Evaluation of Folate Conjugate Uptake and Transport by the Choroid Plexus of Mice

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Purpose. Because the choroid plexus (CP) is enriched in cell surface folate receptors, an investigation was initiated to evaluate whether folate receptor-mediated transcytosis might be exploited to deliver folate conjugates into the brain.

Methods. Balb/c mice were injected with radioactive and fluorescent conjugates of folate to measure and image their uptake by the CP.

Results. Retention of a radioactive folate conjugate, folate-diethylenetriaminepentaacetic acid (DTPA)-^{111}In, into the brain of balb/c mice was observed, although repeated injections or prolonged release via an osmotic pump of the compound did not result in increased brain uptake. Uptake of an ^{125}I-labeled anti-folate receptor antibody into the brain was very low, and no competition was observed with unlabeled antibody. Imaging of brain thin-sections and whole brain tissue from a mouse injected with folate-fluorescein revealed strong fluorescence in the CP, but virtually no where else in the brain.

Conclusions. Both fluorescence and radioimaging results demonstrate specific uptake of small molecular weight folate conjugates into CP cells of the murine brain, but no significant transport of the molecules across the CSF. Furthermore, no uptake of larger folate-linked proteins by choroid plexus cells is observed, suggesting folate conjugate size may strongly influence access to CP folate receptors.

KEY WORDS: folic acid; choroid plexus; folate receptor; fluorescein; blood CSF barrier.

INTRODUCTION

Folates are required for one carbon metabolism of several biomolecules, including the addition of a carbon to deoxyuridine monophosphate to form deoxythymidine monophosphate by N^5, N^{10}-methylene-tetrahydrofolate, the synthesis of methionine from homocysteine by 5-methyltetrahydrofolate, and the synthesis of purines using 10-formyltetrahydrofolate. Because virtually all tissues engage in one or more of these reactions, folates are required by all cell types.

Folates enter cells by two distinct routes. The reduced folate carrier (RFC) is a classic facilitated transport protein with μM affinity that transports reduced folates across the membrane (1). The RFC is found in virtually all cells and neither binds nor transports folate conjugates into cells. Some cells also express the folate receptor (FR) that exhibits pM affinity for folic acid (2). Folate receptors associate with folate and folate conjugates at the cell surface and usher them into the cell by receptor-mediated endocytosis (3). In contrast to the RFC, the FR is expressed at high levels only in the kidney proximal tubules (4–6), the placenta (7), cells of the CP (8), and in a variety of malignant cells (4–6,9–14). FR overexpression on cancer cells has been exploited for targeting imaging and therapeutic agents to these cells (15).

Although folate receptors expressed in the kidney proximal tubules are presumably present to mediate the capture and transcytosis of folates from the urine filtrate back into the blood (16), the function of folate receptors in the CP of the brain has not been thoroughly examined (11,17,18). Because hydrophilic folate conjugates can only enter cells by FR-mediated endocytosis, this work is focused on exploring whether CP folate receptors might serve to transcytose such folate conjugates across the CSF. Resolution of this issue is critical, since the outcome would (i) clarify the physiologic role of the CP folate receptor, (ii) impact the safety of folate targeted cancer therapeutics, and (iii) reveal whether folate conjugation might be exploited to deliver otherwise impermeable drugs, proteins, and genes into the brain.

MATERIALS AND METHODS

Materials

Folic acid, fluorescein isothiocyanate, mouse anti-FITC antibody, anti-mouse IgG-phycoerythrin, fluorescein amine, Sephadex G-15, and phycoerythrin pyridyl-disulfide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methotrexate-fluorescein was purchased from Molecular Probes (Eugene, OR, USA). Folate deficient mouse food was obtained from Harlan (Indianapolis, IN, USA). Pertechnetate was purchased from Syncor (Indianapolis, IN, USA). $^{111}InCl_3$ and $Na^{125}I$ were obtained from DuPont-NEN (Boston, MA, USA). Osmotic pumps were purchased from ALZA (Mountain View, CA, USA). IodoGEN was purchased from Pierce (Rockford, IL, USA). Folate-DTPA, folate-cysteine, and anti-folate receptor antibody were provided by Endocyte (West Lafayette, IN, USA).

Synthesis of Folate-Fluorescein and Folate-Phycoerythrin

To synthesize a high-affinity folate-fluorescein conjugate, folate-(γ)-ethylenediamine (folate-eda) was prepared according to published procedures (19,20). Folate-eda was dissolved in anhydrous DMSO with 3 equivalents of triethylamine, and a 2-fold excess of flourescein-isothiocyanate was then added. After reacting at room temperature for 4 h in the dark, the crude product was precipitated and washed with diethyl ether and then evaporated to dryness under vacuum. Folate-fluorescein was purified by reverse-phase HPLC on a Microsorb preparative C18 column (250 × 22 mm, 10 mm) at a flow rate of 10 mL/min. The mobile phase, consisting of 10 mM NH_4HCO_3 buffer, pH 8 (eluant A) and acetonitrile (eluant B), was maintained at a 94:6 A:B ratio for the first 10 min and then changed to 70:30 A:B in a linear gradient over the next 20 min. Folate-fluorescein eluted at 20 min. The product was confirmed by mass spectroscopy and the biologic activity was confirmed by fluorescence measurement of its binding to

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ABBREVIATIONS: CP, choroid plexus; CSF, blood CSF barrier; FR, folate receptor; DTPA, diethylenetriaminepentaacetic acid.

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cell surface FR on FR-positive M109 tumor cells in culture (data not shown). The structure of folate-fluorescein is shown in Fig. 1.

Folate-phycoerythrin was synthesized by adding a 10-fold excess of folate-cysteine to a solution of phycoerythrin-pyridyl-disulfide in phosphate-buffered saline (PBS), pH 7.4. The solution was allowed to react overnight at 4°C and the labeled protein (Mr ~260 kDa) was purified by gel filtration chromatography using a G-15 desalting column. The folate labeling was confirmed by fluorescence microscopy of M109 cells incubated with folate-phycoerythrin in the presence and absence of 100-fold excess of folic acid. After a 2-h incubation and a 3-fold wash with PBS, the treated cells were intensely fluorescent, while there was little cellular fluorescence in the presence of excess free folate (data not shown).

Radiolabeling

One millicurie of $^{111}InCl_3$ in 0.5 M HCl was added to a solution of 0.3 mg of folate-DTPA in citrate buffer (pH 6.0) and allowed to react for 2 h at room temperature. The radiolabeling purity was determined by silica gel TLC using methanol as the mobile phase, and the TLC was quantified using a Packard Cyclone phosphorimaging instrument. Radiopurity of the preparation was greater than 95%.

Iodination of the anti-folate receptor antibody was achieved using IodoGEN reagent. Placed in an IodoGEN coated test tube for five minutes was 0.75 mCi of $Na^{111}In$ in PBS (pH 7.4). Then, 100 μg of the anti-FR antibody in PBS (pH 7.4) was added to the IodoGEN tube containing the radioactive ^{125}I and the incubation was continued for five minutes. The antibody solution was removed and separated from free ^{125}I on a G-15 column using saline as the eluant.

Brain Uptake Studies of Intravenously Injected or Intraperitonealiy Implanted Pump-Released Folate-Conjugates

All animal studies were carried out in accordance with procedures approved by the Purdue Animal Care and Use Committee and the NIH publication 85-23 (1985), "Principles of Laboratory Animal Care". Balb/c mice were placed on a folate deficient diet for 2 weeks prior to each experiment to lower their serum folate levels into the physiologic concentration range (1–30 nM) (20). For biodistribution studies, mice were injected in the femoral vein either once or at multiple times at 12 h intervals with 15 μCi of folate-DTPA-^{111}In. Four hours later, the mice were sacrificed and their brains were collected and counted. Some mice were also co-injected with 0.1 mg of free folic acid to serve as a competitive inhibitor. Alternatively, mice were injected with 1 μg of the radiolabeled anti-FR antibody in the presence or absence of 10 μg unlabeled antibody using the same procedure. Antibody-treated mice were sacrificed either four or twenty-four hours post-injection and the brain tissues were counted.

For continuous exposure to folate-conjugates, an ALZA osmotic pump that releases at a constant rate of 0.5 μL per hour for 7 days was filled with 200 μL of PBS containing 200 μCi of folate-DTPA-^{111}In. The pump was inserted into the mouse peritoneal cavity by making an incision through the skin and underlying membrane and then closing the opening with sutures. After 7 days the pump was removed under anesthesia, and after an additional 8 hours (to allow for any remaining conjugate to clear from the blood) the mouse was euthanized and analyzed as described earlier. Mice were also given multiple intraperitoneal injections of folate-DTPA-^{111}In (from 2–4 injections, with the total amount injected equaling 200 μCi), and were sacrificed 8 hours after the last injection and then the brains were isolated and counted.

Fluorescent Imaging of Brain Slices and Whole Brain Tissue

Five micrometers of brain slices were prepared from mice injected (femoral vein) with 35 μg of folate-fluorescein with or without excess folate (100-fold excess) and sacrificed 2 hours later. To enhance the fluorescence from fluorescein in the CP tissues, a mouse anti-FITC antibody followed by an anti-mouse-IgG-phycoerythrin conjugate were used to increase the fluorescence for imaging. After thin sections were made, the brain slices were immersed in ice-cold acetone for 10 min and dried. The slices were then washed three times in PBS buffer and reacted with 0.5 μg/mL of a mouse anti-FITC antibody for 30 min at room temperature. The slides were washed three times in PBS and then reacted with an anti-mouse-IgG-phycoerythrin conjugate for 30 min at room temperature. The slides were then washed three times in PBS, covered with glycerin, and then stored at –80°C. The slices were then imaged using a Bio-Rad MRC 1024 UV/Vis System with an Ar-Kr laser. The slides were viewed with a Plan APO 60 × 1.4 objective under oil immersion.

Imaging of whole brain tissue was performed on mice injected with 10 nmol of folate-fluorescein, fluoresceinamine, methotrexate-fluorescein, or with 15 μg of folate-phycoerythrin. Two hours after each injection, the mice were sacrificed and their brains were removed and dissected through the brain midline to access the interior CP tissue. The lateral CP were then exposed by a small incision. Whole-tissue fluorescent imaging of the exposed tissue was performed using an imaging system consisting of an argon laser (Spectra-Physics, Mountain View, CA, USA) operating at 488 nm and a total laser power of 200 mW at the sample surface. Fluorescence was monitored by a colored CCD camera (JAI CV-53200N)

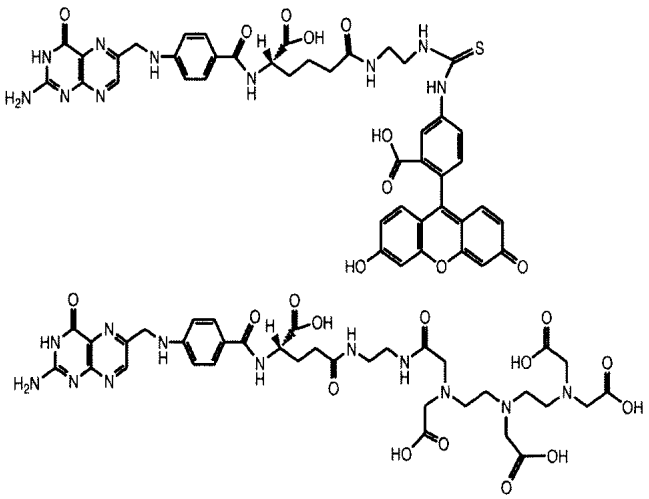

Fig. 1. Structure of folate-DTPA and folate-fluorescein. The folate-fluorescein was synthesized using folate-γ-ethylenediamine. Folate-DTPA was provided by Endocyte Inc. (IN, USA).

(Edmund Industrial Optics, Barrington, NJ, USA) with a sensing area of 768 × 494 pixels with a pixel size of 8.4 × 9.8 μm^2. The CCD camera was equipped with a f/5.6 152–457 mm ×10 CCD zoom lens and a band pass filter at 515–585 nm (Intor Inc., Socorro, NM, USA). The images were digitally acquired using Snappy software v. 4.0 (Play Inc., Sacramento, CA, USA).

RESULTS

Uptake of Radiolabeled Folate-Conjugates by the Brain

To learn whether a low molecular weight folate conjugate might access the known population of folate receptors in the CP, Balb/c mice were injected with 15 μCi of folate-DPTA-^{111}In in the presence or absence of excess folate and evaluated 4 h later for competitive retention of radioactivity in the brain. Although the absolute level of folate conjugate retention was low compared to uptake previously noted in tumor and kidney tissue, at least 90% of the uptake was competitive, suggesting some of the folate receptors present in the brain are accessible to low molecular weight folate conjugates in the blood (Fig. 2). Because no microscopy could be performed in these samples, it was not possible to assess whether transport into the brain was actually achieved.

A biodistribution analysis of radiolabeled antibodies to the folate receptor was also performed to determine whether larger molecules could also access the FR present in the brain. For this purpose, mice were injected with the radiolabeled antibody with and without a 10-fold excess of unlabeled antibody, and the mice were sacrificed at 4 and 24 hours post-injection. Although anti-FR antibody levels were initially very high, within 24 hours antibody retention was negligible. Furthermore, no competition was seen with unlabeled antibody at either time point, suggesting that the initial radioactivity in the brain was most likely caused by antibodies that had not cleared from the circulatory system (Fig. 2). It would, therefore, appear that the extravasation/diffusion pathway leading to CP folate receptors couldn't readily accommodate an IgG-sized molecule.

Finally, although the CP takes up only low levels of small molecular weight folate conjugates, it is still possible that multiple transcytotic events could transport considerable numbers of these conjugates into the brain. To explore this issue, it was necessary to maintain a reasonable level of the low molecular weight conjugates in circulation for an extended period to allow time for measurable accumulation in the brain to occur. For this purpose, folate-DPTA-^{111}In was either given in multiple injections or the radioconjugate was continuously administered for 1 week by implantation of an Alza pump (see Methods). Regardless of the route of continuous exposure, no net accumulation of folate conjugate was observed, despite the fact that receptor saturating levels were maintained in circulation for the entire week by the Alza pump (Fig. 3). Although an efflux pump could conceivably be transporting all internalized folate conjugates back out of the brain, we favor the conclusion that the CP folate receptor is simply inactive in transcytosing the conjugates into the brain.

Fluorescent Imaging of Brain Slices and Whole Brain Tissue

To further characterize folate conjugate access to FR sites in the CP, a second set of folate conjugates was examined following intravenous administration, only this time their location in the brain was determined by fluorescence microscopy and *in situ* fluorescence imaging.

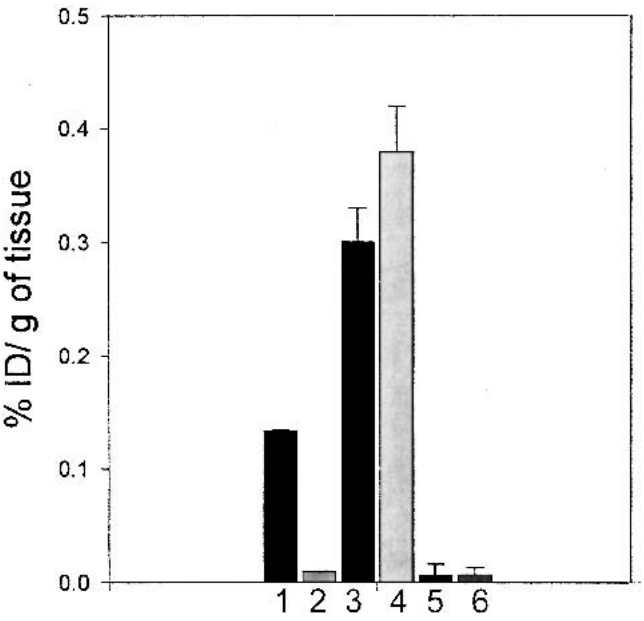

Fig. 2. Uptake of radiolabeled folate conjugates by the brain. 1) Folate-DTPA-^{111}In after 4 h. 2) Folate-DTPA-^{111}In with a co-injection of 100x excess free folic acid. 3) ^{125}I-labeled anti-FR antibody after 4 h. 4) ^{125}I-labeled anti-FR antibody after 4 h with a 10x co-injection of unlabeled antibody. 5) ^{125}I-labeled anti-FR antibody after 24 h. 6) ^{125}I-labeled anti-FR antibody after 24 h with a 10x co-injection of unlabeled antibody. The data are presented as %ID/ g of tissue and each bar represents the average of 3 mice. Mean ± SD

Folate-fluorescein was injected into the femoral vein of mice with and without excess folic acid (100-fold excess), and 2 hours later the mice were sacrificed and the brain tissue

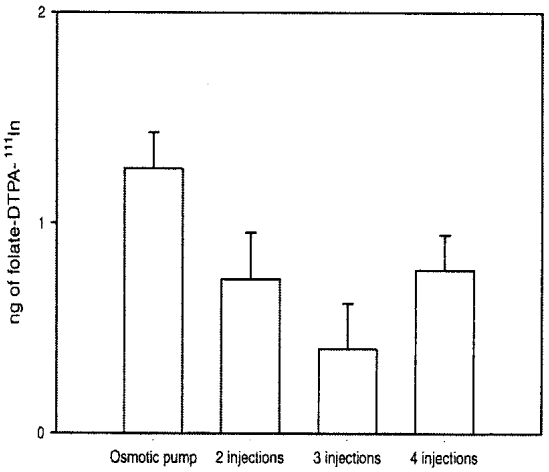

Fig. 3. Brain uptake of folate-DTPA-^{111}In from either multiple injections or from a 1-week slow-releasing osmotic pump. One group of mice was implanted with an osmotic pump containing 200 μCi of folate-DTPA-^{111}In. The pump was removed 7 d later and the mice were sacrificed 8 h after pump removal. Other groups of mice were given multiple intraperitoneal injections with the total amount equaling 200 μCi of folate-DTPA-^{111}In and were sacrificed 8 h later and the brains counted. The data are presented as mean +/– standard deviation of three mice.

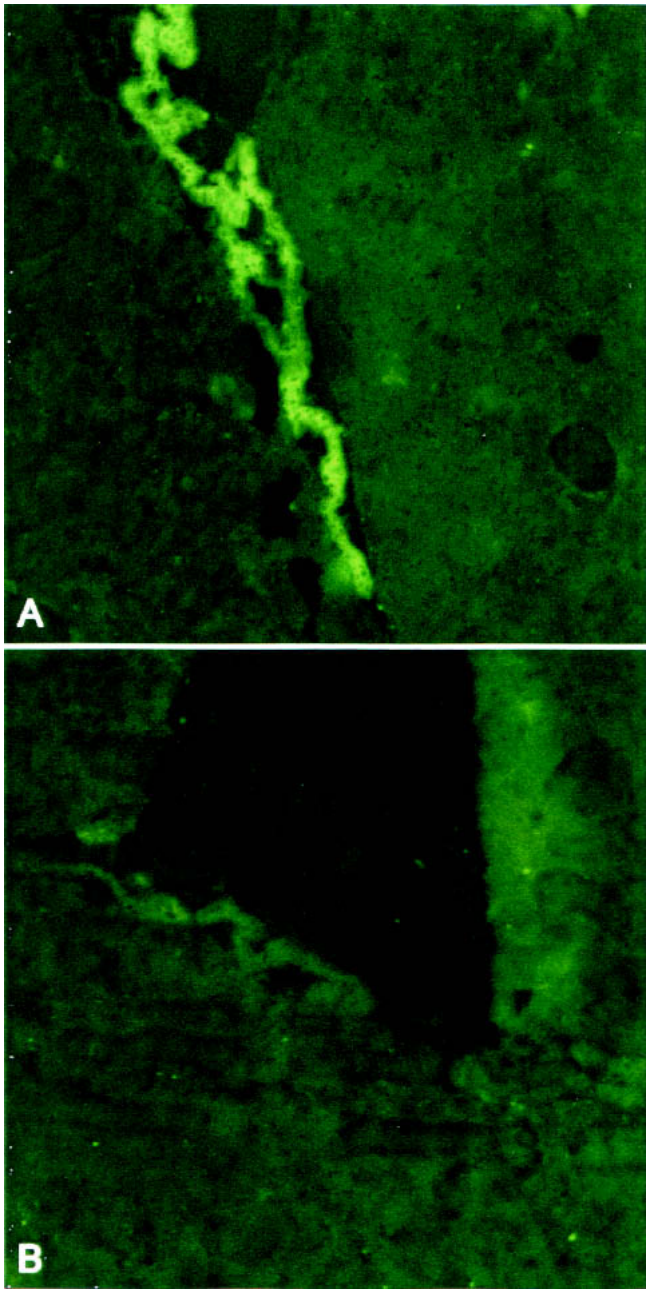

Fig. 4. Uptake of folate-fluorescein into the choroid plexus. Mice were either injected with 35 μg of folate-fluorescein or also with a 100-fold molar excess of folic acid. The mice were sacrificed 2 h later, the brain was removed and 5 μm slices were prepared. The slices were then incubated with a mouse anti-FITC antibody followed by an anti-mouse-antibody-phycoerythrin conjugate to enhance the fluorescent signal. The slices were then imaged by confocal microscopy. (A) CP tissue from mouse injected with folate-fluorescein. (B) CP tissue from a mouse injected with folate-fluorescein plus 100-fold excess of free folic acid.

isolated. Five-micrometer slices of the brain tissue were then prepared and the fluorescence enhanced using a staining procedure employing a mouse anti-FITC antibody followed by an anti-mouse IgG-phycoerythrin conjugate. The resulting confocal micrographs revealed fluorescence on all cells of the CP, with virtually no fluorescence in other regions of the brain (Fig. 4A). Importantly, images of brain tissue of mice

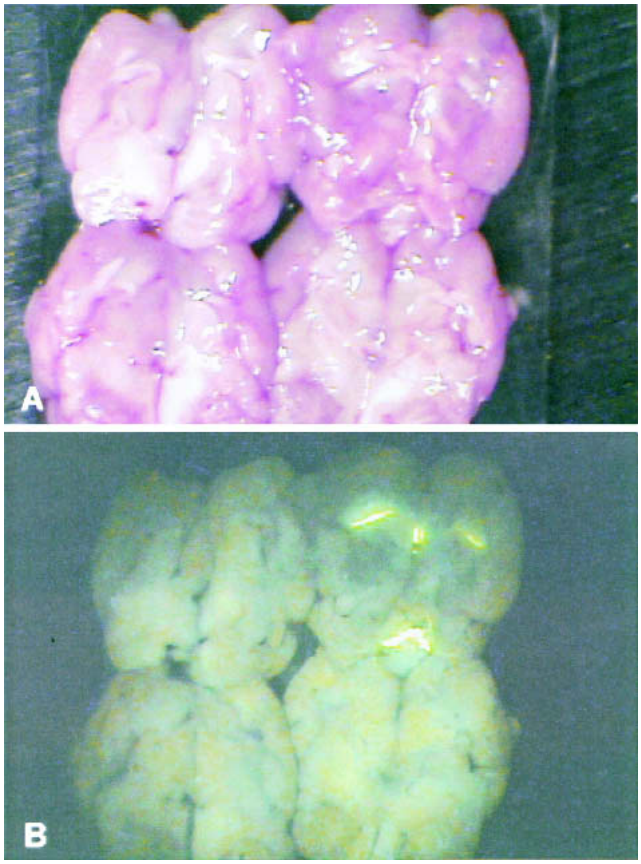

Fig. 5. Imaging of brain tissue after injection of dye molecules. Mice were injected with the dyes in the femoral vein and sacrificed 2 h later. Their brains were dissected, sliced through the midline to expose the interior, and illuminated with (A) white light, (B) and argon laser light at 488 nm for fluorescence imaging. The brains were isolated from mice injected with folate-flourescein (top right), folate-phycoerythrin (bottom right), methotrexate-fluorescein (bottom left), and fluorescein amine (top left).

co-injected with excess free folate (100-fold excess) showed significantly lower fluorescence in the CP tissue (Fig. 4B), suggesting the CP constitutes the major competing site of folate-conjugate retention. Uptake of folate-conjugates was also confirmed by whole brain imaging. Brain tissue from a mouse injected with folate-fluorescein and sacrificed 2 hours later was also sliced through the midline of the brain and opened up at the lateral CP for viewing. As anticipated, high fluorescence was seen in all four CP (Fig. 5). Furthermore, imaging of a mouse injected with nontargeted fluorescein-amine or methotrexate-fluorescein, a folate analog with low affinity for the FR (2), did not show any enrichment in the CP (Fig. 5). These results confirm that the CP cells are able to concentrate small molecular weight folate conjugates from the blood in a manner that can be competed by free folic acid.

To further establish that large protein conjugates of folic acid do not have access to the folate receptors of the CP, folate-phycoerythrin (15 μg) was injected into the femoral vein of mice and the whole brains were again imaged two hours later. Significantly, these images did not show any fluorescence localization into the CP (Fig. 5). These data confirm that large protein molecule cannot diffuse to the FRs on the choroid plexus.

DISCUSSION

The results of this study show that the folate receptor is involved in the retention of low molecular weight folate conjugates by the CP. The biodistribution of folate-DTPA showed a significant drop in brain uptake when co-injected with excess folate. Although the amount of whole brain uptake is low compared to tumor tissue and kidney (21), the amount of CP tissue uptake must be higher since the measurements were made on whole brain tissue and not isolated CP tissue. Thin-section images of the CP tissue after folate-fluorescein injection show fluorescence throughout the cells of the CP, which suggests that the folate-fluorescein may be internalized into the cell and not just binding to the receptors on the membrane. This also suggests that the folate receptor is involved in folate retention, as the RFC is not able to bind and internalize folate conjugates (15). These results are confirmed by whole brain images from mice injected with folate-fluorescein, which showed fluorescence throughout the CP tissue.

Although the results of Patrick *et al.* (22) show localization of the folate receptor primarily on the apical surface of the CP cells, with very little on the basolateral (blood facing) side of the cells, results by Wu *et al.* (23) demonstrate that folate can inhibit the uptake of ^{3}H-5-methyltetrahydrofolate into the CP. These latter data suggest that the folate receptor is somehow involved in the uptake of ^{3}H-methytetrahydrofolate. The results from this study reveal that some of the FRs are accessible on the blood side of the CSF. Two explanations can be offered to reconcile these disparate observations. First, a small fraction of the CP folate receptor could be available on the basolateral side of the CP but at too low a level to be detected by staining with an antibody to the folate receptor (22). Second, a CSF folate transporter/carrier might exist that can be blocked by folic acid. Since the CP cells are believed to comprise the CSF in the CP (24), it is unlikely that folate would be able to diffuse across the CP. This is also confirmed in our study, since little or no folate conjugates were seen in areas of the brain outside of the CP, and there was almost no significant accumulation of folate-DTPA-^{111}In after slow release into the mouse over the course of a week. The lack of transport of folates across the CP or a greater efflux/influx was also confirmed by multiple injections and prolonged release of the folate-conjugate, since the amount delivered by these methods was not significantly greater than the same dose delivered with fewer injections.

The results of this study suggest that the design of folate-based therapies should consider the effects of the therapeutic agent on the CP, at least for small-molecular weight agents. Large molecular weight folate-linked therapeutic agents, such as proteins, are found to be inaccessible to the CP cells and so pose no threat of damaging the cells. Furthermore, since the cells of the CP are not a rapidly dividing cell population, any small molecular weight therapeutic agent that selectively harms dividing cells would be optimal for tumor cell killing, meanwhile avoiding any harm to cells of the CP. In contrast, a therapeutic agent that kills both dividing and non-dividing cells could potentially destroy the CP cells and therefore disrupt the CSF. Evaluation of this possible limitation of folate-targeted cancer therapies will obviously have to await further investigation.

Although these results show that there is little transport across the brain, the uptake of folate conjugates into the CP could possibly open up several therapeutic strategies for CP tumors that over-express the folate receptor. One use would be in the targeting of folate based therapeutic agents against virus induced ependymomas. Tumors of the CP break down the blood-brain barrier, and Roy *et al.* have shown that folate-targeted immune complexes can be used for therapy against these cancers (25). The use of a small molecular weight folate toxic agent would be ideal, because the tumor penetration would be expected to be greater than a large molecular weight antibody conjugate.

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