidase and possesses zonulae occludentes (Møllgård & Saunders, 1986; Stewart & Hayakawa, 1994). It is also impermeable to ionic lanthanum (E17) and shows moderately high electrical resistance (mean of 310 Ω cm^{-2}) (Butt et al. 1990). The number of fenestrae in rat brain endothelial cells declines rapidly from E11 so that they are absent by E15–17 (Yoshida et al. 1988; Stewart & Hayakawa, (1994). Since transcellular transport becomes necessary when brain endothelial cells form epithelium-like zonulae occludentes, the very early (E 16) appearance of the transporter protein GLUT-1 at the rat BBB was not unexpected and is supported by earlier observations (Harik et al. 1993). In the mouse GLUT-1 has been demonstrated in brain endothelial cells at E 12 together with a permeability barrier to horseradish peroxidase (Bauer et al. 1995). The glucose requirements of the brain are very high since glucose supports virtually all cerebral functions and GLUT-1 is responsible for at least 90% of glucose transport across the BBB (Pardridge & Boado, 1993). Hence brain endothelial cells acquire early a saturable, stereospecific glucose transport system since the paracellular diffusion route becomes eliminated. By contrast capillaries of circumventricular organs which lack BBB properties do not express GLUT-1 (Rahner-Welsch et al. 1995). The GLUT-1 isoform acquired by endothelial cells at the BBB is identical to the glucose transporter type 1 (GLUT-1) of human red blood cells (Laterra & Goldstein, 1993). A differential distribution of GLUT-1 between luminal and abluminal brain endothelial cell surfaces was not apparent from the techniques used in the present study but was inferred from an immunogold study where 45% of endothelial cell anti-GLUT-1 labelling was present on the abluminal membrane and 11% on the luminal membrane (Pardridge & Boado, 1993).

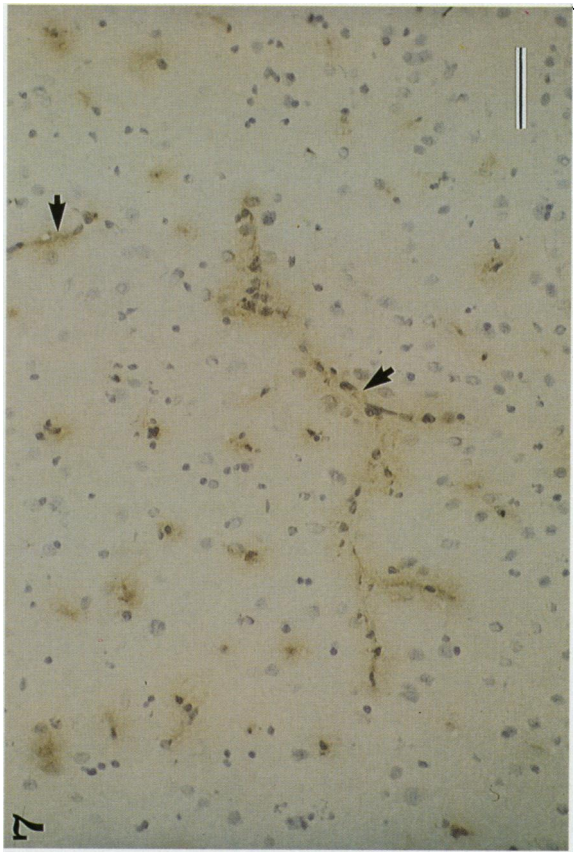
The appearance of EBA during the early postnatal period (Sternberger & Sternberger, 1987; and this study) confirms that molecular maturation of brain endothelial cells continues after birth in the rat. This is in accordance with other data. For example the interendothelial cell clefts and zonulae occludentes of rat brain undergo maturation during fetal and postnatal life (Schulze & Firth, 1992). Similarly, the transendothelial electrical resistance of rat pial vessels is still increasing postnatally (Butt et al. 1990). The implication is that the underlying molecular mechanisms involved are continuing to change. This morphological and electrophysiological evidence supports the view that the maturation of the BBB represents a continuum rather than a milestone event (Harik et al. 1993). The antigen EBA was named *endothelial barrier antigen* because it was associated with those CNS endothelia that have a selective permeability barrier property (Sternberger & Sternberger, 1987). However, since the function of EBA is unknown (Sternberger & Sternberger, 1987; Lawrenson et al. 1995*b*), the appearance of the antigen postnatally does not yet necessarily confirm that the barrier property itself of the endothelial cells is changing. However, in experimental allergic encephalomyelitis (EAE) rat microvessels in lesion areas lose their anti-EBA reactivity (Sternberger et al. 1989). In stab wound injury of the brain, microvessels which are involved similarly become EBA-negative concomitant with losing their BBB properties (Rosenstein et al. 1992). Microvessels in transplants of fetal cortex into adult brain lack EBA expression at 2 wk but are immunopositive at 4 wk (Rosenstein et al. 1992). Conversely, in peripheral nerve endoneurial microvessels, which show barrier properties but lack perivascular astrocytes, EBA is only weakly present or absent (Lawrenson et al. 1995*b*). Furthermore EBA has been reported in nonbarrier ocular microvessels in the choroid and ciliary body as well as in microvessels, such as those in the retina, which possess barrier properties (Lawrenson et al. 1995*a*). The functional significance of the distribution of EBA therefore requires clarification, including its appearance in the ontogeny of brain endothelial cells.

Like EBA, s-laminin is a late BBB marker. No immunoreactivity was present at 1 d post partum, only weakly present at d 7 and not until d 15 were adult levels of intensity detected. S-laminin is a









Figs 5 to 8. For legends see opposite.

homologue of the B1 subunit of laminin and is restricted to a subset of extracellular matrices including the basal laminae of microvessels in the brain (Hunter et al. 1992). The expression of s-laminin is apparently also controlled independently, though in coordination with other laminin constituents (Hunter et al. 1992). Several extracellular matrix proteins contain active sites that mediate intercellular interactions. S-laminin fragments are selectively adhesive for neurons and inhibit neurite outgrowth presumably via neuronal cell surface receptors (Hunter et al. 1989; Porter et al. 1995). Any analogous influences of s-laminin on endothelial cells and pericytes at the BBB have not yet been investigated. Hunter et al. (1992) also described s-laminin as appearing during the first 2 postnatal weeks and as being coextensive with laminin in adult brain capillaries. While s-laminin is a late marker in cortical capillaries this does not reflect the general situation for CNS s-laminin, since the molecule is present in basal laminae of the pia and choroid plexus from as early as E 13 and transiently in the neuropil of the cortical subplate at E 15 (Hunter et al. 1992).

The embryonic appearance of OX-47 has apparently not been examined previously and was detected at E 16 in the rat embryo. OX-47 is homologous to the chick protein HT7 which has been much more extensively investigated. The amino acid sequence identity between OX-47 and HT7 is 94%. HT7 is a very early BBB marker even when compared with the early embryonic brain endothelial cell markers, alkaline phosphatase and transferrin receptor which appear slightly later (Seulberger & Risau, 1993). It is first detectable at E 9–10 in chick development, and becomes more extensive from E 11 onwards (Risau et al. 1986). It occurs on the luminal and abluminal plasma membranes of endothelial cells with BBB properties but not in endothelial cells of brain circumventricular organs which lack a BBB (Albrecht et al. 1990; Seulberger & Risau, 1993). In the chick the first CNS vessels to appear, as early as E 4, are permeable to horseradish peroxidase, with barrier properties to the protein tracer developing around E 13 (Wakai & Hirokawa, 1978). Risau and colleagues (Risau et al. 1986; Seulberger & Risau, 1993) have found that chick brain endothelial cells start to express a number of proteins at around this time indicating a role, as yet unknown, in BBB function. HT7, occurring in erythroblasts as well as brain endothelial cells, is a member of the Ig superfamily some of whose functions are known. Also HT7 is found in most epithelial cells, including the epithelium of the choroid plexus (blood-CSF barrier) and pigmented epithelium of the retina (blood-retinal barrier), indicating a role in transport processes or cell adhesion (Seulberger & Risau, 1993). OX-47 antigen, like HT7, is located on endothelial cells of the BBB and on most epithelial cells (Seulberger & Risau, 1993) and in addition is present at low levels on lymphocytes, its expression being greatly increased on their activation and transformation into lymphoblasts (Fossum et al. 1991).

The evidence is now overwhelming that astrocytes represent an essential prerequisite for the induction and maintenance of the BBB. Consequently, a number of enzymes and antigens characteristic of brain endothelial cells have been assessed for their induction and maintenance by astrocytes (Abbott et al. 1992; Cancilla et al. 1993). The enzyme γ-glutamyl transpeptidase (GGTP) is present in brain endothelial cells when they are in contact with astrocytes in vivo and in vitro. Its down-regulation on removal of astrocytes can be reversed with astrocyte-conditioned medium. The neutral amino acid transport A system (alanine) is modified by the presence of astrocytes. Alkaline phosphatase, expressed on both luminal and abluminal plasma membranes, can be induced in endothelial cells by astrocytes or astrocyte-conditioned medium. Na^+-K^+-ATPase, occurring mainly on the abluminal front, is upregulated by astrocytoma cells but not by astrocyte-conditioned medium. Of the BBB markers used in the present study, GLUT-1 is expressed in coculture of fetal brain endothelial cells and astrocytes (Hurwitz et al. 1993) and glucose uptake can be increased by up to 50% by astrocyte-conditioned medium, perhaps by inducing the synthesis of additional glucose carriers (Maxwell et al. 1989; Cancilla et al. 1993). However GLUT-1 cannot apparently be reinduced in brain endothelial cells in culture by astrocytes or astrocyte-conditioned medium (Risau, 1991). Thus while CNS factors appear not to have a role in inducing the high expression of

Fig. 5. GLUT-1 strong peroxidase immunolabelling of pial and cerebral cortical microvessels in E 16 rat brain. Bar, 1.7 μm.

Fig. 6. GLUT-1 strong peroxidase immunolabelling of pial and cerebral cortical microvessels in adult rat brain. Bar, 1.7 μm.

Fig. 7. S-laminin peroxidase immunolabelling of microvessels (arrows) in rat cerebral cortex. Bar, 1 μm.

Fig. 8. S-laminin peroxidase immunolabelling of a pial microvessel in adult rat brain. Note the label is localised to the basement membrane (arrowheads) of the endothelial cell and perivascular space while the smooth muscle (sm) is unlabelled. Bar, 1 μm.

GLUT-1, such factors are probably important in maintaining the high level of GLUT-1 in brain endothelial cells (Harik et al. 1993). OX-47, and the chick homologue HT7, are not downregulated in endothelial cells in culture, in both primary and passage cells, indicating that their expression may not be astrocyte-dependent, but there is good evidence that they are modulated in vivo (Risau, 1991). HT7 can be induced in endothelial cells of nonneural origin (from chorioallantoic vessels) by astrocyte-conditioned medium (Lobrinus et al. 1992). Astrocytes themselves produce s-laminin (Hunter et al. 1992). Since the astrocyte-endothelial cell influence is bidirectional (Goldstein, 1988; Abbott et al. 1992; Cancilla et al. 1993), the interaction between the 2 cells and the involvement of s-laminin requires investigation. Whether EBA expression is astrocyte-dependent is apparently not known, though it is absent from some regions of the normal and pathological nervous system where astrocytes are present and conversely is present weakly in some PNS endoneurial vessels where there are no astrocytes (Sternberger & Sternberger, 1987; Sternberger et al. 1989; Lawrenson et al. 1995*b*). However, pial vessels show heterogeneity for EBA expression (Cassella et al. 1996, and unpublished observations). Commonly, microvessels adjacent to the glia limitans are EBA-positive while more distal microvessels are negative. For larger vessels labelling occurs unilaterally where the vessel wall is in contact with the glial membrane.

In summary, the 4 BBB antigens investigated in the present study, including early and late phase markers, appeared contemporaneously in pial and parenchymal microvessels. The extent to which astrocyte-derived factors are involved with these markers still requires further clarification since in vivo such factors may act via intermediary molecules or in concert with other factors (Risau, 1991; Lobrinus et al. 1992; Cancilla et al. 1993). The interactions between the astrocyte and endothelial cell are complex. However, we can say at this stage that, whether diffusible astrocytic factors are involved or not, the 4 antigens described appear in development in pial vessels which lack an astrocyte ensheathment, at the same time as in cerebral cortical vessels contiguous with astrocytes.

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