ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 52 (2001) 1–11

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

# Histochemical characterization of primary capillary endothelial cells from porcine brains using monoclonal antibodies and fluorescein isothiocyanate-labelled lectins: implications for drug delivery

Dagmar Fischer, Thomas Kissel*

*Department of Pharmaceutics and Biopharmacy, Philipps University of Marburg, Marburg, Germany*

Received 14 September 2000; accepted in revised form 26 March 2001

**Abstract**

Primary endothelial cells isolated from cerebral microvessels by combined mechanical and enzymatic treatment from porcine brains were characterized with regard to identity, purity and membrane surface characteristics. Cells were grown in culture to adherent monolayers and characterized morphologically and histochemically by their binding for fluorescently-labelled lectins and monoclonal antibodies detected by indirect immunofluorescence. The binding patterns of the cells were compared with the affinity of frozen tissue sections of porcine brain cortex for the markers. Endothelial cells in culture were characterized by the binding of von Willebrand factor, vimentin and fibronectin antibodies. They failed to react with anti-glial fibrillary acid protein, anti-galactocerebroside C and anti-neurofilament 160 antibodies characteristic for astrocytes, oligodendrocytes and neurons, respectively. Cell cultures were stained by the lectins, wheat germ agglutinin, horse gram agglutinin and soybean agglutinin, demonstrating the presence of *N*-acetylglucosamine and *N*-acetylgalactosamine residues on membrane surface. Binding sites for concanavalin A and peanut agglutinin characteristic for mannose and galactose could not be detected. Cell age and differentiation had no effect on lectin and antibody staining. Cell cultures gave staining results similar to those of microvessels in frozen tissue sections. The results of morphology, antibody and lectin staining pattern indicate that our in vitro endothelial cell culture model retained many histological characteristics observed for capillary microvessels in vivo and appears to be suitable for studying uptake and targeting properties of drug carrier systems with regard to the blood–brain barrier. 

*Keywords*: Primary capillary endothelial cells; Blood–brain barrier; Histochemical characterization; Lectin binding; Monoclonal antibodies

## 1. Introduction

Brain capillaries possess particular morphological and enzymatic features which distinguish them from blood vessels in other organs. Their endothelial cells lack fenestrations, have few pinocytotic vesicles and form very tight junctions which are responsible for the formation of the blood–brain barrier, effectively restricting the movement of most molecules from blood to the brain [1]. These capillaries are completely surrounded by astrocytes which were assumed to contribute to the barrier properties. To overcome the limited access of drugs to the brain tissue, several methods have been employed to enhance blood–brain barrier penetration [2].

Due to difficulties in investigating the structure and function of this barrier in vivo, attempts have been made to employ in vitro models mimicking barrier characteristics. Blood–brain barrier cell culture models based on primary cultures and cell lines have been used for the study of drug transport, metabolism and biological processes on the cellular level [3–5]. Cell lines can easily and reproducibly be cultivated, but often lose typical morphological or biochemical characteristics in culture [6]. In contrast, primary cell cultures are more difficult to cultivate, but seem to retain more properties of the original tissue [6].

The apparent differences between the permeability properties of cultured brain endothelial cell monolayers and the blood–brain barrier in vivo are usually attributed to the incomplete development of tight junctions and the absence of natural trophic factors stimulating barrier characteristics

* Corresponding author. Department of Pharmaceutics and Biopharmacy, Philipps University of Marburg, Ketzerbach 63, 35032 Marburg, Germany. Tel: +49-6421-2825880; fax: +49-6421-2827016.

*E-mail address:* kissel@mailer.uni-marburg.de (T. Kissel).

*Abbreviations*: ALP, alkaline phosphatase; BSA, bovine serum albumin; Con A, concanavalin A; DBA, horse gram agglutinin; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GGTP, γ-glutamyl transpeptidase; GalC, galactocerebroside C; GFAP, glial fibrillary acidic protein; NF 160, neurofilament 160; PBS, phosphate-buffered saline; PNA, peanut agglutinin; SBA, soybean agglutinin; vWF, von Willebrand factor; WGA, wheat germ agglutinin.

0939-6411/01/$ - see front matter 
PII: S0939-6411(01)00159-X

[7]. Consequently, transcellular drug transport or barrier properties of the blood–brain barrier in vivo are difficult to compare with in vitro systems. Monolayers of brain capillary endothelial cells enable the interactions between the luminal surface and drug carrier systems regarding targeting and uptake mechanisms to be examined. Cell-surface associated molecules and receptors are important for the characterization of cell culture systems with respect to identity, purity and differentiation, but are also fundamental components of specific targeting, attachment and uptake of carrier systems for the transport of drugs across the blood–brain barrier.

The main criterion to assess whether cultured cells are of endothelial origin is the detection of von Willebrand factor (vWF), a large glycoprotein of complex multimeric structure synthesized by endothelial cells and concentrated in Weibel–Palade bodies [8]. Several enzymes are also characteristic for endothelial cells. Histochemical studies suggested that alkaline phosphatase (ALP) [4] and γ-glutamyl transpeptidase (GGTP) [9] may be located primarily within cerebral capillaries. Furthermore, the purity of cell cultures can be determined by the exclusion of any contamination with other cerebral cells, such as astrocytes, oligodendrocytes, neurons and smooth muscle cells, by immunofluorescent staining of their cell type specific markers [10].

The glycocalyx of endothelial cells extending into the capillary lumen is also assumed to be a part of the barrier function [11]. The surface membrane glycoproteins may play an important role in structure, function and characterization of the blood–brain barrier and can be detected by lectin staining [12]. Lectins can be used as tools for histochemical and morphological studies of normal and pathological cells and tissues, e.g. in cancer therapy [13]. Furthermore, the interaction of drug carriers with cell membranes can be enhanced by lectin coating of the particles [14].

It was the aim of this study to establish an in vitro cell culture model of blood–brain barrier to characterize sites for active targeting of micro- and nanoparticulate systems. Therefore, primary capillary endothelial cells were isolated by combined mechanical and enzymatic techniques from porcine brains. Cell cultures were characterized by: (i), staining with monoclonal antibodies directed against surface antigens or cytoskeleton elements of different cerebral cell types; and (ii), fluorescein isothiocyanate (FITC)-labelled lectins to reveal exposed carbohydrate residues on plasma membranes. Staining patterns of frozen brain tissue sections were used as controls.

## 2. Materials and methods

### 2.1. Isolation of capillary endothelial cells from porcine brain

Primary capillary endothelial cells were isolated from brains removed from freshly slaughtered pigs according to the method of Bowman [15] and Mischek [16] with minor modifications. Briefly, the grey matter of two fresh porcine brains was aseptically minced and collected in M199 (Gibco, Eggenstein, Germany) supplemented with 2% penicillin/streptomycin (10.000 E/10.000 μg/ml; Gibco) to a final volume of 100 ml/brain. After digestion in 1% (w/v) dispase II from *Bacillus polymyxa* (Boehringer Mannheim, Mannheim, Germany) for 3 h at 37°C, cerebral microvessels were collected by centrifugation ($5800 \times g$, 10 min, 4°C) with a 15% dextran (MW, 71 000; Sigma, Deisenhofen, Germany) solution. The pellet of crude capillaries was resuspended in M199 and digested with 0.1% collagenase/dispase II (from *Vibrio alginolyticus*/*Bacillus polymyxa*; Boehringer Mannheim) for 3–5 h at 37°C. Cells were sedimented at $1000 \times g$ for 10 min. To separate red blood cells, cell debris and fat, the pellet was centrifuged on a discontinuous Percoll-gradient prepared from 1.07 and 1.03 g/cm$^3$ solutions (Pharmacia, Freiburg, Germany) at $1300 \times g$ for 10 min. Endothelial cells, mainly present in cell clusters at the gradient interface, were aspirated, washed in M199 and resuspended in M199 supplemented with 10% foetal calf serum (FCS; Biozol, Eching, Germany), 2% penicillin/streptomycin solution and 2 mM glutamine (Gibco) for cultivation.

### 2.2. Culture of endothelial cells

Cell viability was determined by the Trypan-Blue exclusion test [17] and propidium iodide staining [18]. For antibody and lectin staining experiments, endothelial cells from one brain were seeded on 250 cm$^2$ of 12-well cell culture multidishes (Nunc, Wiesbaden, Germany), coated with 0.2 mg/ml rat tail collagen type I (Serva, Heidelberg, Germany). Cells were cultured in M199 supplemented with 10% FCS, 2 mM glutamine, 2% penicillin/streptomycin solution at 37°C, 10% $CO_2$ and 98% relative humidity. Cell cultures were used for experiments after 2, 4 and 6 days of incubation. Contaminating cell debris and non-adherent single cells were removed by washing with medium 48 h after seeding. Afterwards, the medium was changed every 2–3 days. Attachment, proliferation and morphology of the cells were observed through an inverse phase contrast microscope (Nikon) with a ×100 magnification.

### 2.3. Preparation of frozen tissue sections

Brains, removed from freshly slaughtered pigs, were snap-frozen in isopentane at −70°C for 5–10 min. Pieces of the cerebral cortex were cut into 20 μm thick sections using the microtome 1207 (Leitz, Wetzlar, Germany), placed on microscope slides and stored at −70°C until use.

### 2.4. Antibody staining

The antibody lyophilisate of the neural cell typing set (Boehringer Mannheim) was reconstituted in bidistilled water to a final concentration of 10 μg/ml. The rabbit

anti-human vWF antibody (Dako Diagnostika GmbH, Hamburg, Germany) was diluted at 1:200 for use. An affinity purified anti-mouse-Ig antibody, $F(ab')_2$ fragment, from sheep (Boehringer Mannheim) conjugated with 5(6)-carboxy-fluorescein-*N*-hydroxysuccinimide ester (FLUOS) was used as a secondary antibody for indirect immunofluorescence in a concentration of 20 μg/ml in phosphate-buffered saline (PBS; pH 7.4) supplemented with 0.5% bovine serum albumin (BSA; Sigma).

Methanol precooled to −20°C was used for fixation of frozen tissue sections. Samples to be stained with anti-galactocerebroside C (GalC) were fixed by incubation in 5% (v/v) acetic acid in ethanol for 10 min at −20°C. After repeated washings with PBS, non-specific binding was blocked by incubation with 1% (w/v) BSA in PBS at 37°C for 30 min. Unbound albumin was removed by PBS washings, and sections were incubated with the primary antibodies at room temperature for 1 h. Thereafter, the slides were rinsed three times with PBS and incubated with the FITC-labelled secondary antibody for 30 min in the dark. They were rinsed again, embedded in PBS/glycerol (2 + 1) and examined under a Zeiss fluorescent microscope with an excitatory wavelength of 485 nm and an emission wavelength of 510 nm (BP 485, FT 510 filterset 17, Zeiss, Germany) using ×312 and ×500 magnifications. Microscopic photographs were taken using a Contac RTS II camera (Germany) and Fuji 100 ASA films. Endothelial monolayers were rinsed three times with PBS, fixed with methanol and stained with the antibodies as described above for tissue sections. Negative controls were similarly prepared using PBS instead of a primary antibody.

### 2.5. Lectin histochemistry

FITC-labelled wheat germ agglutinin (WGA), soybean agglutinin (SBA), and *Dolichos biflorus* (horse gram) agglutinin (DBA) were obtained from Sigma, and peanut agglutinin (PNA) and concanavalin A (Con A) were obtained from ICN (Eschwege, Germany). Lectins were diluted in PBS (pH 7.2) supplemented with 0.1 mM of calcium chloride, magnesium chloride and manganese chloride (lectin buffer) to a final concentration of 100 μg/ml.

Endothelial cell monolayers were washed twice with lectin buffer and incubated with lectin solutions for 30 min at room temperature under light exclusion. To remove excess lectin, cells were rinsed three times with lectin buffer and examined immediately under the Zeiss fluorescence microscope as described for antibody staining. Cryostat tissue sections were fixed in 4% formaldehyde in PBS overnight, rinsed with lectin buffer and stained as described for cell monolayers. Afterwards, they were dehydrated in a graded series of ethanol, embedded in Corbit–balsam and examined by fluorescence microscopy as described above.

Specificity of lectin binding was verified by competitive inhibition with the appropriate saccharides (Merck, Darmstadt, Germany) prior to incubation. One hundred microliters of a 0.4 M sugar solution was incubated with 100 μl lectin solution for 48 h at room temperature before adding to the cells or slides. For perforation of plasma membranes, cell cultures were fixed in 4% formaldehyde in PBS for 10 min at 0°C, rinsed and treated with 1% Triton X-100 (Gibco) on ice for 5 min. After repeated washings with PBS, lectin staining was performed as described above. To remove terminal sialic acid residues, samples were digested with neuraminidase (*Clostridium perfringens*, Boehringer Mannheim), 0.025 U/ml, in 0.2 M citrate–maleate buffer containing 0.9 mM calcium chloride for 24 h at 37°C prior to incubation with lectins.

## 3. Results

### 3.1. Light microscopy of primary capillary endothelial cells

Immediately after seeding, capillary endothelial cells from porcine brains associated with cell clusters which attached to the collagen-coated surfaces within 12–24 h (Fig. 1A). The viability of the isolated cells was determined to be approximately 90%. After 3–4 days in culture, elongated cells grew out in colonies (Fig. 1B) and reached confluence within 6–8 days postseeding. With increasing cultivation time, cell clusters disappeared and morphologically homogeneous growth could be observed. The typical morphology of a confluent endothelial cell monolayer is shown in Fig. 1C. Confluence was reached after cessation of cell division, and cell death occurred after 10–12 days in culture.

### 3.2. Antibody staining

Cellular heterogeneity of the CNS is a problem for the isolation of a well-defined population of capillary endothelial cells. To characterize cell monolayers from pig brain endothelial cells with respect to identity and purity, a panel of six monoclonal antibodies for indirect immunofluorescent labelling technique was used. The specificity of antibody binding was demonstrated by the staining of frozen porcine cortical brain sections and is shown in Table 1. Negative controls incubated with PBS instead of a primary antibody showed no fluorescence (data not shown).

#### 3.2.1. Antibody staining of frozen tissue sections

Anti-NF160 antibodies directed against a 160 kDa neurofilament gave a slightly different staining pattern for white and grey matter due to their different compositions (Fig. 2A). With this staining, two types of neuronal processes could be distinguished in the cortical sections due to their structural properties: short processes with extensive arborizations and long, low branched processes. Whereas the filaments of the grey matter displayed a disordered network, in the white matter, the processes were more parallel and well-ordered in orientation. Neurofilament staining could not be observed in blood vessels.

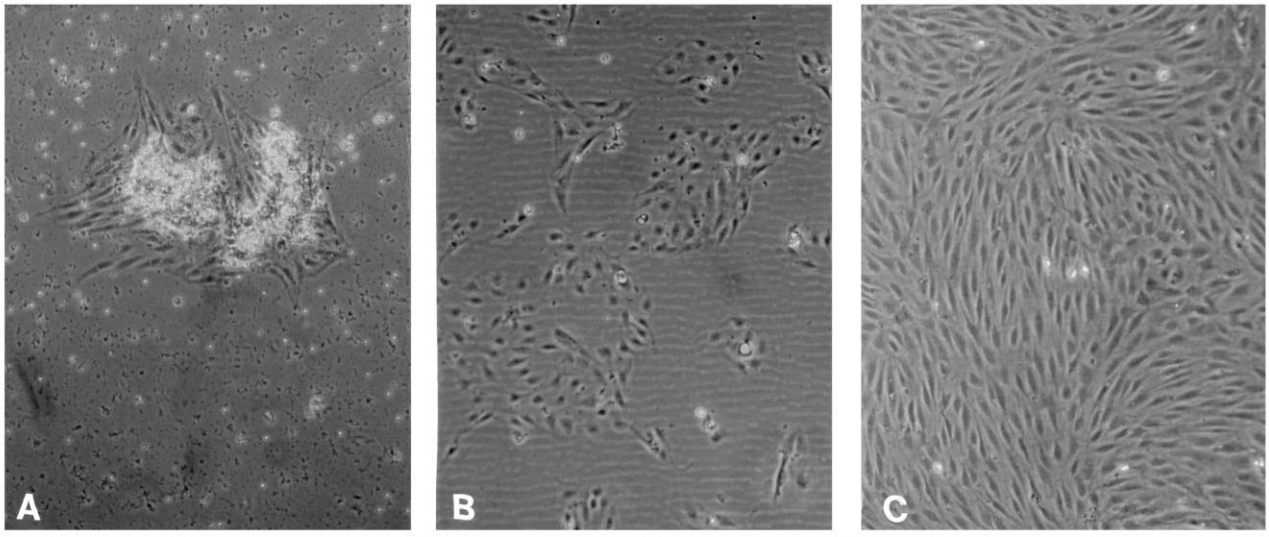


Fig. 1. Phase contrast micrographs of primary endothelial cell cultures isolated from porcine brain microvessels: (A), 2; (B), 4; and (C), 6 days after seeding (100×).

Astrocytes, characterized by the presence of glial fibrillary acidic protein (GFAP), a 50 kDa intermediate filament (Fig. 2B), showed extensive fluorescence of their star-like perikaryons with the exception of the nucleus. Astrocytes of the grey matter displayed small cell bodies with a few, short, highly branched cytoplasmatic processes. In contrast, astrocytes of the white matter were characterized by many long, but poorly ramified processes. No staining could be observed in blood vessels, indicating the absence of GFAP in endothelial cells. To detect the localization of oligodendrocytes, antibodies against galactocerebroside (anti-GalC), a surface membrane glycolipid, were used (Fig. 2C). Both grey and white matter contained similar small, roughly globular cells with non-fluorescent nuclei. In contrast to astrocytes, only a few, low branched processes were seen. Cerebral microvessels were not stained by the anti-GalC antibody.

Since the occurrence of the intermediate filament, vimentin, is not restricted to the endothelial cells of the blood vessels, an intensive fluorescence of different cerebral cell types could be observed (Fig. 2D). White and grey matter showed comparable staining patterns. The entire outer surface of the vessel basement membrane was covered with foot processes from astrocytes. In all cells, vimentin staining was restricted to the cytoplasma, with exclusion of the nucleus. Indirect immunofluorescent staining with anti-fibronectin antibodies revealed the presence of fibronectin in grey and white matter with a similar pattern as indicated by fibrillary structures (Fig. 2E). Under the chosen conditions, different cell types could not be morphologically distinguished.

Fluorescent blood vessels stained by the anti-vWF antibody were detected in macro- and microvascular vessels in cortical sections (Fig. 2F). Immunofluorescence staining revealed a punctiform distribution, indicating the accumulation of vWF in vesicular cytoplasmatic structures. Using the monoclonal rabbit anti-human vWF antibody, the fluorescence intensity was significantly weaker compared with all other antibody staining described above. A human anti-Factor VIIIC antibody (Boehringer Mannheim) did not interact with cortical structures and gave no detectable fluorescence signal (data not shown).

### *3.2.2. Antibody staining of cultured endothelial cells*

After 2, 4 and 6 days, endothelial cell monolayers did not display any affinity for anti-GFAP, anti-GalC and anti-neurofilament 160 (NF 160) antibodies (data not shown). They showed fluorescence intensities which were not significantly different from the control values. Therefore, contamination with astrocytes, oligodendrocytes and neurons could be excluded. Cells stained histochemically positive for

Table 1
Origin, major sugar specificities of the lectins and the binding inhibitors used in this study

| Cell type | NF 160 | GFAP | GalC | Vimentin | Fibronectin | vWF |
|---|---|---|---|---|---|---|
| Neurons | + | − | − | − | − | − |
| Astrocytes | − | + | − | + | − | − |
| Oligodendrocytes | − | − | + | − | − | − |
| Fibroblasts | − | − | − | + | + | − |
| Endothelial cells | − | − | − | + | + | + |

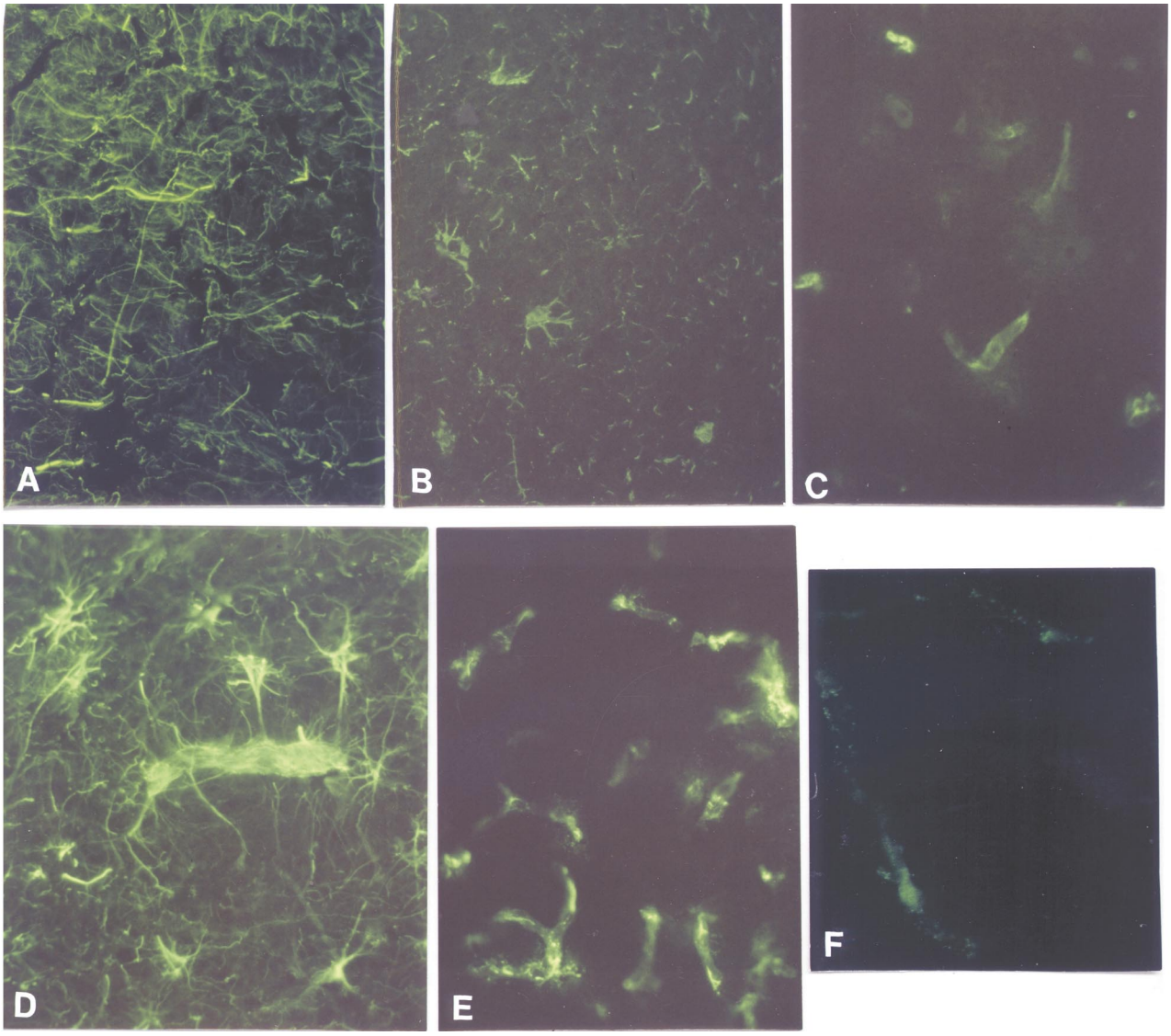


Fig. 2. Indirect immunostaining of frozen tissue sections from porcine brains using the neural cell typing set (Boehringer Mannheim; 312.5 ×). (A) Anti-NF 160; (B), anti-GFAP; (C), anti-GalC; (D), anti-vimentin; (E), anti-fibronectin; (F), anti-vWF.

vimentin, demonstrating a typical intracellular filamentous network staining with exclusion of the nucleus (Fig. 3A). Staining with anti-fibronectin antibodies revealed an adhesive web of fibronectin, mainly underlying the endothelial cells and binding them to their substrate (Fig. 3B). Fluorescently-labelled fibrils are primarily accumulated in the region of cell clusters, an area of extensive growth and cell migration. Positive immunostaining for vWF antigen was present diffusely in the cytoplasma of cultured cells, although the staining intensity was significantly weaker compared with the other antibody reactions (Fig. 3C). The observed staining patterns for the different antibodies were independent of the age and differentiation of the cells.

### 3.3. Lectin staining

Five different FITC-labelled lectins were selected to characterize the distribution of carbohydrate residues on cell surfaces of cultured endothelial cells from porcine brain capillaries. In vitro cell culture data were compared with the lectin staining pattern on the surface of cerebrovascular vessels in vivo by incubation of fixed sections of pig brain slides with lectins. The origin, carbohydrate specificity and inhibitory monosaccharides of the lectins are listed in Table 2. The specificity of lectin binding obtained was verified by competitive inhibition with appropriate sugars. Incubations with FITC-lectins inhibited by the competing sugars failed to demonstrate lectin binding on endothelial cells. Using the lectins at concentrations of 0.1 and 0.5 μg/ml revealed similar results.

#### 3.3.1. Lectin staining of tissue sections

Visualization of the lectins bound to the cortex cryosections provided a variety of staining patterns that were speci-

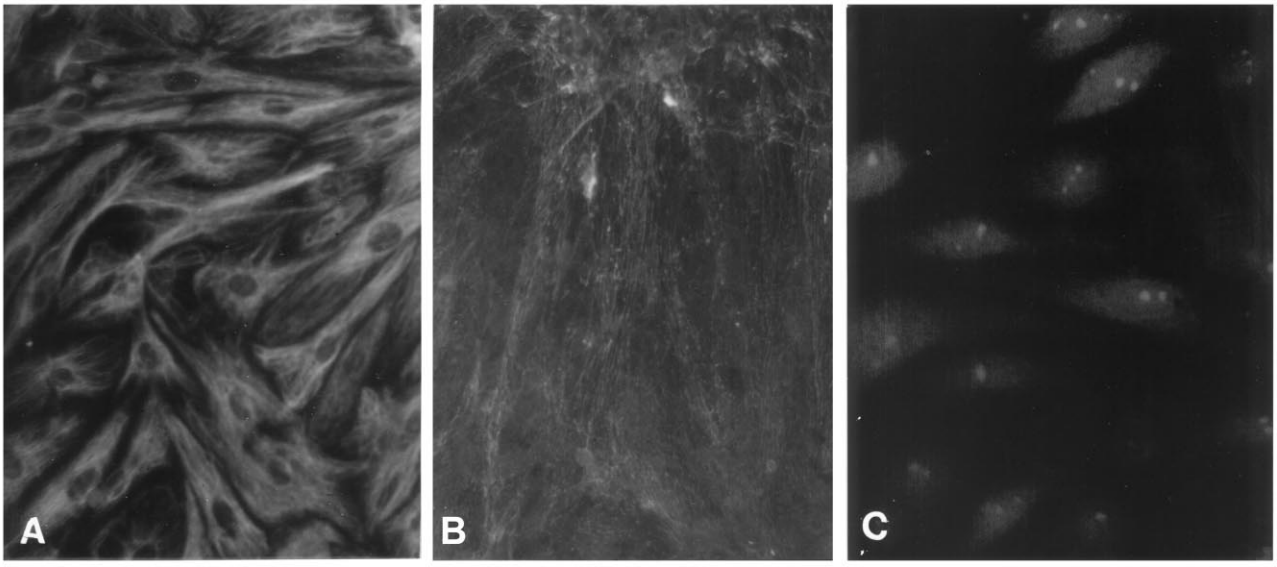


Fig. 3. Staining of endothelial cell monolayers from porcine brain with antibodies of the neural cell typing set (Boehringer Mannheim): (A,B), 312.5 ×; (C), 500×. Monolayers stained positive for: (A), anti-vimentin; (B), anti-fibronectin; and (C), anti-vWF.

fic for each lectin. WGA was the most effective lectin to label cortex sections from porcine brains (Fig. 4A). Since binding sites for WGA appeared to be evenly and strongly distributed on both grey and white matter, the fluorescence of single structures such as blood vessels could not be distinguished. SBA (Fig. 4D) and DBA (Fig. 4E) showed intensive vessel staining with moderate (SBA) or no (DBA) staining of other cerebral structures. Binding of PNA (Fig. 4B) and Con A (Fig. 4C) could not be observed under our experimental conditions. Neuraminidase digestion did not alter the binding patterns of WGA, SBA and Con A. Using DBA, the background fluorescence increased by the removal of terminal sialic acid residues. Pretreatment of the slides with neuraminidase results in exposure of PNA-binding sites (data not shown).

### *3.3.2. Lectin staining of cultured cells*

The surface lectin staining pattern of the in vitro endothelial cell monolayers was determined after 2, 4 and 6 days in culture. None of these staining patterns changed with increasing culture duration. To allow the staining of intracellular structures like nuclei, golgi complex or cytoplasmatic vesicles, the monolayers were fixed and the plasma membrane perforated by 1% Triton X-100 treatment.

When applied to unfixed cells, SBA extensively stained the monolayers and appeared to be especially associated with intercellular contacts (Fig. 5A). Pretreatment with Triton X-100 resulted in an increase of fluorescence in the cytoplasmatic region without nuclear staining (Fig. 5B). The fluorescence intensity of DBA was comparable with SBA staining (Fig. 5C). No difference between fixed and unfixed monolayers could be detected. The most intense staining of plasma membranes was observed with WGA (Fig. 6A). Additionally, after longer incubation periods (45 min), in all cells, the fluorescence appeared to be localized in discrete cytoplasmatic vesicles, as indicated by an intense perinuclear punctate fluorescent pattern suggesting a vesicular uptake mechanism for the WGA (Fig. 6B). After fixing the monolayer, the fluorescence intensity increased and cell nuclei became labelled (Fig. 6C). Whereas unfixed endothelial cells failed in binding Con A (Fig. 5D), after detergent treatment, binding sites for this lectin were detectable in the cytoplasma, but not in the nucleus of the cells (Fig. 5E). Independent of the pretreatment, PNA showed no affinity to the cultured endothelial cells (Fig. 5F). Using endothelial cell monolayers, neuraminidase treatment was not possible since cells were destroyed by the enzymatic treatment during incubation.

Table 2
Specificity of the monoclonal antibodies used in this study

| Lectin | Source | Carbohydrate specificity | Inhibitor |
|---|---|---|---|
| WGA | *Triticum vulgaris* (wheat) | GlcNAc, NANA | *N*-acetylglucosamine |
| SBA | *Glycine max* (soybean) | α-D-GalNAc; β-D-GalNAc, Gal | *N*-acetylgalactosamine |
| DBA | *Dolichos biflorus* (horse gram) | α-D-GalNAc, Gal | *N*-acetylgalactosamine |
| PNA | *Arachis hypogaea* (peanut) | β-D-Gal-D-GalNAc; β-D-GalNAc, Gal | Galactose |
| Con A | *Canavalia ensiformis* (jack bean) | α-Man, α-Glc | Mannose |

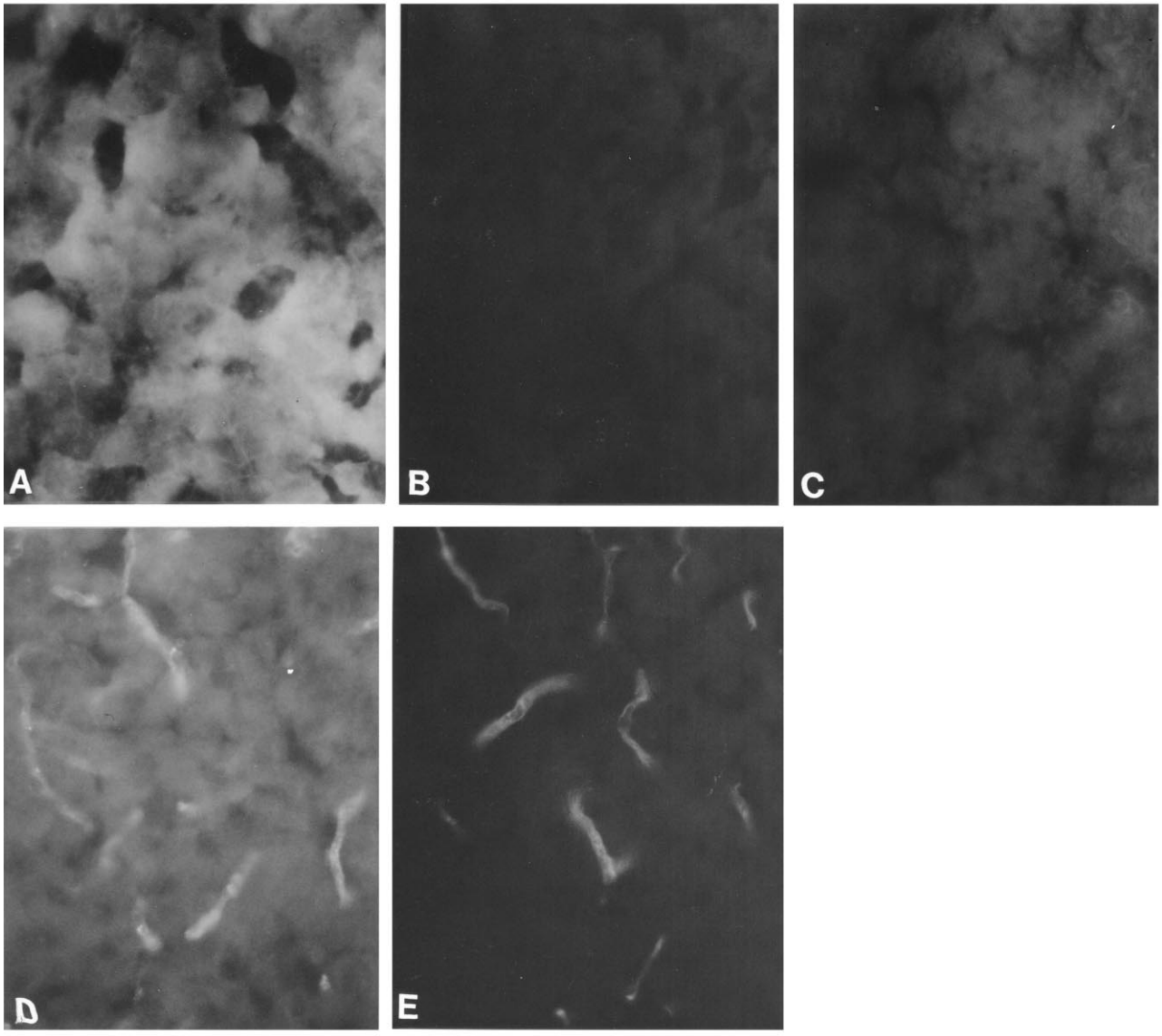


Fig. 4. FITC-coupled lectin staining of frozen tissue sections from porcine brain cortex. Blood vessels were labelled in the following order: (A) WGA; > (D) SBA; = (E) DBA. No definite affinity to endothelium could be observed for: (B) PNA; and (C) Con A (312.5 ×).

## 4. Discussion

The expression of characteristic blood brain–barrier surface markers offers the opportunity to selectively target drugs and carriers to the brain. We screened an in vitro porcine endothelial cell culture as a model system for the blood brain–barrier for specific recognition signals on the plasma membranes. We were especially interested in the binding of different antibodies and lectins to the surface of these cells. Therefore, techniques characterizing transcellular transport mechanisms (e.g. transendothelial electrical resistance [7]) or the evidence of intracellular components, such as ALP, GGTP [16], were not taken into consideration. These studies are currently under investigation. Cell culture data were compared with the results from cortical sections from pig brains and gave corresponding results. All staining experiments were run in triplicate using three separate cultures of endothelial cells from porcine brains and gave corresponding and reproducible results.

Since the endothelial monolayer did not display any affinity for antibodies directed against GFAP, NF 160 and GalC, contamination with astrocytes, neurons and oligodendrocytes could be excluded. Contaminating cells were removed by the different enzymatic digestion steps and by mechanical dispersion. Furthermore, the culture conditions chosen for the cultivation of endothelial cells were not suitable for the attachment, survival and growth of some of these cells [19]. To confirm that the cultured cells were of endothelial origin, they were stained by indirect immunofluorescence for vWF. Antibody staining was detectable, but the intensities were significantly lower than those for all other antigens used in our experiments, presumably depend-

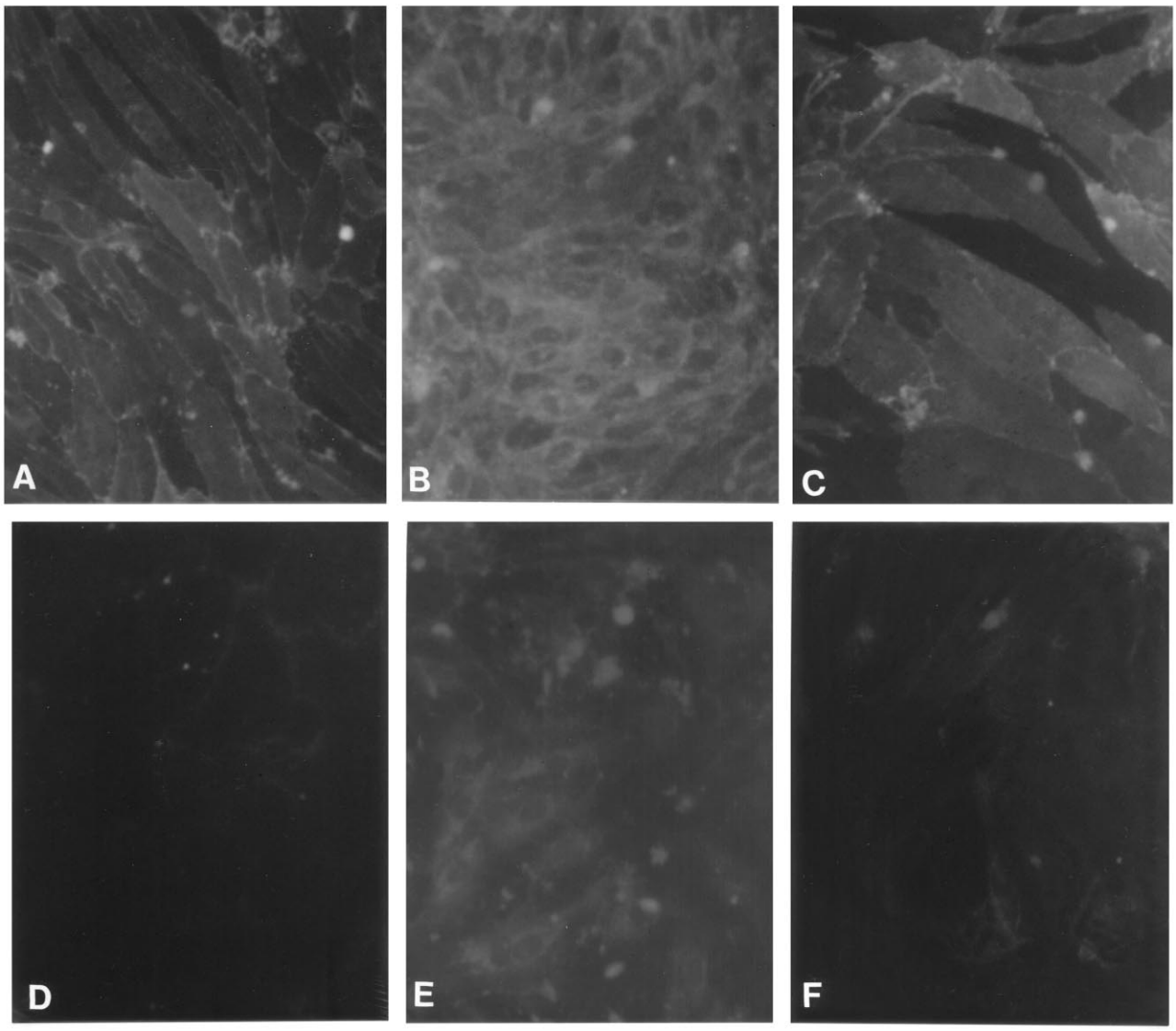


Fig. 5. Lectin staining of endothelial cell monolayers: (A,B,D–F) 312.5×; (C) 500×. (A) SBA; (B) SBA with Triton X-100 pretreatment; (C) DBA, (D) Con A; (E) Con A with Triton X-100 pretreatment; (F) PNA.

ing on the choice of species, fixation conditions and the preparation of the endothelial cells. Several authors hypothesized that the expression of vWF in culture seems to depend on the capillary isolation procedure as well as the species of the laboratory animal and the selected antibody. Mischek et al. [16] reported the presence of the glycoprotein in brain capillaries in vivo, but no reaction of porcine endothelial cells to vWF in vitro. Since all endothelial cells express vWF, vWF-antibodies are not suitable for the specific targeting of brain capillaries [20]. Additionally, after systemic application, the disturbance of physiological functions by blocking vWF with an antibody could not be excluded and limits the use of this marker for targeting.

Endothelial cells and fibroblasts produce the cellular, non-soluble, oligomeric form of fibronectin, which is mainly localized on the cell surface and mediates in vitro adhesion of the cells to their substrate. However, FCS is also a rich source of fibronectin. The contribution of serum fibronectin to the staining intensity vis-à-vis fibronectin produced by the cells, needs further investigation. Ultrastructural studies revealed that endothelial cells, fibroblasts, embryonic neurons and epithelial cells express vimentin elements [21,22], as one of the major parts of their cytoskeleton. Since both vimentin and fibronectin are present on different types of cells, they are not suitable potential addresses for selective brain targeting. Additionally, due to its intracellular localization, vimentin is not accessible for interactions with carrier systems.

Several microscopic studies have demonstrated lectin binding to the peripheral, non-cerebral endothelium [23] of the intestinal mucosa, pancreas [24], bone marrow sinusoidals [25] and the fenestrated vessels of glomeruli [26]. The special morphology and permeability of cerebral endothelia are unlike those of the peripheral organs which

facilitate a controlled transport of substances into the brain [1]. An increase in cerebrovascular permeability was found to be associated with an alteration in surface charge in vivo [27]. Vorbrodt et al. [12] observed changes in lectin receptor distribution within vessel walls correlated with blood–brain barrier damage, raising the question of the role of membrane glycoproteins in barrier function. Similar observations were reported by Szumanska et al. [28] and Mann et al. [29].

In this study, due to the intensity of fluorescence labelling, capillary endothelial cells from porcine brains with lectins yielded the following rank order: WGA > SBA = DBA. No specific affinity could be demonstrated for Con A and PNA. Therefore, the monolayers displayed lectin-binding patterns compatible with accessible α-D-*N*-acetylated glycoproteins (SBA, DBA), whereas β-D-*N*-acetylated sugars (PNA) were found to be less abundant. The staining pattern observed with Con A strongly suggests the absence of mannose containing glycoproteins. In accordance with Fatehi et al. [11], lectin staining was independent of age and differentiation of the endothelial cells. However, an in vivo study by Vorbrodt et al. [12] found a remodelling of the surface glycoprotein layer during blood–brain barrier maturation between the 12th and 24th day of life in mice, coinciding with a decrease in blood–brain barrier permeability. Changes in the ultrastructural localization of lectin binding sites in the developing brain microvasculature in vivo were also described by Nico et al. [30].

According to previous observations for rat brain sections [31] and human endothelial cell cultures, PNA neither displayed an affinity for microvessels in vivo nor for endothelial cells in vitro. PNA receptors seemed to be associated more with arterioles than with capillaries [11]. Furthermore, the major difference between non-cerebral and cerebral endothelia was that PNA binding could not be observed on the cerebral endothelium of rats [31]. Whereas isolated canine capillaries showed weak SBA binding, both isolated endothelial cells from bovine brains [32] and in our study from pig brains showed intense staining. SBA and PNA fluorescence were intensified by neuraminidase digestion, resulting in the removal of sialic acid residues and following exposure of D-gal(1-3)D-gal-NAc sequences. Similar results were obtained with choroid plexus endothelium [24] and cultivated rat capillary endothelial cells [11].

DBA binding of our monolayers correlated with the observations of Fatehi et al. [11] and Nag et al. [31] in vivo using dogs and rats. In vivo, WGA revealed binding to the endothelium of all types of blood vessels. Furthermore, cell nuclei were stained by WGA, illustrating the localization of *N*-acetylglucosamine residues in the nucleus.

Apart from WGA, in several studies, strong staining of vessels was also observed for *Ricinus communis* agglutinin I (RCA-I) and *Ulex europaeus* I (UEA-I) [11,31,33,34]. In contrast to WGA, which strongly stained many cells in cortical sections, RCA-I strongly stained vessels and capillaries with little or no staining of other cortical cells. Therefore, RCA-I has been proposed to be the preferable marker for endothelial cells. In our study, Con A failed to stain endothelial cells both in vivo and in vitro, although several authors reported intense vessel staining of Con A [11,33]. This may probably be due to different fixation procedures or species-dependent effects.

Together with previously published data, lectins for the characterization of porcine capillary endothelium can be categorized in three groups: (i), WGA, UEA-I and RCA-I with the highest affinity for endothelium could be used to

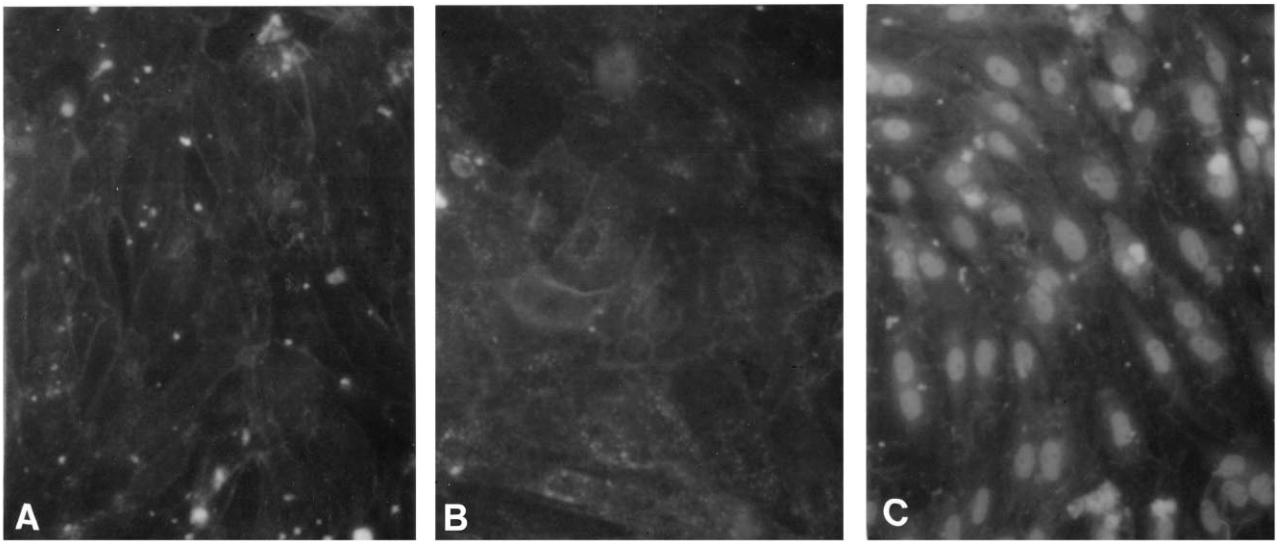


Fig. 6. WGA staining of capillary endothelial cells (321.5 × ). (A) Endothelial cells showed intensive fluorescence after FITC–WGA treatment. (B) Forty-five minute incubations resulted in granular vesicular perinuclear structures, suggesting an endocytic uptake mechanism. (C) After fixation of the monolayers, fluorescence increased and cell nuclei became labelled.

determine the endothelial origin of cells; whereas (ii), SBA and DBA showed weaker staining; and (iii), PNA and Con A binding did not occur on cerebral endothelium.

Plant lectins demonstrated some potential to mediate cytoadhesion and cytoinvasion of drugs and drug carrier systems in the gastrointestinal tract [35] and airway epithelium [36]. Based on genetic engineering techniques, natural lectins can be specifically modified to increase yield and purity, to enhance binding and uptake and to reduce cytotoxicity. Broadwell et al. [37] reported absorptive mediated uptake and transcytosis for horse radish peroxidase (HRP)–WGA in rat endothelium. After endocytosis, the conjugate and its metabolites accumulated in endosomes, golgi complex and the cytoplasma of pericytes. In our experiments, WGA-mediated fluorescence was also found to be localized in cytoplasmatic perinuclear vesicles, presumably indicating an endocytic uptake mechanism. WGA was found to enhance the uptake of HIV-1 gp120, usually slowly crossing the blood–brain barrier, into different peripheral tissues, but the greatest percentage increase occurred for the brain without disrupting the barrier function [38]. Five other lectins tested had little or no effect on brain uptake, suggesting a key role for sugars in binding WGA. Additionally, compared with RCA-I and UEA-I, WGA demonstrated a favourable toxicity profile. Based on our experiments, WGA seems to be the most promising ligand for a targeting or the enhancement of uptake of substances to the brain. Covalently or electrostatically attached to the surface of particulate drug carriers, WGA seems to be a promising ligand to trigger the interaction with cells forming the blood–brain barrier.

## 5. Conclusion

The selected antibodies were suitable for the characterization of the identity and purity of the cells, but not as ligands for active targeting due to the intracellular localization of their targets or the negative side-effects on physiological functions. Some of the lectins were also identified as useful tools to determine the identity and purity of the cells, and additionally, the suitability for drug targeting. WGA seems to be a very promising candidate due to its high affinity for the cerebral capillary endothelium compared with other lectins tested for active targeting and its low cytotoxicity. Whereas SBA and DBA showed only weak staining, PNA and Con A binding could not be observed. Additionally, WGA was found to mediate endocytosis and transcytosis of substances. Our findings were in good agreement with data published for other endothelial cell culture systems of the blood–brain barrier. The results compared with cortical brain sections also demonstrated that characteristics of the brain capillary endothelium were preserved in our cell culture model, indicating its suitability for studying the uptake and targeting properties of drug carrier systems with regard to the blood–brain barrier.

## Acknowledgements

The authors would like to thank Professor Dr H.J. Galla (University of Münster, Germany), Dr U. Gath, Dr A. Volz (both Boehringer Ingelheim, Germany) and Dr B. Kummer (Bonn, Germany) for the introduction in the preparation of capillary endothelial cells and S. Engel (University of Marburg, Germany) for the preparation of the frozen tissue sections. The authors are grateful to Dr B. Ahlemeyer (University of Marburg, Germany) who kindly provided us with the anti-vWF antibodies.

## References

[1] G.W. Goldstein, A.L. Betz, The blood–brain barrier, Sci. Am. 9 (1991) 70–79.
[2] U. Bickel, T. Yoshikawa, W.M. Pardridge, Delivery of peptides and proteins through the blood–brain barrier, Adv. Drug Deliv. Rev. 10 (1993) 205–245.
[3] U. Tontsch, H.C. Bauer, Isolation, characterization and long-term cultivation of porcine and murine cerebral capillary endothelial cells, Microvasc. Res. 37 (1989) 148–161.
[4] L. Dallaire, L. Tremblay, R. Beliveau, Purification and characterization of metabolically active capillaries of the blood–brain barrier, Biochem. J. 276 (1991) 745–752.
[5] R. Cecchelli, B. Dehouck, L. Descamps, L. Fenart, V. Buee-Scherrer, C. Duhem, M. Lundquis, G. Torpier, M.P. Dehouck, In vitro model for evaluating drug transport across the blood–brain barrier, Adv. Drug Deliv. 36 (1999) 165–178.
[6] C. Rollinson, N.J. Lane, N.J. Abbott, Characterization of an immortalized cell line of brain endothelial cells, RBE4, 2nd International Symposium on 'Drug Transport to the Brain' Amsterdam, 1995.
[7] M.P. Dehouck, S. Meresse, B. Dehouck, J.C. Fruchart, R. Cecchelli, In vitro reconstituted blood–brain barrier, J. Controlled Release 21 (1992) 81–92.
[8] E.R. Weibel, G.E. Palade, New cytoplasmic components in arterial endothelia, J. Cell Biol. 23 (1964) 101–112.
[9] L.E. DeBault, P.A. Cancilla, γ-Glutamyltranspeptidase in isolated brain endothelial cells: induction by glial cells in vitro, Science 207 (1980) 653–655.
[10] G.J. Siegel, B.W. Agranoff, R.W. Albers, P.B. Molinoff (Eds.), Basic Neurochemistry Raven Press, New York, 1993.
[11] M.I. Fatehi, D.Z. Gerhart, T.G. Myers, L.R. Drewes, Characterization of the blood–brain barrier: glycoconjugate receptors of 14 lectins in canine brain, cultured endothelial cells and blotted membrane proteins, Brain Res. 415 (1987) 30–39.
[12] A.W. Vorbrodt, A.S. Lossinsky, D.H. Dobrogowska, H.M. Wisniewski, Distribution of anionic sites and glycoconjugates on the endothelial surfaces of the developing blood–brain barrier, Dev. Brain Res. 29 (1986) 69–79.
[13] H.J. Gabius, S. Gabius, Lectins and Cancer, Springer–Verlag, Heidelberg, 1991.
[14] M. Monsigny, A.C. Roche, P. Midoux, R. Mayer, Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes, Adv. Drug Deliv. Rev. 14 (1994) 1–24.
[15] P.D. Bowman, S.R. Ennis, K.E. Rarey, A.L. Betz, G.W. Goldstein, Brain microvessel endothelial cells in tissue culture: a model for study of blood–brain barrier permeability, Ann. Neurol. 14 (1983) 396–402.
[16] U. Mischek, J. Meyer, H.J. Galla, Characterization of GGTP activity of cultured endothelial cells from porcine brain capillaries, Cell Tissue Res. 256 (1989) 221–226.
[17] T. Lindl, J. Bauer, Zell- und Gewebekultur, Gustav Fischer Verlag, Stuttgart, 1993.

[18] B.J. Kroesen, G. Mesander, J.G. ter Haar, T.H. The, L. de Leij, Direct visualization and quantification of cellular cytotoxicity using two colour fluorescence, J. Immunol. Methods 156 (1992) 47–52.
[19] G. Banker, K. Goslin (Eds.), Culturing Nerve Cells, A Bradford Book MIT Press, Cambridge, MA, 1991.
[20] E.A. Jaffe, Endothelial cells and the biology of factor VIII, N. Engl. J. Med. 296 (1977) 377–383.
[21] E. Ruoslahti, M.D. Pierschbacher, New perspectives in cell adhesion: RGD and integrins, Science 238 (1987) 491–493.
[22] N.J. Abbott, C.C.W. Hughes, P.A. Revest, J. Greenwood, Development and characterisation of a rat brain capillary endothelial culture: towards an in vitro blood–brain barrier, J. Cell Sci. 103 (1992) 23–37.
[23] H. Holthöfer, I. Virtanen, A.L. Kariniemi, M. Hormia, E. Linder, A. Miettinen, *Ulex europaeus* I lectin as a marker for vascular endothelium in human tissues, Lab. Invest. 47 (1982) 60.
[24] M. Simionescu, N. Simionescu, G.E. Palade, Differentiated microdomains on the luminal surface of capillary endothelium: distribution of lectin receptors, J. Cell Biol. 94 (1982) 406–413.
[25] R.M. Pino, Ultrastructural localization of lectin receptors on the bone-marrow sinusoidal endothelium of the rat, Am. J. Anat. 169 (1984) 259–272.
[26] R. Bretton, J. Bariety, A comparative ultrastructural localization of concanavalin A, wheat germ and *Ricinus communis* on glomeruli of normal rat kidney, J. Histochem. Cytochem. 24 (1976) 1093–1100.
[27] S. Nag, Cerebral endothelial surface charge in hypertension, Acta Neuropathol. 63 (1984) 276–281.
[28] G. Szumanska, J. Albrecht, Lectin histochemistry of the rat brain following thioacetamide-induced hepatic failure, Mol. Chem. Neuropathol. 32 (1997) 163–177.
[29] D.M.A. Mann, M.S. Purkiss, R.E. Bonshek, D. Jones, A.M.T. Brown, R.W. Stoddart, Lectin histochemistry of cerebral microvessels in ageing, Alzheimer's disease and Down's syndrome, Neurobiol. Aging 13 (1991) 137–143.
[30] B. Nico, F. Quondamatteo, D. Ribatti, M. Bertossi, G. Russo, R. Herken, L. Roncali, Ultrastructural localization of lectin binding sites in the developing brain microvasculature, Anat. Embryol. 197 (1998) 305–315.
[31] S. Nag, Ultrastructural localization on monosaccharide residues on cerebral endothelium, Lab. Invest. 52 (1985) 553–558.
[32] W.M. Pardrigde, Peptide Drug Delivery to the Brain, Raven Press, New York, 1991.
[33] A.W. Vorbrodt, D.H. Dobrogowska, A.S. Losinsky, H.M. Wisniewski, Ultrastructural localization of lectin receptors on the luminal and abluminal aspects of brain micro-blood vessels, J. Histochem. Cytochem. 34 (1986) 251–261.
[34] K. Lamszus, N.O. Schmidt, S. Ergün, M. Westphal, Isolation and culture of human neuromicrovascular endothelial cells for the study of angiogenesis in vitro, J. Neurosci. Res. 55 (1999) 370–381.
[35] M. Wirth, A. Fuchs, M. Wolf, B. Ertl, F. Gabor, Lectin-mediated drug targeting: preparation, binding characteristics, and antiproliferative activity of wheat germ agglutinin conjugated doxorubicin on Caco-2 cells, Pharm. Res. 15 (1998) 1031–1037.
[36] B. Lundt, U. Brockstedt, K. Kristensson, Lectin-binding pattern of neuroepithelial and respiratory epithelial cells in the mouse nasal cavity, Histochem. J. 21 (1989) 33–43.
[37] R.D. Broadwell, Transcytosis of macromolecules through the blood–brain barrier: a cell biological perspective and critical appraisal, Acta Neuropathol. 79 (1989) 117–128.
[38] W.A. Banks, A.J. Kastin, Characterization of lectin-mediated brain uptake of HIV-1 GP 120, J. Neurosci. Res. 54 (1998) 522–529.