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14. ABSTRACT Multiple sclerosis (MS) is a chronic, autoimmune neurodegenerative disease. Epstein-Barr virus (EBV) infection is associated with MS pathogenesis. However, mechanism for the EBV-MS connection is unclear. The blood–brain barrier (BBB) is a separation of circulating blood and the brain extracellular fluid in the central nervous system. BBB contains both endothelial cells as well as astrocytes. Interestingly EBV is able to infect both kinds of cells. Because EBV is able to transfer infection from one cell type to another cell type, it is thus hypothesized that EBV uses endothelial cells to infect astrocytes in the BBB, generate serials of cytokines that may eventually cause a leakage in the BBB. An in vitro model for human BBB will be established and infected the endothelial side of the BBB with EBV. Whether BBB is infected by the virus, the expression profiles of cytokines and other cellular genes, as well as the integrity of the BBB will be determined. Because both cytokine production and leakage of BBB are critical events for MS, this line of experiments may provide an evidence to support the etiological role of EBV in MS disease.					
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Introduction:

Multiple sclerosis (MS) is a chronic, autoimmune neurodegenerative disease. Many aspects of MS, including its cause, are not well understood. Patients with MS seem to have genetic vulnerability to certain environmental factors such as Epstein-Barr virus (EBV) infections. EBV is a herpesvirus that infects many cell types and associated with other autoimmune diseases. The blood-brain barrier (BBB) is a separation of circulating blood and the brain extracellular fluid in the central nervous system (CNS). The BBB barrier includes endothelial cells, a thick basement membrane, and astrocytes. The BBB leakage is a critical event in MS. Astrocytes maybe one of the major cytokine producers in MS. We hypothesize that EBV uses endothelial cells to infect/bind to astrocytes in BBB, generate serials of cytokines including tumor necrosis factor alpha (TNF-a) and interleukin6 (IL-6) in astrocytes. The production of cytokines may eventually cause a leakage in BBB and allowing other cells/materials into the CNS, leading to nerve cell damages, and eventually MS.

Body:

All research was done at the University of Nebraska-Lincoln. The objective of this project is to determine if EBV infects astrocytes in the human blood brain barrier (BBB), leading to cytokine production, BBB damage, and eventually multiple sclerosis.

Task1: Determine the extent to which EBV infects astrocytes in BBB, leading to cytokine production. EBV may use endothelial cells to infect/bind to astrocytes in human BBB, generating serials of cytokines including tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6) in astrocytes.

Subtask a: Establish a human BBB model in vitro. We were trying to use primary human brain microvascular endothelial cells and primary astrocytes, and establish a BBB that closely mimics the “in vivo” situation by culturing brain capillary endothelial cells on one side of a filter and astrocytes on the other as shown in the following Figure 1.

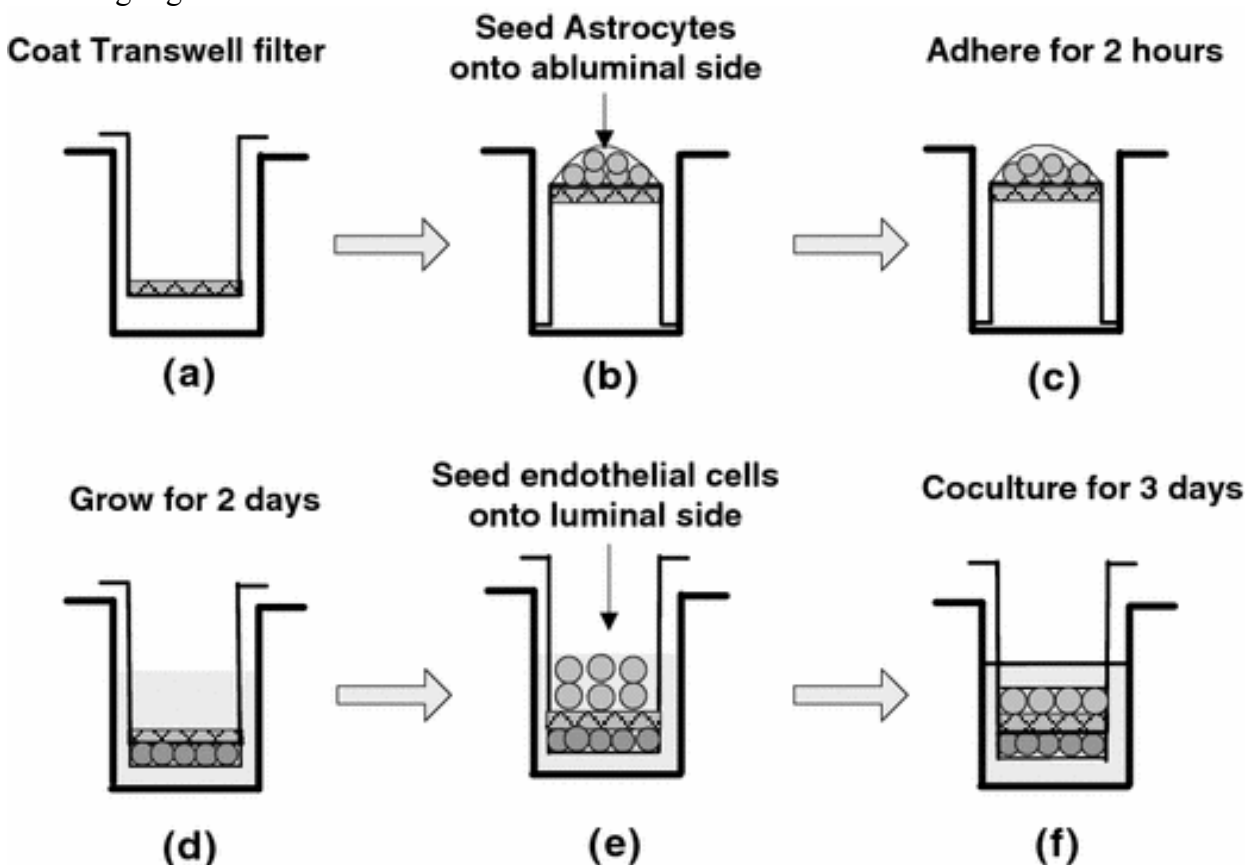


Figure 1: the experimental procedure for establishing a human BBB in vitro.

We used Matrigel and a mixture of collagen types I and IV as the basement membrane substitutes for our in vitro BBB models. For Matrigel coating, Matrigel was diluted in Dulbecco's PBS (DPBS) to a concentration of 476 µg/mL. The Transwell filter was reversed and 200 µL of diluted Matrigel solution was added to the abluminal side of each Transwell filter. The Transwell plate containing the filters was placed in a 37 °C, 5% CO₂-95% air incubator for 3 h for the Matrigel to gel. For collagen mixture coating, types I and IV were diluted in DPBS to 0.5 mg/mL and their pH brought to 7.4 with NaOH and HCl respectively. The two collagens were then mixed thoroughly (40:60, collagen I: collagen IV, v/v) and 200 µL of the mixture was added to the luminal side of each Transwell filter. The Transwell plates with filters were incubated at 37 °C for 1 h for the mixture to gel. After that, the Matrigel and collagen mixture coated Transwell filters were placed into a sterile hood and allowed to air dry for two days. Once dry, the coated filters were rehydrated in DMEM+10% FBS for 1 h in a 37 °C, 5%CO₂-95% air incubator prior to cell seeding. Transwell filter with 0.4 µm diameter pores was used as a scaffold for the co-culturing primary human brain endothelial cells and primary human astrocytes on different sides of the filter. The brain microvascular endothelial cells (MEC) were first seeded onto the abluminal side of the inverted Transwell filter at a density of $3-10 \times 10^4$ cells per filter (b), allowed to adhere for 2 h (c), then the filter was flipped back and the astrocytes were cultured for 2 days in Astrocyte Medium supplemented with 10% FBS and 1% PS (d). At the end of the second day, Human Brain Microvascular Endothelial Cells (HBMEC) were seeded onto the luminal side of Transwell filter at a density of $6-10 \times 10^4$ cells per filter (e) and cocultured with astrocytes for an additional 3–4 days in Endothelial Cell Medium or Astrocyte medium (f). After several days, we then examined the cell attachments to both sides: astrocytes always had big problems! Cells could only cover the surface sparsely: it was independent of the amount of cells introduced. Endothelial cells are better. We were following the published report on the BBB in vitro modeling (Li et al., 2010). Unfortunately, we were unable to establish an “unleaky” BBB in the laboratory. We have the following explanation for the failure: 1) Human systems are different from rodent system. All established BBB models are using rodent system; 2) Human astrocytes may be too old. The human astrocytes were obtained from adult brain tissue, according to the suppliers. In rodent system, the astrocytes were obtained from 8-10 days old mice (Li et al., 2010). 3) We also did to put the endothelial cells first, and then human astrocytes. Both gave similar results.

Subtask 1b: Infect endothelial side of BBB with free virus. Because we failed to establish a functional human BBB model in vitro, we did not test if the EBV infects the human brain microvascular endothelial cells. The results showed that EBV can barely infect the cell type. We could not detect any infection by the EBV by Western blot, or GFP expression (Our EBV has a green fluorescent protein inside the genome). However, it has to be stressed that infection of one cell type may not represent the in vivo situation of the BBB.

Task2: Determine the extent to which EBV damages BBB. As the human BBB model cannot be established, currently we have not addressed this task yet. We are using the leftover money to circumvent the current problems and test if EBV infects other brain cells.

KEY RESEARCH ACCOMPLISHMENTS:

- None. Human blood-brain barrier (BBB) in vitro model was not established and we are sorry.

REPORTABLE OUTCOMES:

No Publication/Presentation directly resulting from the award.

CONCLUSION:

There is one major experiment done as proposed in the grant: to establish and infect the human BBB model and test the gene expression as well as BBB integrity in vitro. This study may establish a role of EBV on MS or

other CNS diseases via modulation of BBB as both cytokine production and leakage of BBB are critical events for MS. The proposal is targeting virus infection on both events.

We have done the experiments: human BBB model has serious problems. The integrity of the BBB could not be established in our laboratory. This may mean the human cells are different from rodent cells, as rodent BBB has been reported, but not humans.

As the quality of the BBB have some issues, we now contrite on alternative approaches. We found that the infection of human brain endothelial cells by EBV seems very difficult. It may mean that EBV may use other routes to gain the entrance of the brain. We are currently doing to use EBV to infect astrocytes in the absence and presence of endothelial cells and test if the endothelial cells affects the capability of EBV to infect astrocytes and furthermore the expression of host genes, especially cytokine genes in astrocytes. The results will hint the role of EBV, once they are in the broken BBB, for the activation of cytokine genes.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

Li, G., Simon, M. J., Cancel, L. M., Shi, Z. D., Ji, X., Tarbell, J. M., Morrison, B., 3rd, and Fu, B. M. (2010). Permeability of endothelial and astrocyte cocultures: in vitro blood-brain barrier models for drug delivery studies. *Ann Biomed Eng* **38**(8), 2499-511.

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