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High levels of P-glycoprotein detected in isolated brain capillaries

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P-glycoprotein (P-gp) is a highly-conserved membrane protein expressed in various multidrug-resistant cell lines. P-glycoprotein was detected in capillaries isolated from human, beef and rat brains with a Western immunoblotting procedure using the monoclonal antibody C219 (mAb C219) specific for P-gp. The mAb C219 detected a 180 kDa protein in brain capillaries isolated from all three species. The largest amount of antigen was detected in capillaries isolated from human brain. Specific binding was assessed by competitive inhibition of mAb C219 binding by the synthetic epitope VQEALD. The glycoprotein nature of the brain capillary proteins was confirmed by its sensitivity to *N*-glycanase treatment, which reduced their apparent molecular mass by 5 to 10 kDa. In addition, immunohistochemical studies using the antibodies C219, JSB-1 and C494 were performed. Bovine and rat capillaries showed reactivity only with the mAb C219. Heavy staining of human brain capillaries was observed with both antibodies C219 and JSB-1, while only weak staining was observed with antibody C494. These results clearly show that P-glycoprotein is strongly expressed at the blood-brain barrier (BBB) site and suggest that this protein may play a physiological role in regulating the access of certain molecules to the central nervous system, or in the secretory functions of the BBB.

Introduction

The development of resistance to multiple chemotherapeutic drugs is a major obstacle in the clinical treatment of many human cancers. Cultured cells grown in media containing selected drugs can acquire cross-resistance to a remarkably wide range of hydrophobic compounds which share no obvious structural or functional similarities [1]. A multidrug resistance phenotype (MDR) has been described, which results from the amplification of members of a small gene family and the increased synthesis of a 4.5–6.0-kb mRNA [2–4]. These alterations are associated with the overexpression of a glycoprotein (P-glycoprotein or P-gp) of relative molecular mass 170–180 kDa [5,6]. P-gp is likely to function as an energy-dependent drug-efflux pump [7,8] and shares notable similarities with bacterial transport proteins [9–11].

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Abbreviations: BBB, blood brain barrier; BSA, bovine serum albumin; BBM, brush-border membrane; CHO, Chinese hamster ovary; mAb, monoclonal antibody; P-gp, P-glycoprotein; PBS, phosphate-buffered saline; PB, physiological buffer; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

The human [9] and mouse [10] P-glycoproteins consist of homologous sequences of 1280 and 1276 amino acid respectively, with 12 predicted transmembrane segments and some potential N-terminal glycosylation sites [12,13]. P-gp is encoded by a small gene family comprising three *mdr* genes in rodents and two *mdr* genes in human [10,14,15]. In human, only the *mdr1* gene has been reported to be expressed in cancer cells [16]. Single amino-acid substitutions in human *mdr1* [17] can alter the activity and substrate specificity of this efflux pump. Recently, a possible role for the human *mdr3* gene has been proposed to explain resistance to cytotoxic drugs in B-cell lymphocytic leukemias [18].

The *mdr* gene products are expressed not only in multidrug resistant tumor cells but also in normal tissues such as hepatocytes, the brush-border membrane of renal proximal tubules, adrenal glands, intestine, pancreas and gravid uterus [19–21]. Immunohistochemical studies using different antibodies demonstrated the presence of P-glycoprotein in brain capillaries [22–25]. In this paper, we have used the monoclonal antibody C219 in Western blot analysis to demonstrate the presence of P-gp in isolated brain capillaries from different species, and shown the glycosylated nature of the antigen detected. Immunohistochemical studies performed with three different antibodies (C219, JSB-1

and C494) were used to further investigate the nature of the *mdr* gene products detected in these isolated brain capillaries.

Materials and Methods

Chemicals. Dextran T-70 was obtained from Pharmacia LKB Biotechnology (Montréal, Québec, Canada). Reagents for electrophoresis were products of Bio-Rad (Mississauga, Ontario, Canada). Monoclonal antibodies C219 and C494 were purchased from Signet Labs (D'edham Mass, Ontario, Canada) and membrane fractions of the colchicine-resistant CHO cell line CH^RC5 were purchased from Centocor Diagnostics. ^{125}I-labelled goat anti-mouse IgG was purchased from ICN. Antibody JSB-1 was purchased from Sanbio LV (Uden, Holland). Horseradish peroxidase conjugated-rabbit anti-mouse IgG and enhanced chemiluminescence (ECL) reagents were from Amersham (Ontario, Canada). Endoglycosidase F/glycopeptidase F mixture was obtained from Boeringher-Mannheim (Laval, Québec, Canada). All the products required for peptide synthesis were from Applied Biosystems (Mississauga, Ontario, Canada). All other reagents were purchased from Sigma (St. Louis, MO, USA).

Isolation of brain capillaries. Fresh bovine brains were obtained from a local abattoir and were transported to the laboratory in ice-cold physiological buffer (PB) composed of 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose and 15 mM Hepes (pH 7.4). Normal human brains were obtained post-mortem from patients less than one year old. The capillaries were isolated the day of the death. Capillaries were also isolated from Sprague-Dawley male rats. For all these preparations, the brains were cleared of meninges, superficial large blood vessels and choroid plexus. The cerebral cortex was homogenized in 5 vols. of PB with a Polytron (Brinkman Instruments, Rexdale, Ontario, Canada). The homogenates were mixed with an equal volume of 26%, 28% and 30% (w/v) Dextran T-70 in PB for bovine, human and murine cortex, respectively. Brain capillaries were then purified according to the procedure of Dallaire et al. [26]. The final pellets containing isolated microvessels were resuspended in PB and stored in liquid nitrogen until use.

Preparation of brush-border membranes. Murine and bovine renal brush-border membranes (BBM) were prepared with the method of Booth and Kenny [27]. Purified membranes were resuspended in 300 mM mannitol, 20 mM Hepes/Tris (pH 7.5), and stored in liquid nitrogen.

Enzymatic assays. γ-Glutamyltranspeptidase activity was assayed using a slight modification of the method described by Orlowski and Meister [28]. Fractions were preincubated for 5 min at 25°C with 0.3% Triton X-100. Enzymatic activity was assayed at 37°C by adding 0.8 ml of a solution containing 5.56 mM L-γ-glutamyl-*p*-nitroanilide, 10 mM $MgCl_2$, 20 mM glycyl-glycine and 100 mM Tris-HCl (pH 9.0). The reaction was allowed to proceed for 5–60 min and was terminated by the addition of 0.1 ml of glacial acetic acid. The mixture was centrifuged at $14000 \times g$ for 5 min and the absorbance of the supernatant was measured at 410 nm. Alkaline phosphatase activity was measured with the method of Kelly and Hamilton [29] to evaluate enrichment factors of BBM fractions prepared from bovine and murine kidney cortex. The mean enrichments for rat and beef brush-border membrane preparations, compared to cortex homogenate, were 11.6 ± 1.4 and 7.4 ± 1.5, respectively. Protein content was determined using the method of Bradford [30].

Detection of P-glycoprotein. P-glycoprotein was detected by Western blot analysis. SDS-PAGE was performed according to the method of Laemmli [31]. Capillaries were resuspended in sample buffer to a final protein concentration of 1 mg/ml and loaded on 7.5% acrylamide/bis-acrylamide (29.2:0.8) gels, with or without prior heating. The proteins were transferred electrophoretically to 0.45 μm-pore-size Immobilon-P membranes (Millipore, MA, USA). Blots were blocked overnight at 4°C, with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 50 mM Tris (pH 7.0), 150 mM NaCl, 2 mM NaN_3, 0.05% (v/v) Tween-20. The membranes, washed three times with 0.1% BSA in TBS, were incubated with the monoclonal antibody C219, 400 ng/ml, for 2 h at 37°C. The blots were then incubated with ^{125}I-labelled goat anti-mouse IgG, at a concentration of 10^6 cpm/ml, for 2 h at room temperature. Both antibodies were diluted in TBS containing 1% BSA. The blots were exposed to Fuji film at −80°C for 1–3 days, and scanned with an LKB Ultroscan XL densitometer. Alternatively, horseradish peroxidase conjugated-rabbit anti-mouse IgG was used as secondary antibody and detection was made with ECL reagents according to the manufacturer's instructions. Molecular mass determination was performed using the following standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa).

Peptide synthesis and competitive binding. The peptide VQEALD was synthesized with an Applied Biosystems synthesizer model 431A (Mississauga, Ontario, Canada) using Fmoc chemistry (9-fluoronylmethyloxycarbonyl). Peptide purity was verified using a semipreparative HPLC column. Specific binding studies were performed by pre-incubating the monoclonal antibody C219 with 0, 1 and 10 mM of the peptide encoding the antibody epitope, for one hour prior to the immunodetection of P-gp in brain capillaries.

Western blot analyses were performed as described above and binding was allowed to proceed for 2 h at 37°C.

Immunohistochemistry. Human, rat and beef cerebral capillaries were smeared on glass slides. They were fixed in cold acetone for 10 min and dried at room temperature. The slides were soaked in phosphate-buffered saline (PBS (pH 7.4)) for 5 min and subsequently incubated overnight at 4°C with the primary antibody (C219, 1/20; C494, 1/100, JSB-1, 1/150). The sections were then incubated sequentially with biotinylated horse anti-mouse secondary antibody for 30 min and the avidin biotin peroxydase complex (Vector Laboratories, Burlingame, CA) for 45 min. The binding of the antibody to tissues was revealed by incubating the slides with 3 amino-9-ethyl-carbazole and counterstaining with Mayer's hematoxilin.

Glycosidase treatment. The enzymatic reactions were carried out in the presence of a cocktail of proteinase inhibitors (2 μg of aprotinin/ml, 10 μg of pepstatin A/ml, 10 μg of chymostastin/ml and 100 μg of bacitracin/ml). Brain capillaries were solubilized with 0.5% SDS and 1% 2-mercaptoethanol at room temperature for 5 min and then diluted 4-fold with 1% octyl β-D-glucopyranoside, 20 mM Tris-HCl (pH 8.0). Endoglycosidase F/glycopeptidase F mixture was then added at 2 U/mg of protein and the digestion was allowed to proceed for 16 h at room temperature. Laemmli sample buffer was then added, without boiling. Western blots were carried out as described previously.

Results

Detected levels of P-glycoprotein

The monoclonal antibody C219 detects P-glycoprotein on immunoblots of plasma membrane proteins prepared from multidrug resistant hamster, mouse and human cell lines [32]. This antibody has been shown to be directed against peptide epitopes located at N-terminal position 568–574 (VQAALD or VQVALD) and C-terminal position 1213–1219 (VQEALD) in the amino-acid sequence of P-gp. We used this antibody to determine the presence of a cross-reactive protein in human, beef and rat brain capillaries (Fig. 1). A detergent extract prepared from a colchicine-resistant CHO cell line (CH^RC5) was used as control [1]. A protein with a molecular mass of 180 kDa could be readily detected in capillaries from the three species. The absorbances of the immunoreactive bands were evaluated with a laser densitometer. A correction was applied to the amount of P-gp detected in the capillaries isolated from the different species (human, beef rat), in order to compare the levels of expression directly. This was performed by dividing the scanned area of the autoradiograms by the enrichment factor of each preparation (Table I). Levels of P-glycoprotein were highest in the human capillaries which contained 3–5-times more P-gp than murine and bovine capillaries.

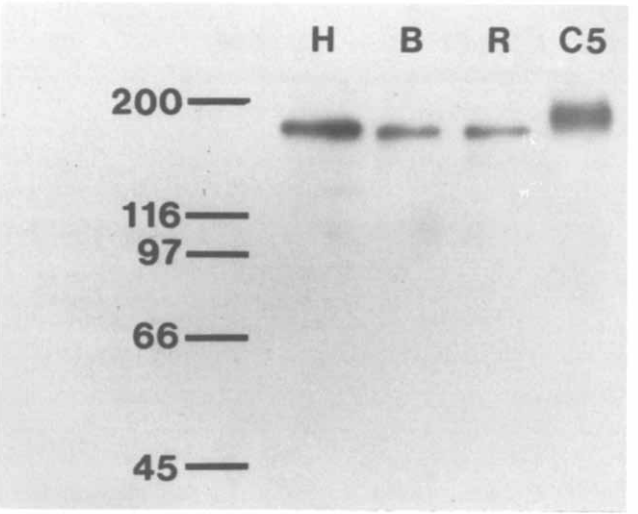

Fig. 1. Detection of P-gp in isolated brain capillaries. Immunoblots were performed as described in Materials and Methods. Protein samples (10 μg) from human (H), beef (B) and rat (R) brain capillaries and a membrane preparation of the CH^RC5 cell line (C5) were resolved by SDS-PAGE using a 7.5% polyacrylamide gel. Monoclonal antibody C219 binding to the antigen was detected using ^{125}I-labelled goat-anti-mouse IgG ($n = 3$).

P-gp detection in renal BBM

A protein with a molecular mass of 155 kDa (Fig. 2) could also be detected in BBM prepared from rat kidney cortex. A longer incubation time with the antibody C219 (16 h) was necessary to detect this cross-reactive protein in rat kidney cortex BBM. Under the same conditions, no reactivity was observed with bovine kidney cortex BBM. Fig. 2 shows that P-gp detected by mAb C219 is considerably more abundant in brain capillaries than in kidney cortex BBM isolated from these two species.

TABLE I

Levels of immunodetected protein in isolated brain capillaries

P-glycoprotein was detected in isolated brain capillaries by Western blot analysis using monoclonal antibody C219. The absorbance of the immunoreactive bands were evaluated with an LKB Ultroscan XL densitometer and corrected for the enrichment of each preparation. Enrichment was the ratio of γ-glutamyltranspeptidase specific activity in isolated brain capillaries and the cortex homogenate. Values represent the means ± S.E. for three preparations.

Preparation	Peak area (Arbitrary units) (PA)	Enrichment factor (EF)	Corrected area (PA/EF)
Human	207.4 ± 2.8	16.5 ± 0.3	12.2 ± 0.2
Beef	55.2 ± 14.7	23.0 ± 1.7	2.5 ± 0.6
Rat	95.2 ± 15.5	23.2 ± 0.8	4.1 ± 0.7

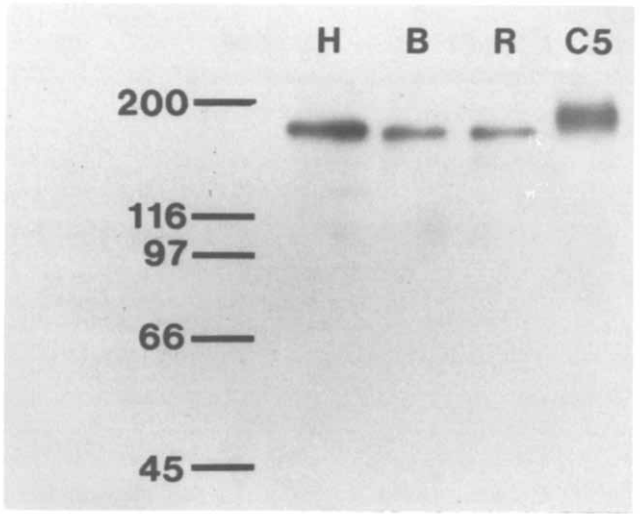

Fig. 2. Comparison of P-gp expression in brain capillaries and in BBM isolated from kidney cortex. Protein samples (20 μg) from rat (R) and beef (B) brain capillaries (CAP) and renal brush border membranes (BBM) were resolved by SDS-PAGE using 7.5% polyacrylamide gel. Immunoblots were performed as described in Materials and Methods and antibody binding was detected using ^{125}I-labelled goat-anti-mouse IgG. The arrows indicate position of the P-glycoprotein from brain capillaries (180 kDa) and rat kidney cortex BBM (155 kDa) ($n = 3$).

C219 binding specificity

The specificity of the monoclonal antibody C219 was assessed by displacement of the antibody binding with the synthetic peptide VQEALD, which represents the C-terminal epitope recognized by mAb C219 [32]. Purity of the synthetic peptide was 93% as analyzed by HPLC (results not shown). Preincubation of the mAb C219 in the presence of 1 and 10 mM peptide resulted in a concentration-dependent inhibition of mAb C219 binding by the specific epitope, with a 40 and a 80% reduction, respectively (Fig. 3).

Linearity of P-gp detection

Because densitometric quantitation of autoradiograms may not give, in some cases, peak areas which are proportional to radioactivity [33], the linear dependence of the peak areas of the autoradiograms quantitated by computer-aided densitometry was assessed as a function of protein concentration, in order to allow quantitative measurements of P-gp in our preparations (Fig. 4).

P-gp immunohistochemical detection

Levels of P-gp-related protein in brain capillaries were further investigated by immunohistochemical studies using antibodies C219, C494 and JSB-1. Antibody JSB-1 recognizes an internal cytoplasmic determinant [34] while monoclonal antibody C494 is a specific immunological probe for the expression of P-gp class I isoform [32]. Human brain capillaries were heavily stained by both antibody C219 and JSB-1 (Fig. 5A and B), while weak staining was observed with the antibody C494 (Fig. 5C). Rat and beef brain capillaries showed weak or no staining with the antibodies C494 and JSB-1, but staining was observed with MAb C219, as summarized in Table II. In these studies, normal frozen human kidneys and CH^{R}C5 cell membranes, which are known to express a large amount of P-gp, were used as positive controls.

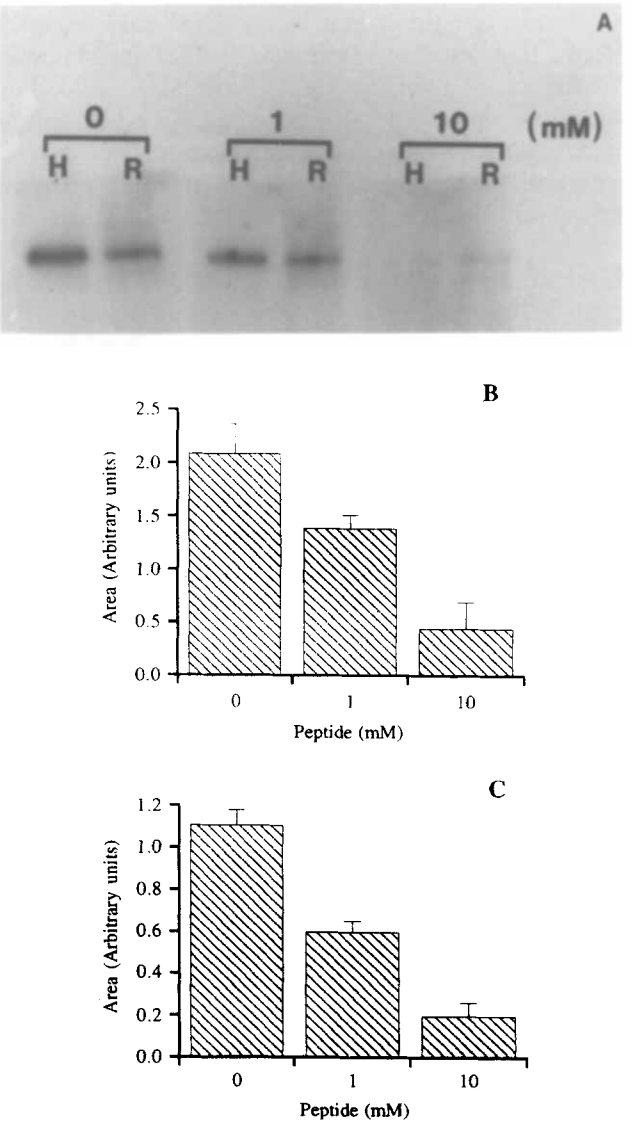

Fig. 3. Specific binding studies were performed by incubating the monoclonal antibody C219 in the presence of 0, 1 and 10 mM of the synthetic peptide VQEALD. Competitive binding of mAb C219 to the peptide and to P-gp present in electrophoretically transferred samples from human (H) and rat (R) isolated brain capillaries was allowed to proceed for 2 h. Immunoblots (A) were performed as described in Materials and Methods. The autoradiograms were scanned with an LKB Ultroscan XL enhanced laser densitometer and the absorbance of the immunoreactive bands detected in human (B) and rat (C) brain capillaries was plot as a function of peptide concentration ($n = 2$).

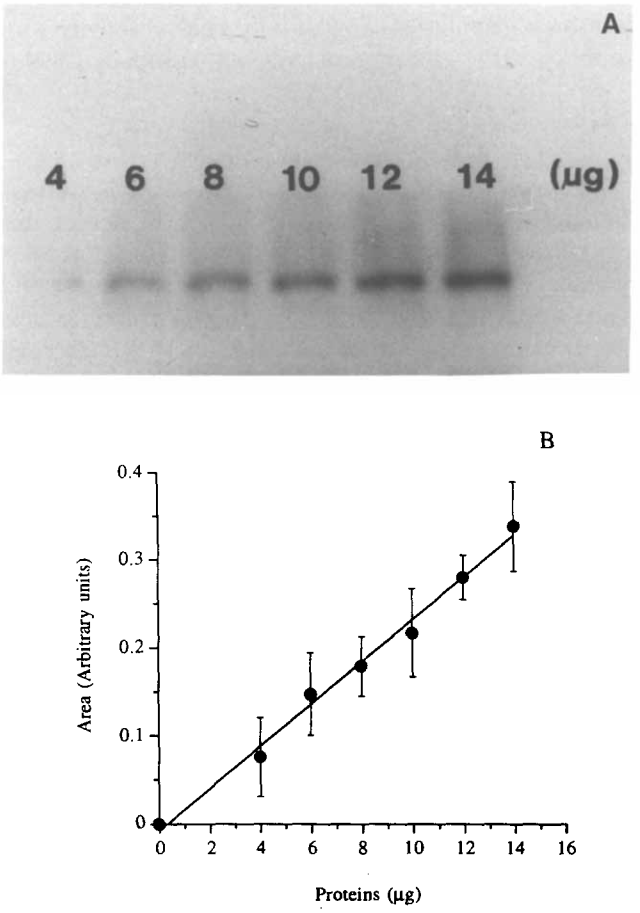

Fig. 4. Linearity of the immunodetection of P-gp in brain capillaries in function of protein concentration. (A) Western blot analysis were performed as described in Materials and Methods on 4–14 μg protein samples from rat brain capillaries. (B) The immunoreactive bands were analysed with a laser densitometer and the absorbance plotted in function of protein concentration ($n = 3$).

P-gp glycosylation characteristics

The level of glycosylation of brain capillaries P-gp was investigated. Brain capillaries were solubilized and treated with a mixture of endoglycosidase F and glycopeptidase F (*N*-glycanase). Following this treatment, the size of P-gp in human, beef and rat brain capillaries was reduced from 180 kDa to 175, 177 and 170 kDa, respectively, as determined by SDS-PAGE (Fig. 6). Under the same conditions, the molecular sizes obtained for the glycosylated and deglycosylated forms

TABLE II

Immunohistochemical detection of P-glycoprotein in isolated brain capillaries

Antibodies C219, JSB-1 and C494 were used to detect P-glycoprotein in isolated brain capillaries as described in Materials and Methods. Intensity of staining: very strong (+ + +), strong (+ +), intermediate (+), weak (±) or negative (−).

Preparation	Reactivity of MAbs		
	C219	JSB-1	C494
Human	+ + +	+ +	±
Beef	+	±	−
Rat	+ +	−	−

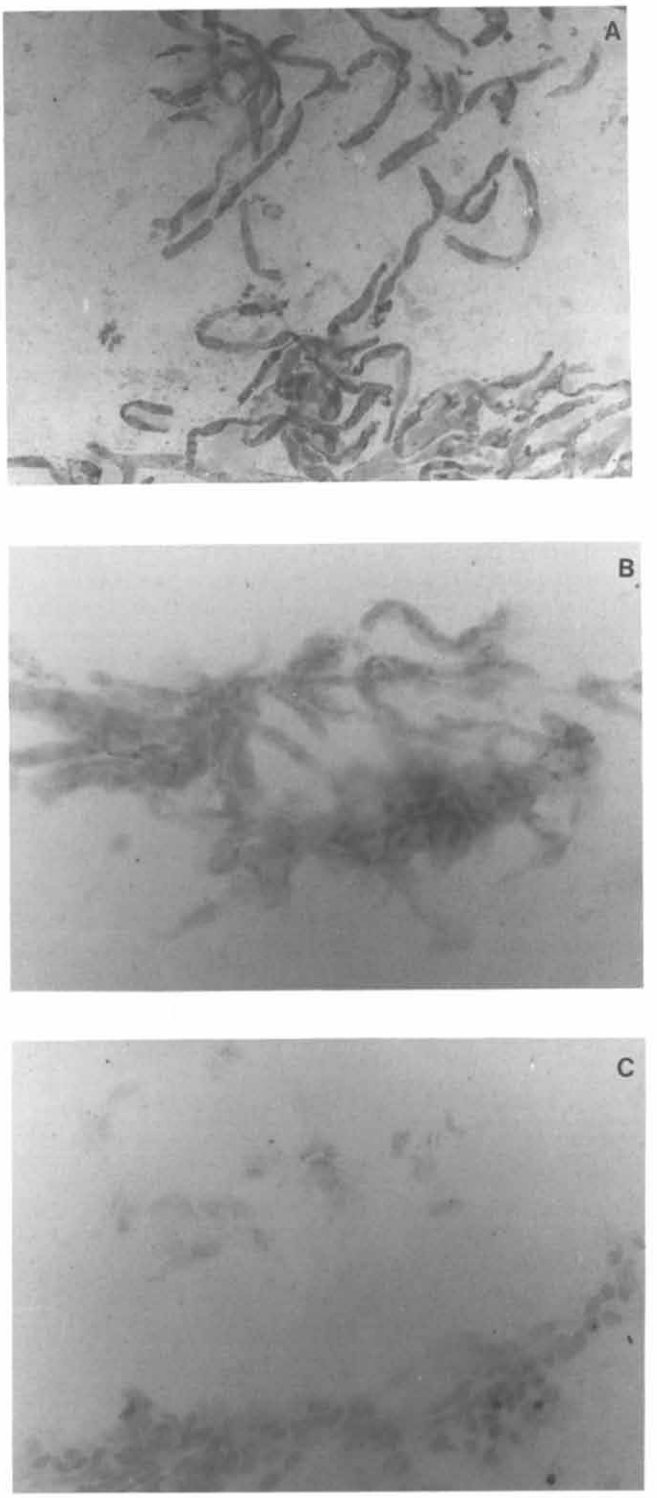

Fig. 5. Immunohistochemical detection of P-glycoprotein in isolated human brain capillaries. Antibodies C219, JSB-1 and C494 were used to localize P-glycoprotein in human isolated brain capillaries as described in Materials and Methods. Strong staining of the human capillaries was observed with both antibodies C219 (A) and JSB-1 (B), and weak staining was observed with antibody C494 (C).

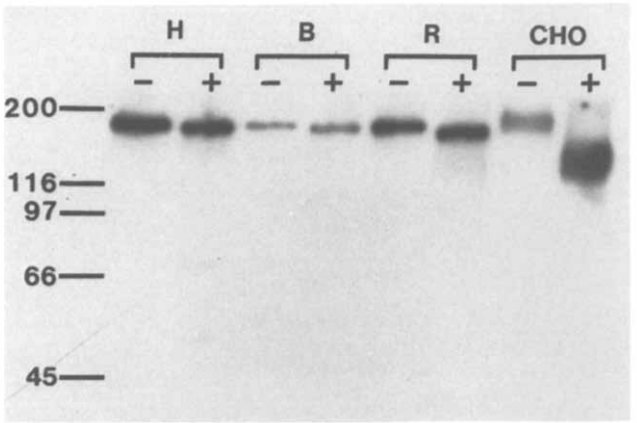

Fig. 6. Effect of *N*-glycanase treatment on the electrophoretic mobility of the P-glycoprotein. All lanes contained 10 μg of protein. Samples were solubilized in 0.5% SDS, 1% 2-mercaptoethanol and then diluted 4-fold with 20 mM Tris-HCl (pH 8.0), 1% octyl β-D-glucopyranoside and incubated at 25°C for 16 h. The samples were incubated with (+) or without (−) 2 U/mg protein endoglycosidase F/glycopeptidase mixture. Enzymatic digestion was performed on isolated capillaries from human (H), beef (B) and rat (R) brains and on a membrane preparation of CH^RC5 cells (CHO). Immunoblots were performed as described in Materials and Methods ($n = 3$).

of the P-glycoprotein from the CH^RC5 cell line were much more distinct; 180 and 120 kDa.

P-gp detection with C494 and JSB-1

Western blot analysis was performed using antibodies C494 and JSB-1 (Fig. 7). These antibodies have been reported to be specific for human and Chinese hamster class-I isoforms [40]. Bovine and murine capillaries showed no reactivity with both antibodies. Human brain capillaries showed only weak reactivity with antibody JSB-1 and no reactivity with antibody C494.

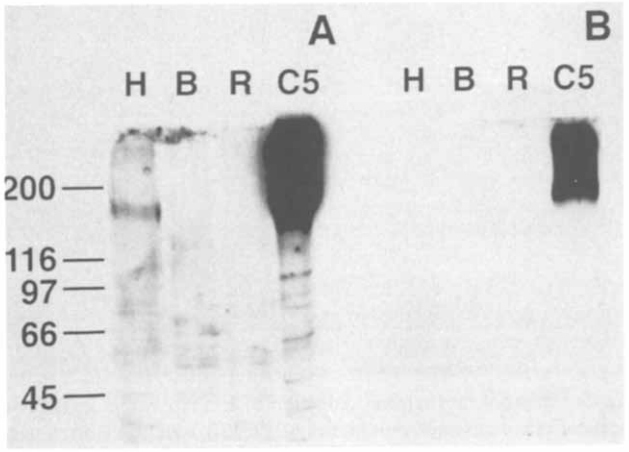

Fig. 7. Detection of P-gp with mAbs C494 and JSB-1. Immunoblots were performed as described in Materials and Methods. Protein samples (20 μg) from human (H), beef (B) and rat (R) brain capillaries and a membrane preparation of the CH^RC5 cell line (C5) were resolved by SDS-PAGE using a 7.5% polyacrylamide gel. Monoclonal antibodies JSB-1 (A) and C494 (B) binding to the antigen was detected using a horseradish peroxidase-conjugated rabbit-anti-mouse IgG and ECL reagents, according to the manufacturer's instructions ($n = 2$).

Discussion

The monoclonal antibody C219 used in this study is specific for P-glycoprotein and is able to detect this protein on immunoblots of plasma membrane proteins prepared from multidrug resistant mouse, hamster and human cell lines [32]. Both of mAb C219 binding sites are located on the cytoplasmic side of P-glycoprotein. We have detected the presence of a cross-reacting protein (180 kDa) by Western blot analysis in human, beef and rat isolated brain capillaries. This antigen co-migrates with the protein detected in the CH^RC5 cell line, which corresponds to the P-glycoprotein that is greatly enriched in the plasma membrane of this drug resistant cell line [5]. The immunologically reactive protein of same molecular mass detected in brain capillaries is likely to be P-glycoprotein, in agreement with the immunohistochemical detection of P-gp reported in this tissue [22,23]. Furthermore, the binding specificity of mAb C219 was assessed by competition with the specific epitope VQEALD which is recognized by this antibody. These results rule out the possibility of a crossreaction of mAb C219 with the muscle protein myosin or unrelated immunoreactivity of the antibody preparation caused by contamination of some of the reagent lots, as previously reported [25,35].

P-glycoprotein is expressed at high level in isolated brain capillaries. Our results show that the detected levels of P-gp vary from one species to another, even after correction for enrichment factors of each preparation (Table I). Isolated brain capillaries contain much more of the immunoreactive protein than BBM prepared from renal cortex from the same species. A molecular mass of 155 kDa has been evaluated for the P-gp detected in rat renal cortex BBM, which is in agreement with the molecular mass already reported for P-gp expressed in this tissue [36].

The glycosylated nature of the antigen detected in the isolated brain capillaries was confirmed by the *N*-glycanase treatment. These enzymes which remove the carbohydrate moieties from *N*-linked glycoproteins [37] induced a small shift (5–10 kDa) in the electrophoretical mobility of the P-gp detected in the isolated brain capillaries, compared to a larger (60 kDa) reduction in apparent size for the P-gp from the CHO extract. Similar values have already been reported for the deglycosylated form of P-gp expressed in this cell line [38]. Therefore, the results obtained with the capillaries preparations cannot be due to lack of enzyme activity, since the same glycanase treatment was applied to all samples. A lack of accessibility of the immunoreactive protein could be an alternative expla-

nation, since it is postulated that P-gp is localized on the luminal side of the endothelial cells of the BBB [22]. This eventuality seems somewhat unlikely, since all the assays were performed in the presence of SDS (0.5%) and *n*-octylglucoside (1%). Glycoproteins are known to migrate aberrantly on SDS gel, the sugar-chain modifying electrophoretical properties of these proteins, and it has already been reported that electrophoretical mobility of P-gp is remarkably dependent on the conditions of analysis [39]. Also, biosynthesis of heterogenous forms of *mdr* gene products has been reported for different multidrug resistant cell lines [38]. Further studies will be necessary to characterize these particular migration properties of the P-gp expressed in the isolated brain capillaries.

The immunohistochemical studies performed with antibody C219 indicated a strong staining of the brain capillaries, in agreement with the results obtained with Western blot analysis. Staining with C494, which is specific for class I isoform, was either negative (rat and beef capillaries) or very weak (human capillaries), suggesting that isoforms other than class I could be predominantly expressed in this tissue. These immunohistological results were confirmed by the Western blot analysis performed with the antibodies C494 and JSB-1. Our results thus differ from those obtained in endothelial cells of Chinese hamster brains, where class I isoform was suggested to be dominant [20]. Our results also show that P-gp is expressed at higher levels in murine and bovine brain capillaries than in kidney cortex BBM isolated from the same species, despite the fact that higher levels of human *mdr1* mRNA have been detected in kidney than in brain samples [19].

In conclusion, our results clearly show that P-gp is endogenously expressed in brain capillaries, and that the level of expression of this glycoprotein varies among species. The strong level of P-glycoprotein expression in this non-cancerous tissue may indicate a physiological role for P-gp in regulating the access of certain molecules to the central nervous system, or in the secretory functions of the blood-brain barrier. P-gp would thus contribute to the barrier function of these endothelial cells. Understanding the role of P-gp in brain capillaries may help to design new drugs to circumvent chemotherapeutic resistance to treatment of infectious diseases and brain tumors.

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