Epstein-Barr Virus dUTPase Induces Neuroinflammatory Mediators: Implications for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

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ABSTRACT

Purpose: Neuroinflammation is a common feature in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), affecting 85%–90% of all patients, yet the underlying mechanism or mechanisms responsible for the initiation and/or promotion of this process is largely unknown. Multiple reports, however, have suggested a role for Epstein-Barr virus (EBV), in particular, in ME/CFS, but its potential role, if any, in the neuroinflammatory process has not been addressed. In support of this premise, studies by our group have found that the EBV protein deoxyuridine triphosphate nucleotidohydrolase (dUTPase) induces anxiety and sickness behaviors in female mice. We also found that a small subset of patients with ME/CFS exhibited prolonged and significantly elevated neutralizing antibodies against EBV dUTPase protein in serum, which inversely correlated with ME/CFS symptoms. A larger ME/CFS case–control cohort study further confirmed that a significant percentage of patients with ME/CFS (30.91%–52.7%) were simultaneously producing antibodies against multiple human herpesviruses-encoded dUTPases and/or human dUTPase. Altogether, these findings suggest that EBV dUTPase protein may be involved in the neuroinflammatory process observed in ME/CFS. Thus, the aim of the present study was to determine whether the EBV dUTPase protein could contribute to neuroinflammation by altering the expression of genes involved with maintaining blood–brain barrier (BBB) integrity and/or modulating synaptic plasticity.

Methods: With the use of human immortalized astrocytes, microglia, and cerebral microvascular endothelial cells, we conducted time-course (0–24 h) experiments with EBV dUTPase protein (10 μg/mL) to determine what effect(s) it may have on the expression of genes involved with BBB permeability, astrocytes and microglia cell function, tryptophan metabolism, and synaptic plasticity by quantitative reverse transcription polymerase chain reaction (qRT-PCR). In parallel, *in vivo* studies were conducted in female C57Bl/6 mice. Mice were injected by the intraperitoneal route with EBV dUTPase protein (10 μg) or vehicle daily for 5 days, and the brains were collected and processed for further qRT-PCR analysis of the *in vivo* effect of the dUTPase on the dopamine/serotonin and γ-aminobutyric acid/glutamate pathways, which are important for brain function, using RT^2 Profiler PCR Arrays.

Findings: EBV dUTPase protein altered the expression *in vitro* (12 of 15 genes and 32 of 1000 proteins examined) and *in vivo* (34 of 84 genes examined) of targets with central roles in BBB integrity/function, fatigue, pain synapse structure, and function, as well as tryptophan, dopamine, and serotonin metabolism.

Implications: The data suggest that in a subset of patients with ME/CFS, the EBV dUTPase could initiate a neuroinflammatory reaction, which contributes to the fatigue, excessive pain, and cognitive impairments observed in these patients. (*Clin Ther.*

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INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a chronic multisystem illness of unconfirmed cause. ME/CFS has largely been diagnosed from Fukuda CFS criteria[1] and/or the Canadian ME/CFS criteria.[2] The Institute of Medicine in 2015 proposed, in addition to a new name of systemic exertion intolerance disease, a new case criteria focused on chronic fatigue, postexertional fatigue, and orthostatic intolerance/cognitive deficits for patients with this disease.[3] Neurocognitive dysfunction has been reported to occur in 85%–90% of patients with ME/CFS. Self-reporting and assessment by objective task performance tests have found that the cognitive problems include declining attention and concentration, slow information processing, and declining memory and learning of complex information.[4]

Several neuroimaging studies that used magnetic resonance imaging, functional magnetic resonance imaging, magnetic resonance spectroscopy, positron emission tomography, and single photon emission computerized tomography have found structural and functional alterations in the brains of patients with ME/CFS.[5–9] Studies that used positron emission tomography[10] and whole-brain magnetic resonance spectroscopy[11] found evidence of widespread neuroinflammation in the brains of patients with ME/CFS, which was associated with the severity of neuropsychologic symptoms. Although numerous models and hypotheses have been proposed to explain the neuroinflammation observed in patients with ME/CFS, the underlying mechanisms that contribute to this neuroinflammatory reaction(s) remain largely undefined.[8,12–15]

In more than one-half of patients with ME/CFS, onset is associated with acute flu-like symptoms,[16] and multiple reports in the literature have suggested a role for viruses in ME/CFS,[17–19] particularly Epstein-Barr virus (EBV). However, further mechanistic studies to address a causal relationship between a virus and ME/CFS are missing and would be of high importance. In support of this premise, several studies have found that EBV can infect and undergo lytic replication in neuronal cell lines as well as primary human neurons[20] and abortively replicates in human astrocytes.[21] Although acute infections of EBV are known to cause neurologic complications in immunocompetent patients with infectious mononucleosis, generally, they are benign. Conversely, in patients co-infected with HIV-1, EBV is associated with primary central nervous system (CNS) lymphoma, which has a poor prognosis.[22] EBV is also a risk factor in the development of multiple sclerosis,[23,24] a claim further supported by a recent study that found significant levels of EBV genomic DNA in B cells, astrocytes, and microglia cells in the brains of patients with multiple sclerosis.[24] These studies focused on the virus, and none have addressed the possibility of a virus-encoded protein rather than the virus itself as the cause or driver of the pathologic features observed in these diseases.

We have previously reported that the EBV deoxyuridine triphosphate nucleotidohydrolase (dUTPase) protein, which is encoded by the *BLLF3* gene and expressed during lytic/abortive-lytic replication of the virus, possesses functions that act as a pathogen-associated molecular pattern for Toll-like receptor (TLR) 2. Engagement of TLR2 by EBV dUTPase leads to the activation of nuclear factor κ light chain enhancer of activated B cells (NF-κB) and subsequent modulation of downstream genes involved in several cellular processes, including chronic inflammation, effector T-cell function, and neurotransmitter function.[25–30] We have also found that the EBV dUTPase can be secreted from B cells in exosomes,[28] which function as intracellular messengers and can cross the blood–brain barrier (BBB). More important, we have found that EBV dUTPase induces anxiety and sickness behaviors in mice[31,32] and that patients with ME/CFS have increased serum levels of antibodies to the EBV

dUTPase.[19] The present study explored the contribution of EBV dUTPase protein, if any, in the neuroimmune dysfunction associated with ME/CFS with the use of *in vitro* and *in vivo* model systems.

METHODS

Reagents

Applied cell extracellular matrix (catalog no. G422), Prigrow I medium (catalog no. TM001), Prigrow III medium (catalog no. TM003), Prigrow IV medium (catalog no. TM004), and PriCoat T25 flasks (catalog no. G299) were purchased from Applied Biological Materials Inc (Richmond, BC, Canada). X-vivo 15 serum-free medium (catalog no. 04-418Q) was purchased from Lonza Inc (Basel, Switzerland). Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 were purchased from PeproTech (Rocky Hill, N J). Fetal bovine serum and SuperScript IV First-Strand Synthesis Kit were purchased from Invitrogen (Carlsbad, Calif). Trizol reagent was purchased from Ambion (Austin, Tex). TaqMan Gene Expression Master Mix was purchased from Applied Biosystems (Foster City, Calif). RNeasy Mini Kit, RNase-Free DNase Set, mouse dopamine (DA)/serotonin (catalog no. PAMM-158Z), mouse γ-aminobutyric acid (GABA)/glutamate (catalog no. PAMM-152Z) RT^2 Profiler PCR Arrays and SYBR green reaction master mix were purchased from Qiagen (Valencia, Calif). Ketamine (100 mg/mL) was obtained from the Wexner Medical Center Pharmacy at The Ohio State University, and xylazine hydrochloride was purchased from Sigma (St. Louis, Mo). The Human L-1000 Antibody Arrays were purchased from RayBiotech (Peachtree Corner, Ga).

Purification of Recombinant EBV dUTPase

Subcloning and purification of recombinant dUTPase protein was performed as previously described.[25,29,30] Recombinant dUTPase protein preparations were tested for the presence of contaminants as described previously[25,29] and were free of detectable levels of lipopolysaccharide, peptidoglycan (SLP-HS), DNA, and RNA. Protein concentration was determined with the Qubit fluorimeter (Invitrogen, Carlsbad, Calif). The purified recombinant EBV dUTPase protein used in these studies was stored at −80 °C in elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 M imidazole; pH 7.4) until further use. Vehicle used in *in vitro* and *in vivo* experiments was elution buffer.

Cells

Immortalized human astrocytes (fetal: SV40 large T antigen; catalog no. T0280), microglia cells (adult; catalog no. T0251), and cerebral microvascular endothelial cells (catalog no. T0259) were obtained from Applied Biological Materials Inc (Richmond, BC, Canada), and were maintained in Prigrow IV (astrocytes), Prigrow III (microglia), and Prigrow I (cerebral microvascular endothelial cells) medium supplemented with 10% fetal bovine serum in a humidified atmosphere at 37 °C and 5% carbon dioxide. Human dendritic cells (hDCs) from healthy female donors were obtained from Astarte Biologics (Bothell, Wash) and cultured in X-Vivo serum-free medium supplemented with 500 U/mL GM-CSF and IL-4.

Human Immortalized Cell Treatments

Cells were seeded at a density of 8×10^4 in 6-well plates coated with applied cell extracellular matrix and cultured in the appropriate Prigrow supplemented medium until they were 70%−90% confluent. Media was then replaced, and cells were treated with EBV dUTPase protein (10 μg/mL) or vehicle for various time points (0, 0.5, 1, 2, 3, 4, 6, and 24 h). After treatments, cells were collected for further processing and mRNA gene expression analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Studies were performed in triplicate a minimum of 3 times.

Human DC Treatment and Protein Array

Cells were seeded at a density of 2.5×10^5 in 24-well plates and cultured in X-Vivo serum-free medium supplemented with 500 U/mL GM-CSF and IL-4. The next day, hDCs were stimulated with EBV dUTPase protein (10 μg/mL) or vehicle for 24 h, as described previously.[27–29] After treatments, cell culture supernatants were collected, the concentration of select immune mediators was measured with a Human L-1000 Antibody Array (RayBiotech), and the fluorescence was captured with an Axon GenePix laser scanner. Positive control spots on the array are standardized amounts of biotinylated immunoglobulin G printed

directly onto the array. Negative control spots on the arrays contain antibody diluent buffer, and their signal intensities represent nonspecific binding of the cyanine 3-Conjugated Streptavidin (background signal). Normalized signal intensity data represent values in which the background signal of negative control spots has been subtracted out and normalized to the mean signal intensity of positive control spots. After normalization, any $\geq$1.5-fold increase or $\leq$0.65-fold decrease in signal intensity for a single analyte between samples was considered a measurable and significant difference in expression, provided that both sets of signals are well above background (mean background [2 SD]; accuracy $\approx$ 95%). Data represent 2 experiments (2 biological replicates/experiment) and are expressed as the fold-change signal intensity for each analyte in dUTPase-treated samples relative to the vehicle control samples.

Mice

Female wild-type C57BL/6J mice (6−8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, Maine) and housed for 7 days for acclimatization before use. Mice were housed in a Biosafety Level 2 barrier facility on a 12-h light/dark cycle and given chow and water *ad libitum*. The facilities are maintained at 22 °C−23 °C and 30%−50% relative humidity. All experiments were conducted in accordance with the Institutional Animal Care and Use Guidelines of The Ohio State University. Mice (n = 40, 10 mice per treatment group) were injected daily with EBV dUTPase protein (10 μg/mL) or vehicle control (100 μL total volume) for 5 days. This dose was selected from our previous studies.[27–32] Mice were then deeply anesthetised with a ketamine/xylazine mixture (ketamine 90 mg/kg and xylazine 8 mg/kg; in 200 μL) administered by intraperitoneal injection. Mice were observed for response to hind paw pinch, and, when no response was observed, surgery was performed to perfuse the brain. The thoracic cavity was opened to expose the heart, and the perfusion needle was inserted into the left ventricle. The mouse was perfused with phosphate-buffered saline for 3−5 min, using a perfusion pump. After perfusion surgery, mice were euthanized by decapitation, and the brain was removed. Brains were stored in RNALater solution for further analysis by qRT-PCR.

Quantitative RT-PCR Analysis

Total RNA was isolated from human cells with the use of Trizol reagent and further cleaned up with RNeasy Mini kit and on column DNAse I treatment. Whole mouse brain tissue samples were homogenized with the Bead Ruptor 12 (Omni International, Inc, Kennesaw, Ga) in 7-mL screw cap tubes that contained DNase/RNase-free 1.4-mm ceramic beads at high speed for 20 s, followed by a centrifugation step at 12,000×*g* for 10 min at 4 °C, according to the manufacture's protocol. Total RNA was then isolated and cleaned up as described above for human cells, and RNA concentration and purity were determined with the NanoDrop 2000. cDNA was synthesized with the SuperScript IV First-Strand Synthesis Kit, and qRT-PCR was performed on a QuantStudio 6 Flex instrument (Applied Biosystems), using Qiagen DA/serotonin (catalog no. PAMM-158Z) and GABA/glutamate (catalog no. PAMM-152A) RT^2 pathway arrays (96-well plate format) and SYBR green chemistry (mouse brain samples) or TaqMan chemistry (immortalized human cell samples) with the use of custom made 96-well plates that contained the following primer/probe target sets. BBB target genes were *TJP2*-Hs00910543_m1, *CGN*-Hs00430426_m1, *CLDN5*-Hs00533949_s1, *OCLN*-Hs00170162_m1, *RAPGEF6*-Hs00255483_m1, *CDH5*-Hs00901465_m1, *IL1B*-Hs00174097_m1, *IL6*-Hs00985639_m1, *TNF*-Hs01113624_g1, *TLR2*-Hs02621280_s1, *NFKB1*-Hs00765730_m1, *CTNNB1*-Hs00355049_m1, and *TBP*-Hs00427620_m1; And astrocytes/microglia target genes were *IDO1*-Hs00984148_m1, *KMO*-Hs00175738_m1, *AADAT*-Hs00212039_m1, *PTGS2*-Hs00153133_m1, *TNC*-Hs01115665_m1, *GPR84*-Hs01874713_s1, *IL1B*-Hs00174097_m1, *IL6*-Hs00985639_m1, *TNF*-Hs01113624_g1, *TLR2*-Hs02621280_s1, *EGR1*-Hs00152928_m1, *VEGFA*-Hs00900055_m1, and *TBP*-Hs00427620_m1. All reactions were run in triplicate in a final volume of 20 μL and repeated at least twice. PCR values were

normalized to internal standards (*B2m* and *Gus B*, mouse brain samples or *GUSB*-Hs00939627_m1 and *HPRT1*-Hs01003267_m1, human samples) and expressed as the mRNA expression levels relative to the control (vehicle control-treated samples). The fold-change/fold-regulation of the expression for each target gene was calculated with the threshold cycle (Ct) values as follows: fold-change ($2^{-\Delta\Delta Ct}$) is the normalized gene expression ($2^{-\Delta Ct}$) in the test sample divided by the normalized gene expression ($2^{-\Delta Ct}$) in the control sample. Fold-regulation represents fold-change results in a biologically meaningful way. Fold-change values > 1 indicate a positive- or an upregulation, and the fold-regulation is equal to the fold-change. Fold-change values < 1 indicate a negative or downregulation, and the fold-regulation is the negative inverse of the fold-change.

The *P* values are calculated, based on a Student's *t* test of the replicate $2^{-\Delta Ct}$) values for each gene in the control group.

Statistical Analysis

GraphPad Prism 7 software (GraphPad Software, La Jolla, Calif) was used for all statistical analyses. Values of $P < 0.05$ were considered statistically significant. To compare 2 groups a Mann-Whitney test was used. RNA and protein experiments were not corrected for multiple comparisons.

RESULTS

Effect of EBV dUTPase on Gene Expression in Human Cerebral Microvascular Endothelial Cells, Astrocytes, and Microglia Cells

We have previously reported that the EBV dUTPase protein increased the expression and secretion of IL-1β, IL-6 and tumor necrosis factor (TNF)-α from hDCs and peripheral blood mononuclear cells in a TLR2-dependent manner.[29] Because IL-1β and IL-6 can disrupt the BBB[33,34] and can affect neurocognitive functions in patients with ME/CFS,[35–38] we asked whether EBV dUTPase protein could modulate the expression of genes important in maintaining BBB integrity and/or synaptic plasticity in immortalized human cerebral microvascular endothelial cells, astrocytes, and microglia cells. As shown in Table I, treatment of human cerebral microvascular endothelial cells with EBV dUTPase protein resulted in a rapid increase in *IL-1β* (10-fold) and *IL-6* (3.5-fold) mRNA expression, beginning at 30 min after treatment and reaching maximum induction levels at 2 h (32-fold and 43-fold for *IL-1β* and *IL-6*, respectively) compared with vehicle-treated control. The increase in *IL-1β* and *IL-6* mRNA expression levels was accompanied by a parallel increase in TLR2 and NF-κB gene expression in these cells. Conversely, EBV dUTPase downregulated the expression of the genes that encoded the tight junction proteins occludin (*OCLN*; 1.62-fold), claudin-5 (*CDH5*; 1.80-fold), and cingulin (*CGN*; 2.24-fold), which reached maximum levels 6 h after treatment.

EBV dUTPase stimulation of microglia cells resulted in the induction of *IL-1β* and *IL-6* mRNA expression, which reached their maximum level of expression at 6 h after treatment (22-fold and 12-fold for *IL-1β* and *IL-6*, respectively). Conversely, although an induction of IL-1β and IL-6 mRNA expression was found in astrocytes, it was not as robust as that observed in cerebral microvascular endothelial cells or microglia cells. The expression of *TLR2* was also upregulated in cerebral microvascular endothelial cells and astrocytes with maximum induction occurring at 24 h after treatment (4.86- and 5.05-fold, respectively); no changes in *TLR2* expression were observed in microglia cells. Furthermore, the dUTPase protein upregulated TNF-α mRNA expression in astrocytes and microglia with maximum expression occurring at 2 h after treatment. The early growth response gene 1 (*Egr-1*) was significantly downregulated in both astrocytes (−2.539-fold) and microglia cells (−5.138-fold), beginning at 3 h after treatment. Of interest, Egr-1 encodes for a transcriptional regulator reported to be involved in neuronal plasticity.[39,40] A significant upregulation of prostaglandin-endoperoxide synthase 2 (*PTGS2*), which encodes for cyclooxygenase 2 (COX2), was observed in astrocytes as early as 0.5 h after treatment, which peaked (6.646-fold) at 2 h after treatment. Although in microglia cells, the dUTPase protein strongly induced the expression of *PTGS2* by 32.969-fold at 2 h after stimulation, it remained significantly increased throughout the treatment period. Similarly, a significant upregulation in the expression of *VEGFA* (vascular endothelial growth factor) and TNC (brain extracellular matrix

Table I. Time course gene expression analysis of Epstein-Barr virus deoxyuridine triphosphate nucleotidohydrolase treatments of immortalized human endothelial cells, astrocytes, and microglia cells by quantitative reverse transcription polymerase chain reaction.

Gene Symbol	0.5 Hour	1 Hour	2 Hours	3 Hours	4 Hours	6 Hours	24 Hours
Immortalized human endothelial cells							
IL1b	10.0364*	15.1413*	31.7606*	18.2101*	16.3126*	13.2937*	10.9182*
IL6	3.5231*	16.9808*	43.3237*	18.653*	10.8122*	10.829*	13.0713*
NFkB	0.9741	1.027	3.2147*	5.2695*	4.1351*	1.7161*	2.818*
TLR2	0.7067	1.027	1.407	2.577*	2.1737*	2.472*	4.8573*
CGN	−1.0639	−1.0492	−1.04	−1.6232	−1.8893*	−2.2359*	−1.2709
OCLN	1.2188	−1.0073	1.4671	−1.1103	−1.2797	−1.6228*	1.5438*
CDH5	−1.763*	−1.5308*	−1.1593	−1.2444	−1.2874	−1.8006*	1.0108
Immortalized human astrocytes							
KMO	−1.0807	−1.1204	−1.1716	−1.0948	−2.9312*	−1.057	1.6771*
PTGS2	2.0392*	2.733*	6.6461*	2.9921*	3.0876*	1.9541*	1.5637
IL1b	1.1165	1.1447	2.619*	1.7597*	2.16*	1.6313*	3.1788*
IL6	1.4196	1.5889*	1.279	2.2214*	2.2207*	1.7734*	−1.4954
AADAT	−1.0021	−1.0094	−1.0007	1.074	1.0497	−1.0461	−1.129
EGR1	2.8501*	4.1641*	−1.0007	−2.5394*	−2.3958*	−2.4906*	1.1173
TLR2	1.0147	1.1282	1.9759*	1.9998*	1.5735*	1.2561	5.0543*
IDO1	−1.1676	−1.0688	1.1451	−1.2108	1.2509	−1.1603	1.0024
TNF	1.2614	4.9161*	1.7777*	1.1527	−1.401	1.4575	−1.1173
VEGFA	1.1732	1.4449	1.3985	1.429	1.0136	1.4687	1.1337
TNC	1.0007	1.1507	1.1822	1.0658	−1.454	−1.2897	−1.0331
Immortalized human microglia cells							
KMO	1.3599	1.3599	1.6121*	2.4753*	1.5756*	1.6262*	1.9897*
PTGS2	1.2146	1.2146	32.9696*	28.7065*	31.8242*	28.6373*	5.5168*
IL1b	1.0673	11.4468*	17.9898*	11.5652*	7.7246*	22.7129*	4.4798*
IL6	−1.0411	4.5281*	12.2746*	10.2858*	8.5455*	12.435*	1.3081
AADAT	1.0244	−1.043	−1.1827	−1.2233	−1.1061	−1.2476	−1.339
EGR1	−1.5767*	−1.1995	−1.0081	−5.1383*	−5.2705*	−6.2691*	−1.1319
TLR2	1.1128	−1.0849	1.6425*	1.7396*	1.6936*	1.1975	1.3081
IDO1	1.2555	−1.5851	−1.1672	1.149	1.0078	2.8985*	3.1619*
TNF	−1.0803	2.0305*	3.8838*	3.1083*	2.0876*	1.2817	1.5184
VEGFA	1.3083	3.0389*	3.071*	2.0312*	1.6698*	3.2235*	1.528
TNC	−1.1904	1.8138*	2.7783*	3.2988*	2.8257*	2.7879*	2.2279*

Data represent mRNA expression levels relative to the vehicle-treated control and expressed as fold-regulation.
* Mean fold-change in expression was significantly different from control by ≥ 1.5 with $P < 0.05$, n = 3.

protein tenascin C) was observed in microglia cells but not in astrocytes. Enzymes involved in kynurenine metabolism were differentially modulated in astrocytes and microglia cells with kynurenine aminotransferase II (*AADAT*) and indoleamine 2.3-dioxygenase (*IDO1*) being unchanged, and kynurenine monooxygenase (*KMO*) was downregulated by 2.93-fold at 4 h after treatment in astrocytes. In microglia cells, a significant increase was found in *KMO* mRNA expression by 2.474-fold

at 3 h after treatment and in *IDO1* at 6 (2.898-fold) and 24 (3.162-fold) hours after treatment. No significant changes in the expression of *AADAT* were observed at any of the time points examined.

EBV dUTPase Induces hDCs to Produce Proteins With Neuroimmune Modulatory Functions

Under physiologic conditions, the presence of DCs in the brain parenchyma was minimal, but their numbers increased during neuroinflammation.[41] We have previously reported that the EBV dUTPase protein increased the expression and secretion of proinflammatory cytokines.[25,28–30] Thus, we next determined whether the EBV dUTPase induced the secretion of additional proteins or other soluble factors in hDCs that could contribute to a neuroinflammatory microenvironment. Briefly, primary hDCs were treated with the EBV dUTPase protein for 24 h, and culture supernatants were collected and analyzed with a human immune Antibody Array (RayBiotech), as described in Methods. As shown in Table II, EBV dUTPase increased the production of >30 proteins, including several neurotrophic factors involved with synaptic transmission, proteins that modulate inflammation and extracellular matrix, compared with vehicle control. Not surprisingly, these proteins have been associated with several neurologic conditions, including anxiety and sickness behaviors, depression, multiple sclerosis, schizophrenia, and Alzheimer's disease.

Effects of EBV dUTPase on Gene Expression in female C57Bl/6 Mouse Brain

To better understand the biological effects of the EBV dUTPase protein on gene modulation in the CNS, C57Bl/6 female mice were injected with dUTPase protein or vehicle daily for 5days. Mice were then sacrificed, and brains were harvested for further processing and gene expression analysis by qRT-PCR, as described in Methods. The results of this study identified 33 differentially expressed genes between control and EBV dUTPase protein-injected mice for each normalized dataset, using the criteria fold change of ≥ 1.5 and $P < 0.05$. As shown in Table III, the EBV dUTPase downregulated significantly the mRNA expression of BBB genes *Cgn* (cingulin), *Tjp2* (Tight junction protein 2), *Rapgef6* (Rap guanine nucleotide exchange factor 6), and *Mmp15* (Metalloprotease 15) and the synaptic plasticity genes *Synpo* (synptopodin), *Lin7B* (Lin 7 homolog B), *Rgs20* (Regulator of G protein signaling), and *Rab33a* (RAB33A member of Ras oncogene family), whereas it upregulated the mRNA expression of Egr1 (Early growth response 1) compared with vehicle control. The dUTPase protein also significantly upregulated the expression of *Gch1* (GTP cyclohydrolase), whereas it downregulated *Gpr84* (G protein-coupled receptor 84), which are genes involved in pain. A downregulation in the expression of *Kmo* (kynurenine-3-monooxygenase), a key gene product in the tryptophan metabolism pathway, *Gpr171* (G protein-coupled receptor 171) and *Tbc1d1* (TBC1 family domain member 1) genes was also observed. Both Gpr171 and Tbc1d1 are important proteins involved in metabolism/energy pathways. More important, the EBV dUTPase affected the DA and serotonin as well as GABA and glutamate pathways in the mouse brain, which are important for brain development and CNS functions, including cognition, emotion processing, and movement. Of interest, among the genes downregulated by the dUTPase in the brain belonging to the DA/serotonin pathways were *Alox12* (arachidonate 12-lipoxygenase), *Arrb1* (β-arrestin 1), *Dbh* (DA β-hydroxylase), *Fos*, *Nr4a1/Nur77* (nuclear receptor 77), *Th* (tyrosine hydroxylase), *Tph2* (tryptophan hydroxylase 2), *Slc6a3*, *Slc6a4*, *Drd1* (DA receptor D1), *Drd5*, *Grk6*, *Pde10a* (phosphodiesterase 10A), *Pik3cg*, and *Plcb1* with the 3 most downregulated genes being *Slc6a4* (DA transporter), followed by *Tph2* and *Th*. Of interest, Th and Tph2 are the rate-limiting enzymes in catecholamine synthesis and serotonin biosynthesis, respectively. In addition, single nucleotide polymorphisms in the *Alox12* gene have been associated with slower activity, loss of energy and tiredness, loss of pleasure. and diminished libido in schizophrenia cohorts.[42] β-Arrestin 1 has been found to have a neuroprotective role and knockout of *Arrb1* was found to exacerbate brain infarction and neurologic deficit in a mouse model of cerebral ischemia.[43] Similarly, several studies have found the important role of the nuclear receptors Nr4a, including Nur77/Nr4a1, in DA neurotransmission in the developing and mature brain.[44] Decreased cortical

Table II. Major proteins modulated by EBV dUTPase in human primary dendritic cells* that alter brain function.

Protein	Fold-Change†	Function	Disease/Symptom Association
ACE-1/-2 (angiotensin-converting enzyme 1 & 2)	3.00/3.00	Zinc metallopeptidases	Alzheimer's disease
ADAMTS-15	330.00	Zinc metallopeptidases	Alzheimer's disease
APP	2.00	Amyloid precursor protein	Alzheimer's disease
BACE-1	4.00	β-site APP cleaving enzyme 1 or β-secretase	Alzheimer's disease
Brain-derived neurotrophic factor (BDNF)	2.00	Supports differentiation, maturation and survival of neurons and synaptic transmission	Alzheimer's disease Depression Schizophrenia
Chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2; GPR44)	3.00	Receptor for prostaglandin D2	Sickness behavior
COX-2	13.00	Cyclooxygenase 2	Inflammation
Dickkopf3 (DKK3)	3.00	Acts as a repressor/activator of WNT/β-catenin signaling	Alzheimer's disease
EN-RAGE (S100A12)	3.00	Ligand for RAGE; pro-inflammatory	Alzheimer's disease
EphB4/A1/A2/B6 (erythropoietin-producing hepatoma receptors)	45.00/3.00/ 3.00/3.00	Receptor tyrosine kinases; neural stem cell differentiation	
Fetulin A	3.00	Pro- and anti-inflammatory	Multiple sclerosis
Frizzled 5	35.00	Receptor for WNT ligands, establishment of neuronal polarity	Alzheimer's disease
Galanin	3.00	Neuropeptide	Nociception; Alzheimer's disease, epilepsy
Growth/differentiation factor 15 (GDF-15)	5.00	Neurotrophic factor	Oral cavity cancer
Glypican-5	8.00	Unknown; reported to regulate WNT and hedgehog pathways	B-cell lymphoma
IDE (insulin degrading enzyme)	2.74	Amyloid-β degradation	Alzheimer's disease
ITM2B	3.00	Regulator role in processing amyloid-β A4	Alzheimer's disease
LDLR (low-density lipoprotein receptor)	1.37	Expressed by adult neurons; binds ApoE	Alzheimer's disease
Lin41/TRIM71	3.00	E3 ubiquitin protein ligase; inhibits translation of EGR1	
MMP 2 (matrix metalloproteinase)	2.00	Calcium-dependent zinc endopeptidase	Alzheimer's disease

(*continued on next page*)

Table II. (*Continued*)

Protein	Fold-Change[†]	Function	Disease/Symptom Association
MMP 9 (matrix metalloproteinase)	6.00	Calcium-dependent zinc endopeptidase	Alzheimer's disease
Netrin-4	4.00	Ligand for Unc-5 homologue 5; promotes terminal branching of axons	
Neuritin (candidate plasticity gene 15; CPG15)	177.00	Neurotrophin synaptic plasticity	Depression
Orexin A/B	130.00/4.00	Neuropeptides important role in hippocampal neurogenesis spatial learning and memory	Depression, learning and memory deficiencies, inflammation
Presenilin 1	2.00	Component of γ-secretase complex	Alzheimer's disease
Presenilin 2	21.00	Component of γ-secretase complex	Alzheimer's disease
ProSAAS	3.00	Precursor protein processed to yield SAAS, GAV, PEN bigLEN, littleLEN	Behaviors, including anxiety, feeding, and stress
RAGE	2.00	Receptor for advanced glycation products	Alzheimer's disease
THY-1 (CD90)	4.00	T cell activation	Tumor suppressor nasopharyngeal carcinoma; axonal regeneration
TIMP-1, 2, 3, 4 (tissue inhibitor of metalloproteinase)	243.00/128.00/ 216.00 6.00	Major endogenous inhibitors of metalloproteinases in tissue	
TMEFF1 (tomorregulin-1)	2.00	Addicsin-associated factor	Schizophrenia
WIF-1	13.00	Inhibitor of WNT signaling pathway	Alzheimer's disease

ADAMTS-15 = ADAM metallopeptidase with thrombospondin type 1 motif 15; APP = β-amyloid precursor protein; BACE = β-secretase 1; bigLEN = synonym for *PCSK1N*; dUTPase = deoxyuridine triphosphate nucleotidohydrolase; EBV = Epstein-Barr virus; EGR1 = early growth response 1; EN-RAGE = extracellular newly identified receptor for advanced glycation end-products binding protein; GAV = Gill-associated virus; GPR44 = putative G protein-coupled receptor 44; ITM2B = integral membrane protein 2B; littleLEN = synonym for *PCSK1N*; PEN = synonym for *PCSK1N*; ProSAAS = synonym for *PCSK1N*; RAGE = receptor for advanced glycation endproducts; SAAS = synonym for *PCSK1N*; TH2 = T helper type 2; THY-1 = Thy-cell surface antigen; TRIM71 = tripartite motif containing 71; WIF-1 = WNT inhibitory factor 1.

* Human primary dendritic cells were stimulated with EBV dUTPase protein (10 μg/mL) or vehicle for 24 h. After treatment, the levels of multiple immune proteins in the culture supernatant was measured with the Human L-1000 Antibody Array (RayBiotech), as described in Methods.

† Normalized signal intensity data for each analyte in EBV dUTPase-treated cells and expressed as fold-change relative to the vehicle control. Normalized data represent values in which the background signal of negative control spots on the array has been subtracted out and normalized to the mean signal intensity of positive control spots. After normalization, any ≥1.5-fold increase or ≤0.65-fold decrease in signal intensity for a single analyte between dUTPase and control samples was considered a measurable and significant difference in expression, provided that both sets of signals are well above background (mean background [2]; accuracy ≈ 95%).

Table III. Major pathways/genes modulated by EBV-dUTPase protein in female C57Bl/6 mouse brain.

Gene Symbol	Gene Name	Fold-Regulation*	*P*	Function
Alox12	Arachidonate 12-lipoxygenase	−1.91324	0.030	Signal transduction phospholipase A2 pathway
Arrb1	Beta-ARRESTIN 1/negative regulators of GPCR	−1.60338	0.012	Signal transduction G-protein coupled receptor regulation
Cgn	cingulin	−1.62	0.049	Blood−brain barrier
Dbh	dopamine β-hydroxylase (DβH) enzyme	−1.7011	0.030	Dopamine metabolism
Drd1	Dopamine receptor D1	−1.75465	0.025	Dopamine receptor
Drd5	Dopamine receptor D5	−1.70713	0.032	Dopamine receptor
Egr1	Early growth response 1	1.74	0.046	Synaptic plasticity; pain
Fos	Transcription factor/essential role in stress resilience	−1.61098	0.033	Signal transduction; cAMP & protein kinase A signaling
Gabrd	$GABA_A$ receptor subunit δ	1.58754	0.017	Neurotransmitter receptor: GABAergic synapse
Gch1	GTP cyclohydrolase	2.94	0.016	Pain; dopamine biosynthesis
Grik4	Glutamate receptor, ionotropic, kainite subunit KA1	2.91804	0.016	Neurotransmitter receptor glutamatergic synapse
Grik5	Glutamate receptor, ionotropic, kainite subunit KA2	1.74256	0.024	Neurotransmitter receptor glutamatergic synapse
Grk6	G protein-coupled receptor kinase 6	−1.5332	0.033	Regulation of dopamine receptors
Gpr84	G protein-coupled receptor 84	−3.00	0.015	Pain
Gpr171	G protein-coupled receptor 171	−1.73	0.022	Energy/metabolism
Il1b	Interleukin-1β	2.0868	0.016	Immune
Kmo	Kynurenine-3-monooxygenase	−2.18	0.050	Tryptophan metabolism
Lin 7B	Lin 7 homolog B	−3.55	0.045	Synaptic plasticity
Mmp15	Metalloprotease 15	−1.83	0.049	Blood−brain barrier
Nr4a1	Nuclear receptor Nur77	−1.58885	0.023	Dopamine & serotonin target
Pde10a	Phosphodiesterase 10A	−1.51367	0.024	Signal transduction Phospholipase A2 pathway
Pik3cg	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	−1.52318	0.031	Signal transduction AKT andPI3 kinase signaling
Plcb1	Phospholipase C β1	−1.52803	0.031	Signal transduction phospholipase C signaling
Rab33a	RAB33A member of Ras oncogene family	−3.67	0.018	Synaptic plasticity
Rapgef6	Rap guanine nucleotide exchange factor 6	−1.61	0.019	Blood−brain barrier
Rgs20	Regulator of G protein signaling	−1.65	0.029	Synaptic plasticity
Slc6a3	Dopamine transporter (DAT1)	−1.5978	0.042	Dopamine transporter
Slc6a4	Solute carrier family 6α member 4/ serotonin transporter	−5.76338	0.033	Serotonin transporter

(*continued on next page*)

Table III. (*Continued*)

Gene Symbol	Gene Name	Fold-Regulation*	*P*	Function
Synpo	Synptopodin	−2.25	0.036	Synaptic plasticity
Tbc1d1	TBC1 family domain member 1	−1.55	0.042	Energy/metabolism
Th	Tyrosine hydroxylase/involved in dopamine synthesis	−2.23353	0.013	Dopamine metabolism
Tjp2	Tight junction protein 2	−2.68	0.023	Blood−brain barrier
Tph2	tryptophan hydroxylase 2	−3.77918	0.010	Serotonin metabolism

Data represent mRNA expression levels relative to the control and expressed as fold-regulation.
AKT = protein kinase B; cAMP = cyclic adenosine monophosphate; dUTPase = deoxyuridine triphosphate nucleotidohydrolase; EBV = Epstein-Barr virus; GABA = γ-aminobutyric acid; GPCR = G protein-coupled receptor; IL = interleukin; PI3 = phosphoinositide 3; TBC1 = TBC1 family domain member 1.
* Mean fold-change in expression significantly different from control by ≥ 1.5, n = 3.

expression of the *Nur77/Nr4a1* has been found in patients with schizophrenia. Furthermore, the dUTPase upregulated the mRNA expression of *Gabrd* ($GABA_A$ receptor subunit δ), *Grik4* (kainic acid receptor subunit KA1), and *Grik5* (kainic acid receptor subunit KA2) genes belonging to the GABA/glutamate pathways as well as *IL-1β* mRNA expression. Increased expression of *Grik4* and *Grik5* has been observed in the hippocampus of patients with refractory temporal lobe epilepsy.[45] These results indicated that EBV dUTPase primarily targeted genes involved in DA and serotonin biosynthesis, causing a significant downregulation in the expression of these genes and thus, suggesting aberrant neurotransmission *in vivo*. EBV dUTPase protein also modulated the expression of genes with key roles in BBB permeability, metabolism/energy, and pain in mouse brain.

DISCUSSION

Numerous studies have proposed various hypotheses and models to explain how neuroinflammation could contribute to the chronic fatigue, postexertional fatigue, and cognitive deficits observed in patients with ME/CFS.[12–15] Although neuroimaging studies have found structural and functional alterations in the brains of patients with ME/CFS, only a single study has presented evidence of an increased activation of astrocytes and microglia in the brain of patients with ME/CFS, suggesting that widespread neuroinflammation was occurring.[10] However, the underlying mechanisms that contribute to this neuroinflammatory reaction or reactions in patients with ME/CFS remain undefined.

We have previously found that a subgroup of patients diagnosed with ME/CFS exhibited a statistically significant elevation in antibodies against the EBV dUTPase protein.[19] In the present study we provide further evidence to support a mechanism by which abortive-lytic reactivation of a systemic latent infection of EBV and subsequent production of dUTPase protein, which occurs in a subgroup of patients with ME/CFS, could contribute to the development of a neuroinflammatory microenvironment in the brain by modulating BBB, microglia cell, and astrocyte gene expression/function, tryptophan, DA and serotonin metabolism, and synaptic plasticity, which in turn may contribute to the increased pain, postexertional fatigue, and cognitive impairments observed in some patients with ME/CFS.

The BBB is composed of endothelial cells of the capillary wall, astrocytes end-feet ensheathing the capillary, and pericytes embedded in the capillary membrane.[46] The function of the BBB is to prevent the free diffusion of substances and the movement of cells from the systemic circulation into the CNS (brain and spinal cord) and, thus, prevent unwanted activation of brain cells. We have previously reported that the EBV dUTPase protein induces the secretion of the proinflammatory cytokines TNF-α, IL-6, and IL-1β in hDCs and

peripheral blood mononuclear cells.[28–30] In this study we found that the EBV dUTPase protein strongly induces the expression of IL-6 and IL-1β in cerebral microvascular endothelial cells and microglia cells as well as TNF-α in both astrocytes and microglia cells. These proinflammatory cytokines and interferon-γ have been reported to disrupt BBB integrity.[34] The EBV dUTPase also downregulated *in vitro* or *in vivo* the expression of genes that encode for the proteins which have critical and direct implications for both forming and maintaining tight junctures between endothelial cells in capillaries comprising the BBB as well as modulating cellular adhesion and the extracellular matrix. Altogether, these data suggest that the EBV dUTPase protein has the capacity to disrupt the BBB, which could result in neuroinflammation and/or neurodegeneration.[46]

In addition, the EBV dUTPase protein also induced a transient increase in the expression of *PTGS2/COX-2* in astrocytes, whereas in microglia cells the dUTPase induced a strong and sustained *PTGS2* expression, suggesting that microglia cells were the primary source of this proinflammatory enzyme. Cox-2 catalyzes the formation of prostaglandin E_2, which is a key mediator of inflammatory responses. Although COX-2 is generally considered to be inducible, it is constitutively expressed in some glutamatergic neurons in the cortex and hippocampus,[47] astrocytes,[48] and microglia.[49] The role of COX-2 as a contributor to neuroinflammatory toxicity in neurodegenerative disorders is well established[50].

Our data also indicate that the EBV dUTPase altered the expression of genes involved with pain (*GPR84*[51] and *GCH1*[52]) and fatigue (*TBC1D1*[53]). Chronic fatigue and pain are characteristic symptoms in patients with ME/CFS.[1–3]

In addition to disrupting the integrity of the BBB and modulating genes involved with inflammatory processes, pain, and fatigue, our data suggest that the EBV dUTPase may alter synaptic plasticity *in vivo*, which is important in learning and memory processes, as indicated by the ability of the dUTPase protein to downregulate the expression of LIN7b, SYNPTO, and RAB33A and upregulate Egr-1 in mouse brain. These genes have critical functions in (1) ensuring proper localization of the GRIN2B subunit of the N-methyl-D-aspartate receptor (NMDAR),[54] (2) long-term potentiation,[55,56] (3) mediating antegrade axonal transport of post-Golgi synaptophysin-positive vesicles and their fusion at growth cones,[57] and (4) NMDAR-mediated downregulation of PSD95 and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) trafficking,[39] all of which are important for synaptic development, plasticity, and functions. AMPAR and NMDAR play critical roles in the plasticity of most excitatory synapses, as indicated by a number of neurologic disorders associated with synaptic dysfunction that have altered NMDAR and AMPAR expression, trafficking, and signaling. These data suggest that the EBV dUTPase is capable of altering synaptic structure and function as well as neuronal communication, which would affect cognitive processes.

Finally, our data found that the EBV dUTPase protein modulates tryptophan, serotonin, and DA metabolism and use *in vitro* and *in vivo*. The EBV dUTPase may alter kynurenine catabolism in microglia *in vitro*, suggesting that there is an increase synthesis of quinolinic acid. Quinolinic acid, an agonist of NMDAR, can cause overstimulation that results in neuronal toxicity.[58] The dopaminergic and serotonergic neurotransmitter systems are reported to play a critical role in the regulation of emotion and mood, and they have been implicated in a spectrum of neuropsychiatric disorders.[59,60]

EBV replicates primarily in the tonsils/nasopharynx and sheds in the saliva.[61] A number of reports have found that physical and/or psychosocial stress induces the reactivation of latent EBV. Burning mouth syndrome (BMS) is a chronic condition characterized by a burning sensation of the oral cavity that affects menopausal or postmenopausal women (50–70 years of age). In addition, these patients exhibit high levels of anxiety and depression.[62,63] Idiopathic BMS can occur spontaneously and without any identifiable precipitating factors. Although the exact mechanism or mechanisms involved in the pathophysiology of idiopathic BMS is unknown, evidence suggests that it may be a neuropathic condition affecting the peripheral nervous system and CNS. Clonazepam, a member of the benzodiazepine family, which is used for treatment of anxiety, is the preferred

treatment option for patients with BMS because of its effect on the peripheral GABA A receptor. It has been found that nerve fibers on the tongue have high expression of GABA A receptors.[64] Our study indicates that EBV dUTPase upregulates a subunit of the GABA A receptor and suggests a possible involvement of EBV in this syndrome, especially in an older population with a decreased immune capacity to control the virus.

CONCLUSIONS

We are proposing that in a subset of patients with ME/CFS there is an increase in abortive lytic replication of EBV, especially in those patients exhibiting a diminished EBV-specific B− and T-cell response,[18] resulting in the increased release of EBV dUTPase possibly in exosomes.[29] Activation of TLR2 by the EBV dUTPase in cerebral microvascular endothelial cells can disrupt the integrity of the BBB by inducing the downregulation of genes in cerebral microvascular endothelial cells that encode for products important for maintaining tight junctions between these cells and simultaneously inducing the upregulation IL-1β, IL-6, and TNF-α proinflammatory cytokines that disrupt the BBB. Disruption of the BBB allows the dUTPase protein to enter the CNS where ligation and activation of TLR2 by the dUTPase on astrocytes,[65] microglia,[66] mast cells,[67] and possibly neurons[68] would result in altered excitatory glutamatergic synapses and expression of genes whose products are involved in fatigue, pain, and cognitive responses all of which are altered in patients with ME/CFS. Recent studies have also found that secretion of IL-33 by microglia cells activates mast cells, resulting in the rapid secretion of TNF and sensitization of meningeal nociceptors.[69] This cross-talk between microglia and mast cells may be an important process for explaining stress-induced neuroinflammation, a common feature in many neurologic conditions such as depression and anxiety.[70] Although these findings are exciting, further studies are necessary to confirm that changes in gene expression equates with changes in protein levels and altered functionality. In addition, multiple test correction was not used, which may affect statistical comparisons. The results of this study provide exciting new data at the molecular level, suggesting a mechanism by which EBV dUTPase may modulate immune activation, disrupt the BBB, and alter the structure/function of neurologic synapses, resulting in loss of neurocognitive functions in a cohort of patients with ME/CFS.

CONFLICTS OF INTEREST

The authors declare no competing interests. The authors have indicated that they have no conflicts of interest regarding the content of this article.

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M.V. Williams analyzed data and wrote paper; B. Cox, and W.P. Lafuse. conducted the experiments, analyzed data, and edited the paper; M.E. Ariza designed and conducted the experiments, analyzed data, and wrote the paper.

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