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Award Number: DAMD17-01-1-0685

TITLE: Blood-Brain Barrier Transport of Uranium

PRINCIPAL INVESTIGATOR: Michael Aschner, Ph.D.

CONTRACTING ORGANIZATION: Wake Forest University School of Medicine
Winston-Salem, North Carolina 27157

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2002	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 01 - 31 Aug 02)
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4. TITLE AND SUBTITLE Blood-Brain Barrier Transport of Uranium	5. FUNDING NUMBERS DAMD17-01-1-0685
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6. AUTHOR(S):
Michael Aschner, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wake Forest University School of Medicine
Winston-Salem, North Carolina 27157

E-Mail: maschner@wfubmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE
20030502 100

13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)
During the first 9 months of work, studies were directed at (1) characterizing the suitability of immortalized Rat Brain Endothelial 4 (RBE4) cells as a model for in vitro blood-brain barrier (BBB) carrying. (2) Transfection of RBE4 cells and Chinese Hamster Cells (CHO) with the Divalent Metal Transporter-1 (DMT-1) gene to enable future studies on the role of this transport in uranium transport. (3) Measurements of uranium uptake in RBE4 cells with Neutron Activation Analysis (NAA). The results presented in this report establish (1) the utility of the model to transport uranium. Treatment of RBE4 cells with Astrocyte Conditioned Medium (ACM) endows the BBB restrictive properties and alters DMT-1 expression profiles, while restricting the transport of polar substances, such as inulin. (2) RBE clones and CHO cells have been successfully transfected, and are presently being sequenced to confirm that they match the original DMT-1. Currently, transfected CHO cells have been selected with G4-18 antibiotic and the forward clones appear to be expressing more DMT-1 protein than reverse or control cells by western blot. The transfected RBE4 cells are in the selection process and will be analyzed as soon as there are enough G4-18 resistant cells. (3) Initial NAA measurements confirm the uptake of uranium into endothelial cells.

14. SUBJECT TERMS
blood-brain barrier, depleted uranium, endothelium, transport

15. NUMBER OF PAGES
18
16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT
Unclassified

18. SECURITY CLASSIFICATION OF THIS PAGE
Unclassified

19. SECURITY CLASSIFICATION OF ABSTRACT
Unclassified

20. LIMITATION OF ABSTRACT
Unlimited

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INTRODUCTION

(Description derived from the original Abstract)

Recent studies of Gulf War veterans with depleted uranium (DU) embedded fragments in their soft tissues point to DU-induced effects on neurobehavioral and cognitive function (McDiarmid et al., 2000). These observations are corroborated by electrophysiological changes in hippocampal slices isolated from rats embedded with DU fragments (Pellmar et al., 1999a; Pellmar et al., 1999b). Notably, studies from the same group also suggest, for the first time, that uranium accumulates within brain tissue (Pellmar et al., 1999a). It is presently *unknown* how uranium is transported into the brain, and there are no pharmacological modalities to reduce its accumulation within the central nervous system (CNS). *The proposal will assess the substrate specificity of uranium transport into the CNS, testing the hypothesis that the divalent metal cation 1 (DMT1), which has an unusually broad substrate range that includes Fe^{2+} , Zn^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} and Pb^{2+} , is also mediating uranium transport into the CNS.* As a first step in testing this hypothesis, we will characterize uranium transport in cultured bovine and rat brain endothelium models of the blood-brain barrier (BBB) (Technical Objective 1.0). Correlative *in vivo* microdialysis studies will delineate the pharmacokinetics of uranium transport across the BBB in rats embedded with DU fragments (Technical Objective 2.0). *The studies will test the hypothesis that a relationship exists between blood and brain uranium concentrations, determining whether rats with the highest blood uranium concentrations also accumulate the highest uranium concentrations in the CNS.* As such, the studies will facilitate risk assessment in Gulf War veterans, and will determine whether veterans with high uranium blood levels are more prone to accumulate uranium in the CNS compared to veterans with low blood uranium levels.

BODY

Stated Technical Objective for Year 1 of this proposal was:

- 1.0 To determine the *in vitro* transport of uranium across the BBB in *in vitro* endothelial cell culture models (RBE4 and bovine brain endothelial cells)

During the first 9 months (since the establishment of an expense account) we have commenced studies on 3 separate fronts.

- (1) Characterization of the suitability of immortalized Rat Brain Endothelial 4 (RBE4) cells as a model for *in vitro* blood-brain barrier (BBB) transport.
- (2) Transfection of RBE4 cells and Chinese Hamster Cells (CHO) with the Divalent Metal Transporter-1 (DMT-1) gene to enable future mechanistic studies on its role in the transport of uranium across membranes.
- (3) Measurements of uranium uptake in RBE4 cells with Neutron Activation Analysis (NAA).

Each of the Technical Objectives will be detailed below:

- (1) Characterization of the suitability of immortalized Rat Brain Endothelial 4 (RBE4) cells as a model for in vitro blood-brain barrier (BBB) transport.

We routinely, and successfully culture RBE4 cells and assess their expression of Factor VIII (results presented in the Preliminary Studies of the original proposal). Briefly, the cells are seeded in a density of 10^3 – 10^4 cells/cm² on filters coated with rat-tail type-I collagen. The cells are grown in culture medium consisting of (α)-minimal essential medium F10 15% fetal bovine serum, penicillin, streptomycin, L-glutamine and 300 μ g/ml G418 (Gibco, Carlsbad, CA/Wake Forest University School of Medicine (WFUSM) tissue culture facility, Winston-Salem, NC) in humidified 5% CO₂/95% air at 37°C.

RBE4 cells are grown on filter inserts and the intactness of the confluent endothelium is measured with ³H-inulin, a polar substance (used as an index for non-specific diffusion). Briefly (see schematic below, Figure 1), the upper luminal well (with insert containing the monolayer) is placed in a lower well. Both wells are filled with the same-buffered medium (defined RBE4 medium) and maintained at 37°C. ³H-inulin is added to the upper well and sampled from the lower well over time. The inset (bottom left of Figure 1) represents a cross section across the filter. Astrocytes can be grown in the bottom well, but we have discovered that the addition of Astrocyte-Conditioned Medium (ACM) is just as effective in inducing the restrictive properties of the cells (see below). We therefore do not culture the astrocytes in the bottom wells.

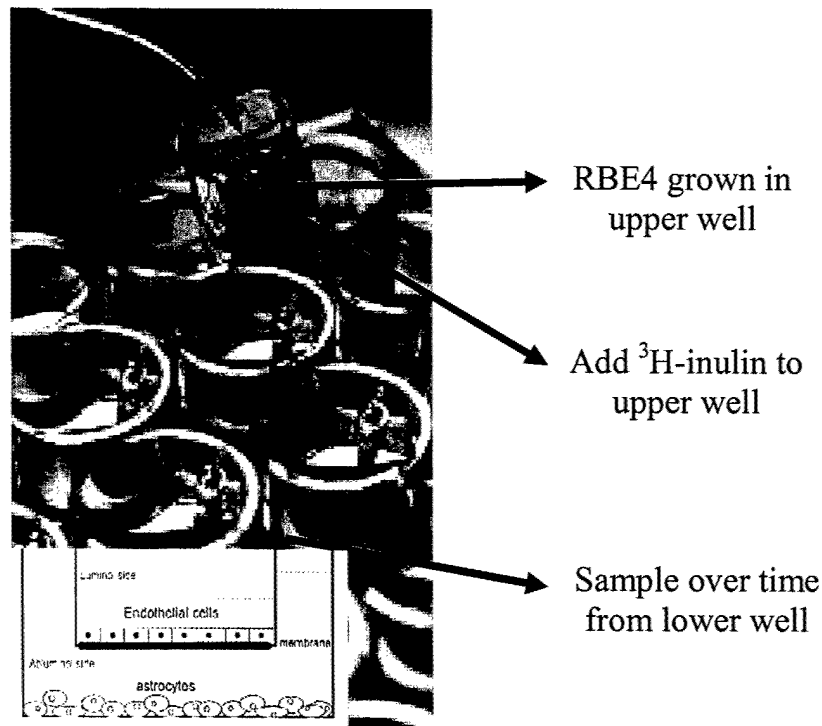


Figure 1 depicts the multi-well system that is employed in transendothelial studies. The cells are grown on filters in the upper well, and radioactive label

is added to the upper well and sampled over time from the bottom well. Inset represents a cross-section through the system (see additional details in text).

$$\%T = \frac{\text{Concentration in bottom well at time-point (Adjusted DPM)}}{\text{Control, initial (Co)}} \times 100$$

Percent transport is calculated from the above equation, where the concentration in the bottom well at a time-point is adjusted at each time point for the volume missing from each successive sampling. The initial control (Co) is the DPM of the measured compound on the luminal (upper-well) topside of the inserted membrane.

$$\text{Apparent Permeability (P}_{\text{app}}) = \frac{\text{Slope of all time-points}}{\text{Area of Filter} \times \text{Control, initial (Co)}}$$

Apparent permeability calculations point to flux and are useful in comparing permeability coefficients between assays and even between models. The data were fitted by a least squares regression analysis and the slope of all time-points was used to calculate P_{app} as a measurement of permeability coefficient. Area of the filter is 4.2 cm^2 .

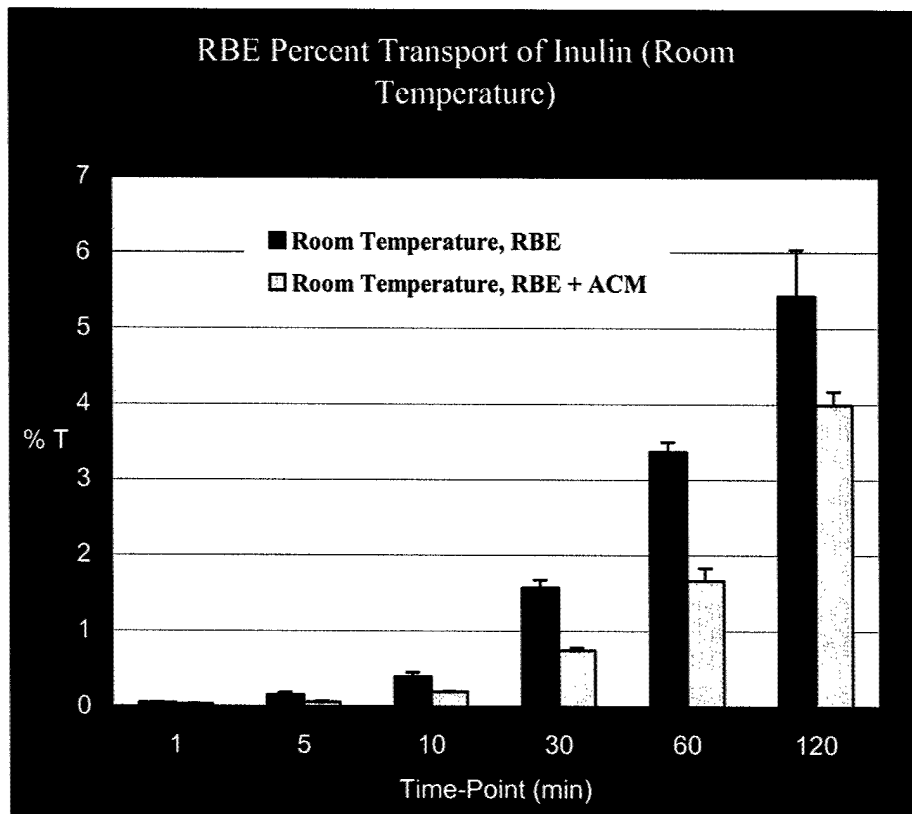


Figure 2 demonstrates that Astrocyte Conditioned Media (ACM) significantly decreases the transendothelial transport (% T on y-axis) of a polar substance, ^3H -inulin. ^3H -inulin is added to the upper-well and sampled over a 120-min time point from the bottom well.

Figure 2 demonstrates that Astrocyte Conditioned Media (ACM) significantly decreases the transendothelial transport (% T on y-axis) of a polar substance, ^3H -inulin, attesting to the ability of ACM to physically tighten the restrictive properties of the blood-brain barrier (BBB). This effect is inherent both to studies conducted at room temperature (25°C) as well as experiments conducted on ice (4°C). Note that is effect is noted immediately upon the addition of inulin and sampling even within 1 min, and throughout the 120 min transendothelial measurements. As shown below (Figure 3), analogous observations are noted for similar experimental conditions for the apparent permeability of ^3H -inulin.

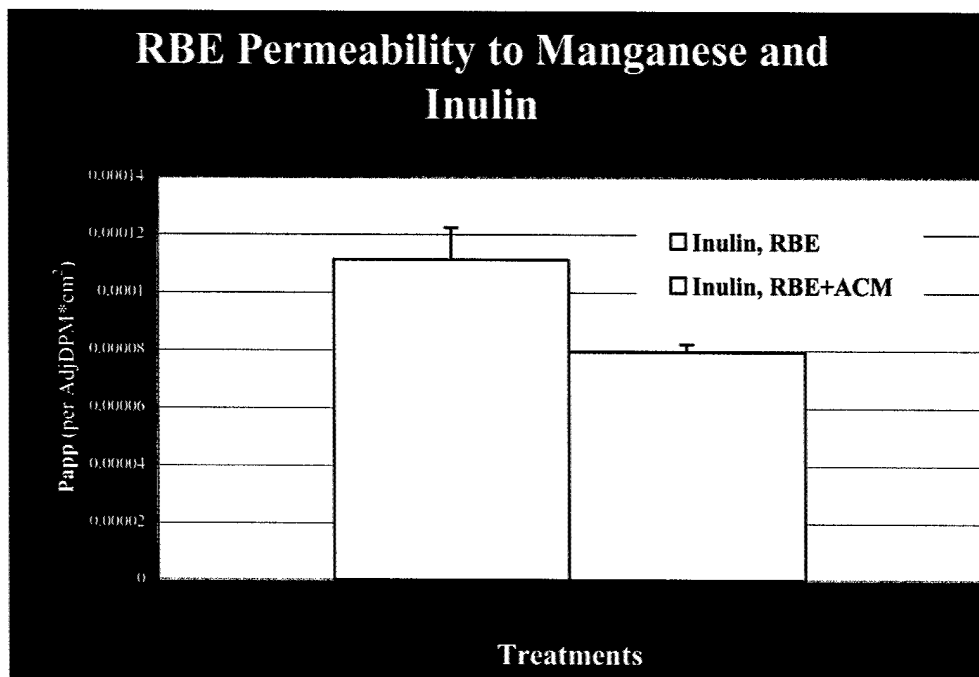


Figure 3 depicts the apparent permeability (P_{app} ; y-axis) of ^3H -inulin across the RBE4 cell monolayer. ^3H -inulin is added to the upper-well and sampled over a 120-min time point from the bottom well [see above for details on calculations of apparent permeability (P_{app})].

Additional studies were carried out to determine whether the increased restrictive barrier properties of the BBB in culture are related to changes in the expression of Cellular Adhesion Molecules (CAM). Cadherins are a family of transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion (Aberle et al., 1996, Gumbiner, 1996). Cadherins are organized in endothelial junctional structures called adherens. Most cadherins form cell-cell adhesion through homophilic interactions, whereby a common cell-adhesion recognition sequence, histidine-alanine-valine (HAV) in the extracellular domain provides specific interaction (Nose et al., 1990). The cytoplasmic domains of cadherins are intracellularly linked to catenins (β -catenin, plakoglobin, p120), which, in turn, promote anchoring to the actin cytoskeleton (Dejana, 1997; Geiger and Ayalon, 1992). The importance of cell adhesion molecules in the maintenance of the cellular integrity of the epithelium is well recognized (Boller et al., 1985; Janzer and Raff, 1987). However, the role of cadherins in maintaining intercellular

junctions between non-epithelial type tissues, such as the endothelium of BBB has been only recently clarified (Lutz and Iahaan, 1997; Pal et al., 1997). Several studies with bovine microvessel endothelial cells (BBMEC) have shown that E-cadherin play an important role in maintenance of tight junction structure across the brain endothelium (Abbruscato and Davis, 1999; Pal et al., 1997).

Methods: Immunohistochemical staining of E-cadherin in RBE4 cells

Immunohistochemical staining of E-cadherin was performed in RBE4 cells grown to confluence on collagen-coated six-well plates (Becton Dickinson Labware, UK) with RBE4 medium or ACM. Confluent monolayers of RBE4 cells were washed (3X) in PBS/Ca²⁺/Mg²⁺, fixed with 4% paraformaldehyde, and washed again with PBS (3X). Cells were permeabilized with methanol (-20°C) for 5 minutes. Removal of endogenous peroxidase was performed by adding 3% hydrogen peroxide for 3 minutes. Cells were subsequently blocked in 5% BSA solution for 30 minutes. Incubations were carried out overnight with polyclonal anti-rabbit antibody conjugated to IgG (Santa Cruz Biotechnology, Inc., CA) at a dilution 1:10, followed by 1 hour incubation with anti-rabbit IgG coupled to horseradish peroxidase (HRP; Santa Cruz Biotechnology, Inc., CA) at a dilution of 1:100.

Methods: Western blot analysis of E-cadherin in REB4 cells

RBE4 cells were grown to confluence on collagen-coated tissue culture flasks as described above with RBE4 medium or ACM. Cells were washed 3 times with 4°C PBS. Protein was isolated from BRE4 cells with 1.5 ml PBS/0.5mM EDTA per flask following centrifugation at 12,000 g for 5 min at 4°C. The supernatant was removed and the pellet was resuspended in 100 µl WANG buffer (25 mM HEPES, pH 7.0, 250 mM sucrose, 100 µM EDTA, 1 µg/ml Leupeptin, 0.5 µg/ml pepstatin A, 1 mM dithiothreitol (DTT), 0.2% Triton-X 100). Subsequently the samples were disrupted by sonication (Vibra Cells, Sonics and Materials, Inc., Danbury, CT; 80% output, 2X, 2 minute each), obtaining a final sample solution. The protein concentration was determined with the BCA protein assay system (Pierce, IL).

An aliquot of 100 µg of protein was concentrated from the imidazole lysis buffer by organic extraction. Sample volumes were brought up to 400 µl with water and an equal volume of methanol (400 µl) was added, followed by 100 µl of chloroform. Samples were vortexed for 20 seconds and centrifuged at 14,000 x g for 3 minutes. The upper layer was removed and discarded. An additional 300 µl of methanol was added to each sample and they were again vortexed and centrifuged. The supernatant was removed and the pellet was air-dried. Each pellet was then dissolved in 100 µl 2% SDS and heated to 65°C. 5 µl Mimura buffer and 2 µl DTT were added to each protein sample, and the samples were boiled for 10 minutes. Bromophenol blue (1 µl of a 50% w/v solution) was added and proteins were separated by denaturing SDS-PAGE using 5% stacking and 6% resolving acrylamide gels in 0.1% SDS, 25 mM Tris, 192 mM glycine buffer. Following fractionation, proteins were electrophoretically transferred to a nitrocellulose membrane (Protran, BA83, Schleicher and Schuell, Keene, NH) in 20% methanol, 0.1% SDS, 25 mM Tris, and 192 mM glycine for 3 hours at 60V. Membranes were blocked with 5% low-fat powdered milk in Tris-buffered saline with Tween (TBST,

0.1% Tween 20; 150 mM NaCl; 20 mM Tris) containing 0.1% gelatin (type B, from ovine skin, Sigma, St. Louis, MO). E-cadherin was detected after overnight incubation with a polyclonal anti-rabbit antibody linked to IgG (Santa Cruz, CA) diluted 1:100 in TBST and 5% milk, and followed by 1 hour incubation with anti-rabbit IgG coupled to HRP (Santa Cruz, CA) diluted 1:1000 (Santa Cruz, CA) in TBST and 5% milk. After extensive washing in TBST, the membranes were developed with an ECL kit and exposure to x-ray film (BioMax MR, Eastman Kodak, Rochester, NY). Films were digitized and band density was determined using the Tina v2.09e computer program (Raytest USA, Inc., Wilmington, NC). For quantitative analysis, the staining intensity for RBE4 cells was standardized to 100%.

Results: Immunohistochemistry localization of E-cadherin in RBE4 cells

Anti-E-cadherin antibody did not positively stain with the extracellular matrix on the flasks (rat tail collagen). Sub-confluent monolayers of RBE4 cells stained positively for E-cadherin. Two days after cell culturing, monolayers of RBE4 cell grown in control medium (Fig. 4A) or ACM (Fig. 4B) showed a different pattern of E-cadherin staining around the cell periphery and throughout the cytoplasm. Monolayers of RBE4 cells grown in control medium showed less ordered and more cell overgrowth. Cells grown in ACM displayed a more intense staining for E-cadherin and a more ordered cobblestone monolayer with flattening cells and minimal cell overgrowth. In addition, RBE4 cells grown in ACM displayed smaller intercellular gaps compared to monolayers grown in control medium (Fig. 4B).

Results: Western blot analysis of E-cadherin in RBE4 cells

The anti-E-cadherin antibody predominantly recognized two bands in the proteins abstracted from cultured RBE4 cells grown in control medium or in ACM (Fig. 4C). One band was recognized at approximately 110 kDa and the other one at approximately 80 kDa (Fig. 4C). In RBE4 cells cultured in ACM, a significant increase was observed in the intensity of molecular mass at 80 kDa compared with RBE4 cells cultured in control medium ($p < 0.001$). No significant change was found in the intensity of the 110 kDa band between RBE4 cells cultured in control medium or in ACM (Fig. 4C).

The 110 kDa band is predominantly recognized by the anti-E-cadherin antibody (Abbruscato and Davis, 1999). The 80 kDa band likely represents multiple glycosylation sites on E-cadherin or fragment formation due to enzymatic digestion. When RBE4 cells were cultured with ACM, there was no apparent change in the intensity of the 110 kDa protein band. However, a significant increase ($p < 0.05$) was found in the intensity of 80 kDa protein band (Figure 4D), suggesting changes in glycosylation sites on E-cadherin (Abbruscato and Davis, 1999). The results corroborate findings from the immunohistochemical experiments where a more intense staining of E-cadherin was noted in RBE4 cells treated with ACM, and suggest that soluble factors in ACM are responsible for the induction of E-cadherin expression in RBE4 cells.

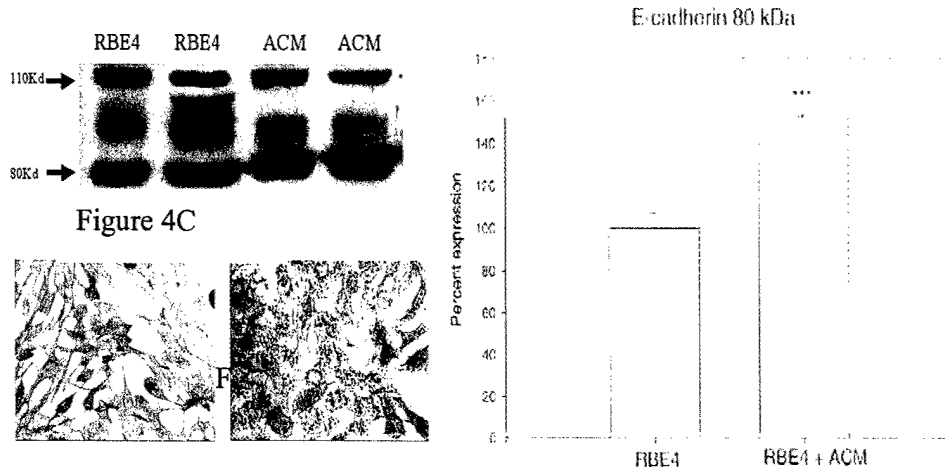


Figure 4D

Figure 4. Immunohistochemical localization of E-cadherin in RBE4 cells cultured with control medium (4A) or in the presence of ACM (4B). RBE4 cells cultured in ACM displayed a increased staining intensity for E-cadherin and smaller intercellular gaps compared with cells cultured in control medium. Western blot analysis of RBE4 cell E-cadherin protein expression (4C). Two bands were recognized at 80 KDa and 110 KDa, respectively, in RBE4 cells cultured in control (RBE4) medium or in the presence of ACM. Figure 4D depicts the image quantification of E-cadherin band intensities. A significant increase was observed in the intensity of the 80 KDa protein band in RBE4 cells cultured in ACM, compared with RBE4 cells cultured in control (RBE4) medium ($p < 0.001$). No significant change was found in the intensity of the 110 kDa band between RBE4 cells cultured in control medium or in ACM. Data represent the means of 4 independent replicates \pm SEM.

In an effort to better characterize the RBE4 BBB model in vitro, additional studies were carried out to determine the effect of ACM added to regular RBE4 cell medium (referred to as alpha medium) on the expression of DMT-1 vs. RBE4 cells incubated in the presence of RBE4 medium alone. The methodology used for these studies is western blotting, and it is identical to the methods described above, substituting for the E-cadherin antibody with an antibody for DMT-1 (a gift from Dr. James Connor at Penn State University, Hershey, PA). As shown in Figure 5, the 24-hour addition of ACM downregulated DMT-1 expression by approximately 60% ($p < 0.05$) consistent with maturation of the BBB and the downregulation of transporter expression.

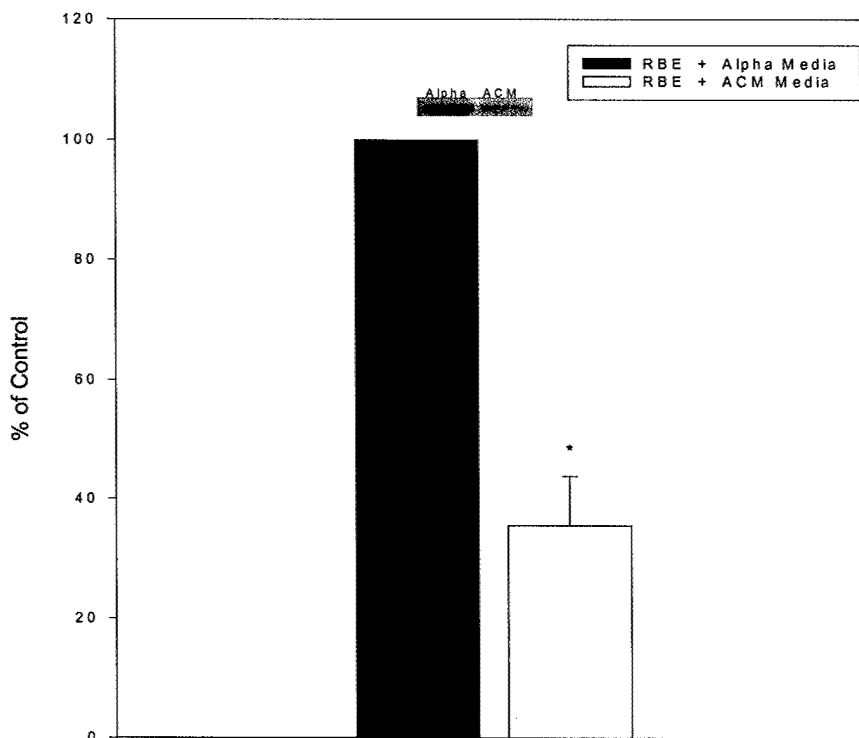


Figure 5. The addition of ACM to RBE4 cells for 24 hours results in downregulated DMT-1 expression ($p < 0.05$). The figure represents image quantification of western blots.

- (2) Transfection of RBE4 cells and Chinese Hamster Cells (CHO) with the Divalent Metal Transporter-1 (DMT-1) gene to enable future studies on the role of this transport in uranium transport.

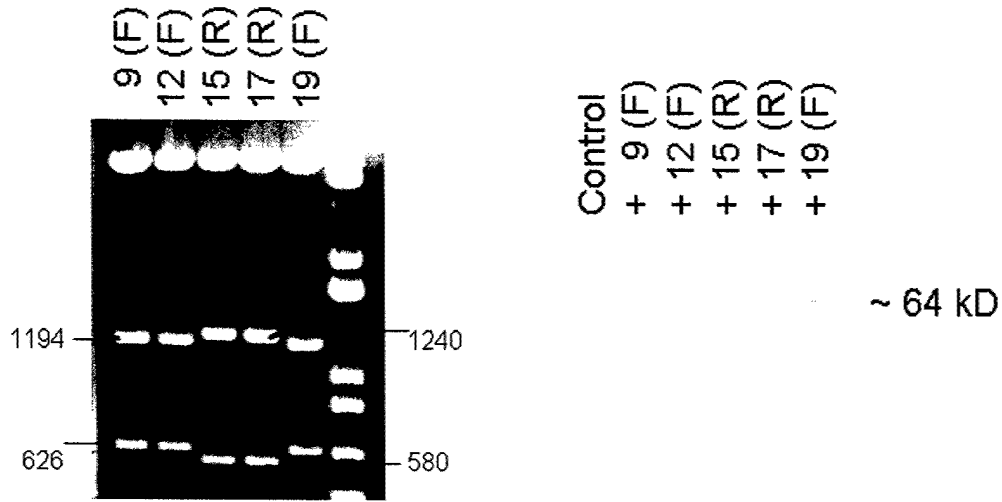
In attempts to construct a mammalian-selectable vector containing the DMT-1 gene, we first obtained the cDNA in pMT2 plasmid from the laboratory of Joseph Bressler (Olivi et al in *AJP – Cell Physiology*, 281, 2001: Involvement of DMT1 in uptake of Cd in MDCK cells: role of protein kinase C). The 1.7-kb cDNA is bordered by EcoRI restriction sites on either end, but the initial attempts to insert it into a mammalian-selectable plasmid (pcDNA3.1 Hygro) did not utilize those EcoRI sites. Instead, primers were designed to PCR-amplify the 1.7-kb cDNA such that there would be two different restriction sites on the ends. This would have allowed only the correct orientation of the DMT-1 cDNA upon ligation into the plasmid. However, although three different primer combinations (containing HindIII/KpnI, HindIII/XhoI, and KpnI/XhoI restriction sites) all amplified the fragment well, the subsequent steps of PCR reaction clean-up, enzymatic digestion, gel purification, and finally ligation into the plasmid presented many obstacles. In short, only two positive clones were obtained, and both were derived from the first combination of primers and enzymes (HindIII/KpnI). One of these clones was determined to contain multiple copies of the gene, so the remaining

clone was the only one sequenced. Although the sequence matched that obtained from the original MT2 vector, neither rat brain endothelial cells (RBE4) nor Chinese hamster ovary cells (CHO) transfected with the new construct showed evidence of producing any more DMT-1 protein than control cells as determined by Western blots.

A new simpler approach to making the construct was initiated in which the original EcoRI restriction sites would be used to insert the DMT-1 gene into the old pcDNA3 plasmid from Invitrogen (which has EcoRI in the multi-cloning site). The cDNA was excised from this plasmid for insertion into pcDNA3, electrophoresed on 1% agarose gel, and purified using Qiagen's gel extraction kit. After ligation into the plasmid, 20 colonies demonstrating ampicillin resistance were selected, grown up, and DNA was isolated using Qiagen's Mini-prep kit. Five of these were clones containing the 1.7-kb DMT-1 insert, and only the orientation was left in question. Digestion with SacI, which cuts the DMT-1 cDNA once, demonstrated that three of these had the cDNA in its proper orientation, and so all three were used (after amplification and Midi-prep) to transfect both RBE4 cells and CHO cells as before. The clones with reverse orientation were also used as negative controls in addition to pcDNA3 vector alone. These clones are being sequenced to confirm that they match the original DMT-1. Currently, transfected CHO cells have been selected with G4-18 antibiotic and the forward clones appear to be expressing more DMT-1 protein than reverse or control cells by Western blot (see Figure 6). The transfected RBE4 cells are in the selection process and will be analyzed as soon as there are enough G4-18 resistant cells.

A northern blot probe for DMT-1 is also being developed based on the paper cited above. Several different radiolabeling techniques are being used to add 32 -P to the 1.7-kb DMT-1 fragment excised from the original pMT2 vector as sent by Dr. Bressler (Johns Hopkins University, Baltimore, MD). With this probe, we will be able to compare the levels of RNA message for DMT-1 to the protein levels.

Cells overexpressing DMT-1 will next be analyzed for transport of uranium.



The clones containing the 1.7-kb DMT-1 cDNA were transfected into CHO cells and selected for resistance to G4-18 (conferred by the neomycin gene in pcDNA3). At left are the plasmids cut with *Sac*I to determine orientation of the gene. Forward (F) orientation should yield overexpression of DMT-1, while reverse (R) clones should serve as negative controls. At right is a Western blot of total protein from untransfected (control) cells and all five transfected cell lines. The primary band at ~ 64 kD agrees with the published size of DMT-1.

Figure 6

- (3) Measurements of uranium uptake in RBE4 cells with Neutron Activation Analysis (NAA).

The studies described herein were carried out to further (1) characterize the RBE4 cell monolayer with respect to DMT-1 protein expression, and (2) as proof-of-concept that the uptake of non-radiolabeled uranium can be used to probe for cellular uptake with the Neutron Activation Analysis (NAA) technique. Expression of DMT-1 proteins was assessed as previously described (see above). In brief, RBE4 cells were probed for the effects of desferrioxamine (DFO) (Sigma, St. Louis, MO), an iron chelator, and the effect of uranium on DMT-1 (Santa Cruz Biotechnologies, Santa Cruz, CA). The cells were treated with 100 uM DFO in the same media they were grown in; α MEM/F10 supplemented with 15% Fetal Bovine Serum, Penicillin, Streptomycin, L-Glutamine and 300ug/ml G418 (Gibco, Carlsbad, CA/WFUSM tissue culture facility, Winston Salem, NC) for 24 hours. The RBE4 cells were then harvested and the protein was quantified using the BCA Assay (Pierce, Rockford, IL). Pre-cast 4-20% gels (BioRad, Hercules, CA) were loaded with 75 ug of protein. The protein bands were detected with Western Lightening Chemiluminescence (Perkin Elmer, Boston, MA). The results (Figure 7) establish a statistically significant upregulation of DMT-1 in RBE4 cells treated with DFO compared with their counter controls treated in RBE4 cell media alone (in the absence of DFO). The effect of uranium treatment (24 hrs. 10, 50, and 100 um) is assessed in ongoing study and the results should be available shortly.

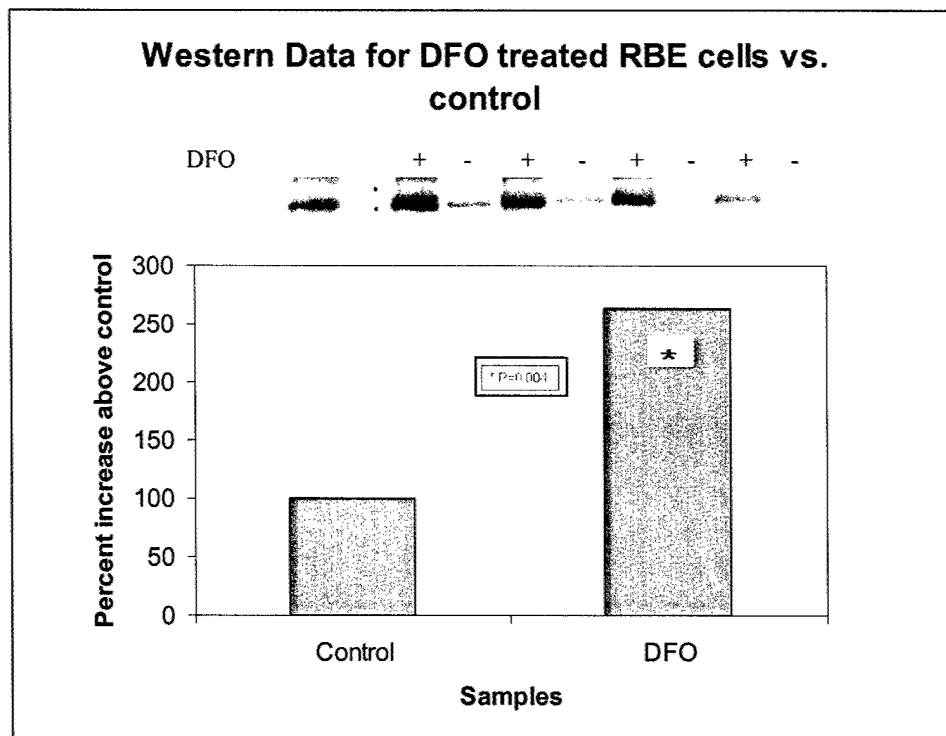


Figure 7. Western blot analysis of DMT-1 in RBE4 cells treated in the presence and absence of desferrioxamine (DFO; 100 uM for 24 hours). Graph depicts the image quantification of the western blots, establishing a statistically

significant upregulation of DMT-1 in RBE4 cells treated with DFO compared with controls treated in RBE4 cell media alone. For additional details see above.

The effect of DFO treatment on the transport of uranium into RBE4 cells was also initiated along with studies on the utility and sensitivity of NAA in measuring uranium cell uptake in RBE4 cells. In preliminary studies, five flasks of RBE4 cells were treated with DFO for 24 hours, while 5 flasks were not (control media alone). After the 24-hour period (aimed at modulating expression levels of DMT-1; See Figure 7), two flasks each were treated with 8 mM uranium oxide (Alpha Aesar, Ward Hill, MA) for 15 and 30 minutes. The 5th flask was the DFO control (30 minutes added incubation in the absence of uranium). Additionally, 5 flasks untreated with DFO were treated with the 8 mM uranium oxide in the same way with the 5th flask being the media control. We consciously opted in these preliminary high concentration of uranium, given that these studies were meant to be a proof-of-concept, namely to establish our ability to detect uranium in the RBE4 cells. It is acknowledged that physiologically these concentrations are not relevant to in vivo exposure scenarios, given the mega-concentration of uranium that we used. The cell lysates were sent to Scott Lassell in the Department of Nuclear Engineering at North Carolina State University in Raleigh for Neutron Activation Analysis (NAA). According to the Nuclear Services experimental parameters: "Samples decayed for one week and [were] counted for 20 minutes each on a gamma spectroscopy system analyzing for Uranium". The results of this experiment are deemed highly exciting, and they demonstrate obvious uptake of uranium in both DFO treated and untreated RBE4 cells (Figure 8). There was no statistically significant difference in the uptake of uranium between the two time periods (15 and 30 min), and an 18.26% decrease in uptake for cells treated with DFO compared to RBE4 cells treated with regular media (Figure 8). While these initial results suggest that DMT-1 might not be involved in uranium transport, it will be obviously necessary to reproduce these studies in cells treated with lower concentrations of uranium, different time points, as well as in studies where the level of expression of DMT-1 is modulated, by DFO (increasing DMT-1 expression), supplementation with iron (decreasing DMT-1 expression), as well as cells transfected with DMT-1 (see section 2 of this progress report for methodology and availability of these cells).

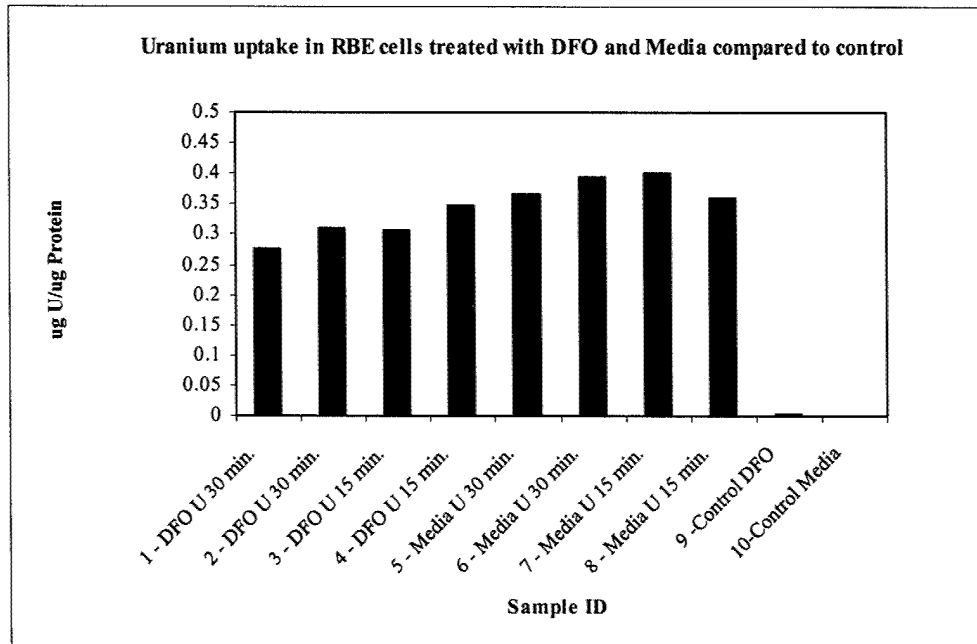


Figure 8. Uranium uptake in RBE4 cells treated with desferrioxamine (DFO; 100 μ M for 24 hours) and control media. For additional details see above.

KEY RESEARCH ACCOMPLISHMENTS

- Rat Brain Endothelial 4 (RBE4) cells were characterized in culture for morphological and functional properties.
- Addition of Astrocyte Conditioned Medium (ACM) restricts the barrier properties of the RBE4 monolayer, as evidenced by attenuated diffusion of 3H-inulin, a polar substance. The process is temperature-dependent.
- ACM upregulates the protein expression of E-cadherin, an adhesion molecule invoked in tightening the morphological characteristics of the blood-brain barrier (BBB)
- ACM downregulates protein expression levels of the Divalent Metal Transporter-1 (DMT-1).
- DMT-1 is expressed in RBE4 cells and its level of expression is modulated by iron (Fe) levels in the culturing medium. Treatment with the Fe chelator, desferrioxamine, (DFO) upregulates the protein expression of DMT-1 in RBE4 cells.
- RBE4 and Chinese Hamster Ovary (CHO) cells have been successfully transfected with DMT-1, allowing for future studies on DMT-1 in uranium transport.

- Uranium is readily taken up by RBE4 cells, as shown by Neutron Activation Analysis (NAA), raising the possibility that it is transported into the central nervous system (CNS).
- Preliminary studies suggest that treatment of astrocytes with DFO, which results in upregulation of DMT-1 expression levels in RBE4 cells does not increase the uptake of uranium into these cells.
- The studies fulfill the technical goals outlined for year 1 of this proposal, positioning the laboratory for controlled studies on the putative role of DMT-1 in the transport of uranium into the brain.

CONCLUSIONS

Taken together, the studies presented in this section clearly substantiate our ability to monitor the upregulation and downregulation of DMT-1 upon pharmacological manipulations, and the uptake of uranium by brain endothelial cells. Although it is yet to be shown, which of the membrane transporter is predominantly associated with the process (clearly multi-transporter involvement remains a possibility), we believe that our preliminary studies over the first few months have established the necessary techniques and proof-of-concept for uranium measurements. *We are well positioned to mechanistically determine if and how uranium is transported across these cells, a critical process in the potential accumulation of uranium in the central nervous system (CNS).*

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