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Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Gulf War Illness patients exhibit increased humoral responses to the Herpesviruses-encoded dUTPase: Implications in disease pathophysiology

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Abstract

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) and Gulf War Illness (GWI) are debilitating diseases with overlapping symptomology and there are currently no validated tests for definitive diagnosis of either syndrome. While there is evidence supporting the premise that some herpesviruses may act as possible triggers of ME/CFS, the involvement of herpesviruses in the pathophysiology of GWI has not been studied in spite of a higher prevalence of ME/CFS in these patients. We have previously demonstrated that the deoxyuridine triphosphate nucleotidohydrolases (dUTPase) encoded by Epstein-Barr virus (EBV), human herpesvirus-6 (HHV-6), and varicella-zoster virus (VZV) possess novel functions in innate and adaptive immunity. The results of this study demonstrate that a significant percentage of patients with ME/CFS (30.91–52.7%) and GWI (29.34%) are simultaneously producing antibodies against multiple human herpesviruses-encoded dUTPases and/or the human dUTPase when compared to controls (17.21%). GWI patients exhibited significantly higher levels of antibodies to the HHV-6 and human dUTPases than controls ($p = 0.0053$ and $p = 0.0036$, respectively), while the ME/CFS cohort had higher anti-EBV-dUTPase antibodies than in both GWI patients ($p = 0.0008$) and

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Authors' contributions

MEA and MVW conceived and designed the study, performed data analysis, drafted the manuscript and revised it. MAF, NGK participated in study design, collected samples, helped draft the manuscript and revised it. PH performed the experiments, participated in data analysis and preparation of the manuscript. ZB collected samples and participated in the preparation of the manuscript. All authors have read and approved the final manuscript.

Ethical information

All study subjects provided written informed consent and the protocol was approved by the Institutional Review Board for Human Subjects at NOVA Southeastern University.

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controls ($p < 0.0001$) as well as significantly higher anti-human dUTPase antibodies than in controls ($p = 0.0241$). These results suggest that screening of patients' sera for the presence of various combinations of anti-dUTPase antibodies could be used as potential biomarkers to help identify/distinguish patients with these syndromes and better direct treatment.

Keywords

Chronic Fatigue Syndrome; Epstein-Barr virus; Human herpesvirus 6; varicella-zoster virus; deoxyuridine triphosphate nucleotidohydrolase; antibodies

Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a chronic multisystem illness of unconfirmed etiology [Institute of Medicine, 2015]. In over half of cases, ME/CFS onset is associated with acute “flu-like” symptoms [Klimas et al., 2015]. There are multiple reports in the literature suggesting a role for viruses, particularly human herpesvirus-6 (HHV-6) [Ablashi et al., 2000; Komaroff, 2006; Chapenko et al., 2012] and Epstein-Barr virus (EBV) [Cameron et al., 2010; Loebel et al., 2014] in ME/CFS. However, the data concerning a causal relationship between a virus and ME/CFS has not been conclusively demonstrated and remains a challenge because of the heterogeneity of the patient population and the realization of possibly multiple etiologies. Surprisingly, none of these studies have approached the possibility that virus encoded proteins rather than the viruses themselves may act as drivers of/contribute to the pathophysiological alterations observed in a subset of patients with ME/CFS.

Approximately one-third of the veterans returning from the Operation Desert Storm conflict, were afflicted with a chronic multisystem disorder known as Gulf War Illness (GWI) [Fukuda et al., 1998]. Characterized by severe and debilitating symptoms including fatigue, musculoskeletal pain and cognitive problems, GWI has no known cure and requires long-term treatment and monitoring [Eisen et al, 2005]. Multiple hypotheses regarding the etiology of these symptoms soon emerged, as these servicemen were subjected to a number of potentially hazardous conditions, including infectious agents, medical prophylaxis, pesticides, depleted uranium, oil well fire smoke, chemical and biological warfare agents and psychological stressors [Steele et al., 2012].

GWI cases exhibit a significant overlap in symptoms with ME/CFS, which include long-term fatigue, sleep disturbances or non-restorative sleep, as well as immune and cognitive dysfunction [Whistler et al., 2009; Institute of Medicine, 2013; Parkitny et al., 2015]. There are currently no validated biomarkers for definitive diagnosis of either syndrome. Thus, there is an urgent need to identify triggers and drivers of ME/CFS and GWI and to understand the molecular mechanisms by which they contribute to the development or progression of these illnesses so more efficient diagnostic tools and effective therapeutics can be developed.

We have previously demonstrated that the deoxyuridine triphosphate nucleotidohydrolase (dUTPase) encoded by EBV, HHV-6 and VZV possess novel immunoregulatory functions

by triggering the activation of toll-like receptor (TLR) 2, which leads to the activation of NF- κ B and subsequent modulation of downstream genes involved in chronic inflammation, effector T-cell function and neurotransmitter function [Glaser et al., 2006; Ariza et al., 2009, 2013, 2014]. We have also shown that the EBV-encoded dUTPase is secreted in exosomes, which function as intracellular messengers, induces the secretion of the pro-inflammatory T_{H1}/T_{H17} cytokines, alters T- and NK cell function *in vitro*, and induces anxiety and sickness behavior in mice [Padgett et al., 2004; Aubrecht et al., 2014]. Perhaps more importantly, was the finding of prolonged and significantly elevated neutralizing antibodies against the EBV-encoded dUTPase, which inversely correlated with ME/CFS symptomology in a small subset of patients with ME/CFS [Lerner et al., 2012].

In this study, we examined, using a newly developed ELISA assay, longitudinal samples from a cohort of patients with ME/CFS enrolled in the “Good Day Bad Day” study and single serum samples from other cohorts of patients diagnosed with GWI or ME/CFS as well as gender, age and ethnicity matched controls. The samples were examined for the presence of IgG antibodies against the HHV-6, EBV and VZV encoded dUTPases as well as the human nuclear dUTPase. The ELISA assay is more advantageous than the previously employed neutralization assays in that it is more rapid, allows for the detection of a wide assortment of antibodies directed against the proteins rather than just those that neutralize enzymatic activity. This is important in the case of dUTPases such as HHV-6, which lack functional enzymatic activity [Ariza et al., 2014]. Altogether, our data is consistent with previous findings supporting aberrant immune responses and a loss in immune competence in ME/CFS and GWI patients. This could result in lytic/abortive replication of these herpesviruses and subsequent expression of virus-encoded dUTPases, which in turn may contribute to the immunopotential of the disease process.

Materials and Methods

Purification of recombinant dUTPase proteins

Subcloning and purification of the recombinant herpesviruses' dUTPase proteins (HHV-6, EBV and VZV), as well as, the human nuclear dUTPase were performed as we have described previously [26–30]. While the HHV-6A U45 gene was cloned from the HHV-6A strain GS, the recombinant protein exhibits greater than 94% identity to the HHV-6B strain Z29 U45 gene. Therefore, it is unlikely that the U45 recombinant protein can distinguish between these strains. The terminology HHV-6 will be employed. All recombinant dUTPase protein preparations were tested for the presence of contaminants as described previously [Glaser et al., 2006; Ariza et al., 2009, 2013, 2014] and were free of detectable levels of LPS, peptidoglycan (SLP-HS), DNA or RNA. Protein concentration was determined using the Qubit fluorimeter (Invitrogen Carlsbad, CA). The purified recombinant dUTPase proteins used in these studies were stored at –80°C until further use.

ELISA assay

ELISAs were performed essentially as described previously [Lai et al., 2012]. Briefly, 96-microtiter well plates (Nunc-Immuno Plate MaxiSorp Surface) were coated overnight at 4 °C with recombinant dUTPase protein at 2.5 μ g/ml in phosphate buffered saline (PBS).

Plates were washed on a Biotek ELx50 plate washer three times with PBS/0.05 % Tween 20 (200 µl) and blocked with blocking buffer (PBS/2.5 % BSA; 100 µl) at room temperature (RT). All serum samples were used at a 1:800 dilution in blocking buffer and incubated for 2 h at RT in duplicate. Plates were washed three times with PBS/0.05% Tween 20 (200 µl) followed by incubation with anti-human-IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma Chemical Co. St. Louis MO) at 1:1000 dilution in blocking buffer at RT for 1 h. Plates were washed six times with PBS/0.05 % Tween 20 and incubated for 15 minutes with 100 µl of tetramethylbenzidine (Invitrogen, Carlsbad, CA). A volume of 50 µl H₂SO₄ (2 M) was added to stop the reaction and plates were read at 490 and 690 nm for background on a Lab Systems Multiskan MCC/340 plate reader using the Genesis v3.05 Life Sciences Ltd software. The background from the 490 nm uncoated wells and PBS-BSA (negative controls) were subtracted from the mean absorbance of the coated wells. ROC curves were constructed to determine optimal threshold points for each antigen using serum obtained from a healthy control donor. Intra-assay variation was determined to be less than 0.02 while inter-assay variation ranged from 0.005 for human to 0.049 for VZV. A positive reaction was defined as a serum sample that led to a signal 3-times over the background OD of the control serum (HHV-6: 0.124 ± 0.045 ; EBV: 0.104 ± 0.025 ; VZV: 0.256 ± 0.016 ; human dUTPase: 0.017 ± 0.005). For the studies involving sera from the Good Day Bad Day study, if any of the four samples from a patient were positive for an anti-dUTPase antibody that individual was considered positive for that particular dUTPase.

The recombinant dUTPase-specific ELISAs were validated using dUTPase-specific antibodies to the EBV, HHV-6 dUTPases and the human nuclear dUTPase and no cross-reactivity was observed. Because of the low protein homology among the herpesviruses' dUTPases (maximum amino acid identity between EBV dUTPase, HHV-6 dUTPase and VZV dUTPase is 24%), it is very unlikely that these dUTPases will bind to each other.

ME/CFS patients

The patients in this study met the Canadian Clinical Case Definition (CCC) [Carruthers et al., 2003] and the Fukuda criteria [Fukuda et al., 1994] and exhibited fatigue, post-exertional malaise, sleep dysfunction and pain; two or more neurological/cognitive manifestations and one or more symptoms from two of the categories of autonomic, neuroendocrine and immune manifestations, as well as illness persisting for at least six months. All study subjects provided written informed consent and the protocol was approved by the Institutional Review Board for Human Subjects at NOVA Southeastern University.

ME/CFS exclusion criteria—Following the recommendations of Reeves et al., 2003, all ME/CFS subjects, selected by their medical history and physical examination, had no history of heart disease, COPD, malignancy, or other systemic disorders including the listed psychiatric exclusions that would be exclusionary for a diagnosis of ME/CFS.

Patients taking medications that have the potential to act as significant immunomodulators were excluded including gamma globulin, steroids and any cytokine or cytokine-blocking therapy. Medications with modest immunologic impact (e.g. anti-depressants) were maintained without change in dosage throughout the study periods.

ME/CFS longitudinal good day bad day study subjects—In 74 subjects with ME/CFS, baseline and 18 month blood samples and clinical data were collected. Two additional blood samples were collected from each subject, one on a day the subject self-described feeling “relatively good” and another one when the subject was having “bad day” (Table 1). There was a minimum of a three-month period between the collection of sera from a good day and a bad day.

ME/CFS single sample study subjects—A single baseline serum sample from 55 ME/CFS subjects in our biobank was selected for testing. There was no overlap between these single sample ME/CFS patients and those in the ME/CFS Good Day Bad Day group. Demographic details of these patients are given in Table 1.

Gulf war illness cohort—Inclusion criteria for GWI was derived from Fukuda et al. [16], and consisted in identifying veterans deployed to the theater of operations between August 8, 1990 and July 31, 1991, with one or more symptoms present after 6 months from at least 2 of the following: fatigue; mood and cognitive complaints; and musculoskeletal complaints. Subjects were in good health prior to 1990 and had no current exclusionary diagnoses. Medications that could have impacted immune function were excluded as were supplements. The use of the Fukuda definition in GWI is supported by Collins et al., 2002. A single baseline serum sample from 45 GWI subjects in our biobank was selected for this study. Demographic details of these patients are given in Table 1.

Healthy controls—Healthy controls were self-defined as healthy, sedentary (no regular exercise program, sedentary employment), and matched to ME/CFS or GWI cases by age (± 5 years), gender, and race/ethnicity (see Table 1).

Herpesvirus serology—The average age of the subjects in this study ranged from 44.09 to 49.5 years and since 70–95% of the adult population has been infected with EBV, HHV-6 and VZV, standard herpesviruses serological assays were not performed.

Statistical analysis

GraphPad Prism 6 software (GraphPad Software, La Jolla, CA) was used for all statistical analyses. For the longitudinal ME/CFS GDBD samples, Pearson’s correlation coefficient was determined. A p value of < 0.05 was used to demonstrate a significant correlation between the expression of the various antibodies within the groups examined. For the GWI study, while only one serum sample was available per patient, the means of the various groups cross-sectional were compared using one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered statistically significant. To compare two groups an unpaired t test was employed. Values of $p < 0.05$ were considered statistically significant.

Results

Evaluation of humoral immune responses to herpesviruses and human dUTPase proteins in ME/CFS good day bad day longitudinal sera

To determine whether there was evidence supporting the hypothesis that herpesviruses-dUTPase proteins were expressed in patients with ME/CFS, we tested longitudinal sera from 74 ME/CFS Good Day-Bad Day (GDBD) patients (Table 1), for antibody responses against EBV, HHV-6 and human nuclear encoded dUTPases by ELISA.

As shown in Table 2, a spectrum of humoral reactivities to the various dUTPases was observed in the ME/CFS GDBD cohort. Of the 74 patients examined, approximately 34% were negative for antibodies to HHV-6, EBV- and the human nuclear encoded dUTPase proteins. While some patients expressed antibodies to only HHV-6 (2.7%) or EBV (5.41%) encoded dUTPases, the majority (48.65%) co-expressed antibodies to both HHV-6 and EBV-encoded dUTPases (Table 2). Furthermore, 55.40% and 54.06% of the total ME/CFS GDBD cohort were positive (single, or double) for HHV-6 and EBV anti-dUTPase antibodies, respectively. While a few patients were positive only for anti-human dUTPase antibodies (6.76%), somewhat surprising was the observation that a significant proportion of the ME/CFS GDBD cohort co-expressed anti-human and anti-HHV-dUTPase antibodies (31.08%) (Table 2).

There was no direct correlation between the levels of anti-dUTPase antibodies and patients' self-determination of whether they were having a good day or a bad day. Twenty-four patients, who were negative for anti-dUTPase antibodies, exhibited no significant change in antibody levels during the test period (Fig. 1A patient 029, and 1B patient 109 are examples). Of the fifty patients who exhibited a statistically significant increase ($p < 0.05$) in the level of one or multiple anti-dUTPase antibodies, 6 (12%) patients exhibited no significant fluctuations in the antibody levels during the test period (Fig. 1C patient 022, and 1D patient 023 are examples). Ten patients (20%) exhibited high levels of antibodies at baseline with a decline over the test period (Fig. 1E patient 055), while 7 patients (14%) exhibited the highest level of antibodies at the conclusion of the test period. Twenty-seven patients (54%) exhibited a peak of anti-dUTPase antibody either on the bad day or on the good day and this was followed by an increase of antibodies at the end of the trial (Fig. 1F-H patients 054, 106 and 114, respectively). Interestingly, there was a statistically significant correlation between the presence of anti-HHV-6 and human dUTPase antibodies in two patients and all three anti-dUTPase antibodies in 21 patients (Pearson correlation coefficient, $p = < 0.05$).

Antibody responses to herpesviruses and human nuclear dUTPase proteins in GWI, ME/CFS and healthy control sera

Because of the overlapping symptoms observed between patients with GWI and ME/CFS [Kipen et al., 1999; McCauley et al., 2002], the higher incidence of ME/CFS in patients with GWI and the lack of information concerning the etiology of GWI, we next examined the potential involvement of herpesviruses in the pathophysiology of GWI. To determine whether there was evidence supporting the hypothesis that herpesviruses-dUTPase proteins

were expressed in patients with GWI, the anti-dUTPase antibody profile to HHV-6, EBV and VZV encoded dUTPases as well as the human dUTPase were determined in a single serum sample from 58 patients diagnosed with GWI and compared to the antibody profiles obtained in patients diagnosed with ME/CFS ($n = 55$) or healthy controls ($n = 151$). As shown in Table 3, a wide-spectrum of anti-dUTPase antibodies were identified in patients (GWI; ME/CFS).

As shown in Fig. 2, there were statistically significant differences in the expression of anti-dUTPase antibodies between the GWI, ME/CFS and control cohorts based upon unpaired t test analyses. The GWI cohort exhibited a statistically significant increase in anti-HHV-6 ($p = 0.0053$; Fig. 2A), anti-VZV ($p = 0.032$; Fig. 2B) and anti-human ($p = 0.0036$; Fig. 3C) dUTPase antibodies when compared to controls while the ME/CFS cohort exhibited a statistically significant increase in anti-EBV ($p = 0.0001$; Fig. 2D) and anti-human ($p = 0.0241$; Fig. 2C) dUTPase antibodies when compared to controls. Interestingly, the only statistically significant difference between the GWI and ME/CFS cohorts was an elevation in anti-EBV dUTPase antibodies in the ME/CFS cohort ($p = 0.0008$; Fig. 2D) and an elevation of anti-VZV dUTPase antibodies in the GWI cohort ($p = 0.0441$; Fig. 2B).

Collectively, these results demonstrate a significant increase in IgG antibodies to EBV, HHV-6, VZV and human nuclear dUTPases suggesting that certain epitopes in the herpesviruses and human nuclear dUTPase proteins may be eliciting an immunological response in some patients with ME/CFS and/or GWI.

Discussion

ME/CFS and GWI are chronic multisystem illnesses with overlapping symptoms including fatigue, neurocognitive dysfunctions and immune activation (elevation of inflammatory markers and elevated cytokine levels). This is further complicated by the fact that GWI patients have an increased prevalence of chronic fatigue compared to the general population [Kipen et al., 1999; McCauley et al. 2002]. Furthermore, there are currently no biomarkers available that are clinically useful for differential diagnoses. Attempts to identify the etiological agents/triggers responsible for these multisystem illnesses have not been successful. Likewise, while numerous studies have demonstrated immune dysfunction in these patients including alterations in cytokine patterns and decreased NK cell function, the factors that act as drivers, which power and maintain these multisystem illnesses are unknown.

Our study demonstrates that a subgroup of patients diagnosed with either ME/CFS or GWI exhibited a statistically significant elevation in antibodies against the HHV-6, EBV and VZV encoded dUTPases, proteins that are only expressed during lytic or abortive lytic replication of these viruses, as well as the human nuclear dUTPase. These results suggest that in this subgroup of patients there is reactivation of multiple herpesviruses; a feature that is also observed in some transplant recipients [Shiley et al., 2010; Inazawa et al., 2015] and patients with drug-induced hypersensitivity syndrome (DIHS/DRESS) [Shiohara et al., 2006; Picard et al., 2010; Chen et al., 2015]. Since GWI patients were exposed to a variety of chemical agents and environmental toxins a DIHS/DRESS-like reaction could contribute to the

reactivation of herpesviruses, especially HHV-6 in these patients. While the mechanism(s) responsible for the simultaneous reactivation of these herpesviruses is unknown, it suggests a loss of immunological control primarily, at the CD8⁺ T-cell level. This is supported in part by a study demonstrating deficient EBV-specific T-cell responses in some ME/CFS patients [Loebel et al., 2014].

Our study demonstrates for the first time, the presence of autoantibodies against the human dUTPase in a subgroup of patients with ME/CFS and GWI. In our study 39.10% of the patients in the GDBD cohort, 14.55% and 18.6% of the ME/CFS and GWI patients, respectively possessed antibodies either alone or in combination with the anti-herpesviruses' dUTPases when compared to the controls (4.64%). It is unlikely that the observed humoral response to the human dUTPase is due to cross-reactivity with the herpesviruses-encoded dUTPases since there is little homology between the homotrimeric human dUTPase and the monomeric herpesviruses dUTPases. Further antibody characterization studies using polyclonal antibodies against the EBV-encoded dUTPase revealed no reaction with the human dUTPase (data not shown). The human nuclear dUTPase, which is released from B cells in exosomes [Buschow et al., 2010], was identified as an autoantigen and potential biomarker for autoimmune hepatitis [Song et al., 2010]. Previous studies have demonstrated the presence of autoantibodies in patients with ME/CFS and in Gulf War veterans against numerous cellular components including nuclear envelope [Konstantinov et al., 1996], muscarinic cholinergic and β -adrenergic receptors [Yamamoto et al., 2012; Loebel et al., 2016] anchorage molecules [Maes et al., 2012], DNA [De Beéck et al., 2012], myelin basic protein and smooth and striated muscle tissue [Vojdani and Thrasher, 2004]. While additional studies are needed to determine the role, if any, of these autoantibodies in the disease process, our study suggest that antibodies against human, EBV, HHV-6 and VZV dUTPases may be useful as biomarkers to differentiate between patients with ME/CFS and GWI.

Numerous studies have suggested a potential role for HHV-6 and/or EBV as triggers for ME/CFS [Ablashi et al., 2000; Chapenko et al., 2012; Loebel et al., 2014; Cameron et al., 2010] and limited studies have suggested the involvement of VZV in ME/CFS [Shapiro, 2009; Tsai et al., 2014]. Conversely, there has only been a single study on GWI patients and herpesviruses suggesting that there was reactivation of multiple herpesviruses (HHV-6, EBV and VZV) in these patients [Vojdani and Thrasher, 2004]. Our studies on ME/CFS are unique in that they focused on determining whether there was an increase in antibodies against a class of dUTPase proteins that have been shown to regulate immune and neurocognitive functions and thus, have the ability/potential to drive/exacerbate the pathophysiological alterations that occur in the course of ME/CFS. Furthermore, we wanted to determine whether a similar antibody profile is observed in patients with GWI, who have a higher prevalence of ME/CFS and who exhibit similar symptomology. The data from this study support the hypothesis that in some patients with ME/CFS and/or GWI there is a complex interaction between various herpesviruses resulting in increased expression of the virus-encoded dUTPases, which in turn drives the disease process. Our working model (Fig. 3), which is a modification of the latent viral immune inflammatory response (LVIIR) model for multisystem illnesses including GWI and ME/CFS [Maloney et al., 2013], shows how disruption of normal immune homeostasis allows for the increased abortive reactivation of

the herpesviruses and release of virus-encoded dUTPases by pyroptosis or in exosomes. While the presence of antibodies to multiple herpesviruses dUTPases as well as the human dUTPase might be useful as potential biomarkers, these antibodies might not be protective unless they blocked the interaction of the dUTPase with TLR2. This may explain the lack of correlation between the presence of antibody and disease symptomology. Additional studies are required to address this possibility as well as to determine whether there is a relationship between disease severity and expression of multiple herpesviruses' encoded dUTPases. This hypothesis, however, is supported by clinical studies demonstrating improvement of symptomology in a subset of patients following long-term therapy with valganciclovir, a potent inhibitor of herpesvirus replication, and relapse upon discontinuation of treatment [Kogelnik et al., 2006; Montoya et al., 2009; 2012 Lerner and Beqaj, 2011; Watt et al., 2012]. Similar studies have not been performed with GWI patients, although long-term valacyclovir treatment of military personnel with chronic pain associated with atypical herpesvirus reactivation improved symptoms [Maloney et al., 2009].

In summary, we have shown that some ME/CFS and GWI patients exhibit a statistically significant increase in IgG antibodies to EBV, HHV-6, VZV and human nuclear dUTPases relative to controls suggesting that the dUTPases encoded by these herpesviruses are expressed at sufficient quantities in these patients to trigger a humoral immune response. This study provides further evidence that the herpesviruses-encoded dUTPases may be involved in the pathophysiology in a subset of patients with ME/CFS and GWI. The data from this study also support the hypothesis that in some patients with ME/CFS and/or GWI there is a complex interaction between various herpesviruses resulting in increased expression of the virus-encoded dUTPases and warrants further studies to address whether the presence of various combinations of antibodies to the herpesviruses encoded dUTPases could be used as potential biomarkers with clinical application for directing the use of specific long-term antiviral chemotherapy. (3484 words max 4000)

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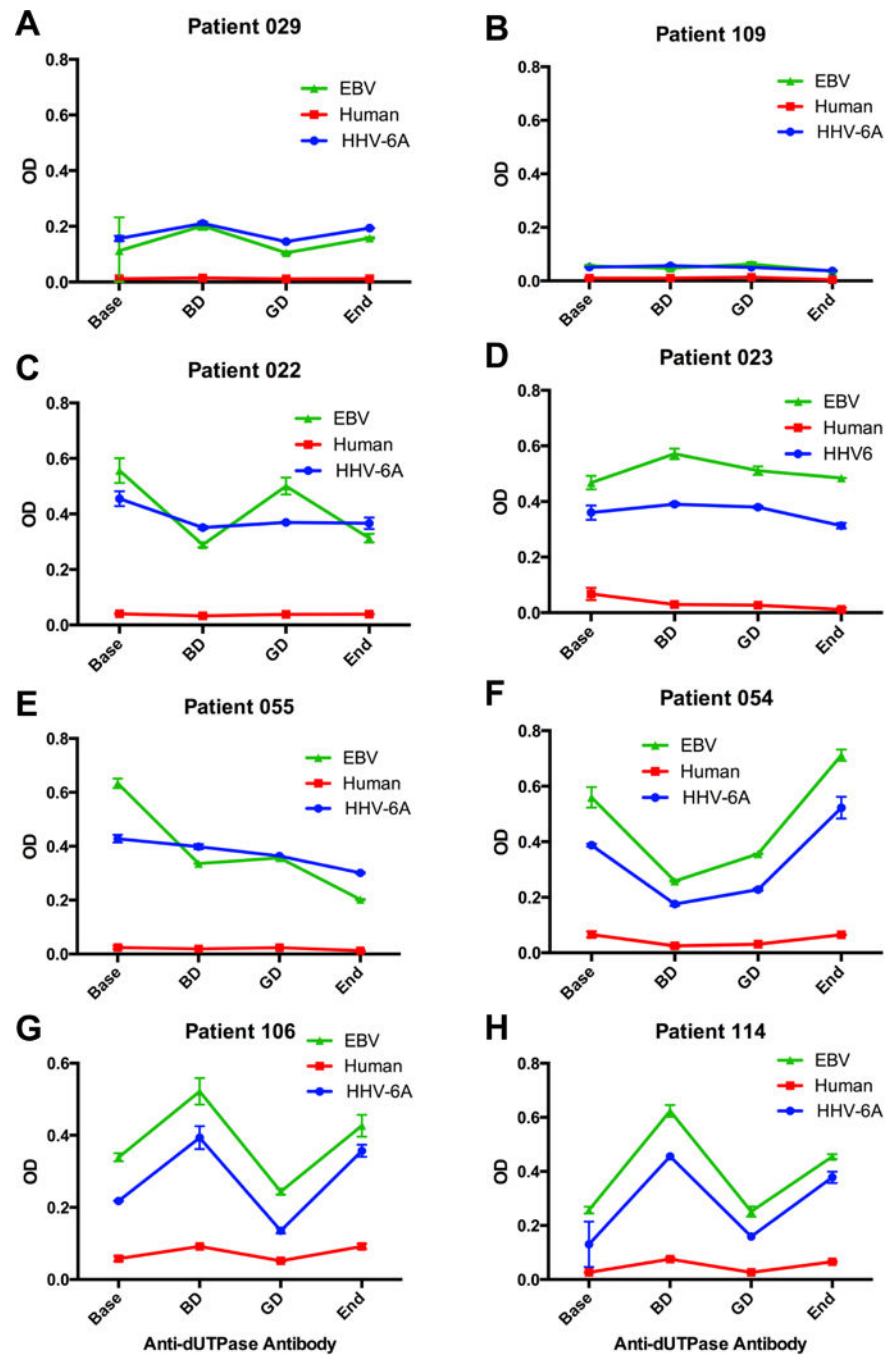


Fig. 1. Anti-dUTPase antibody patterns in ME/CFS good day bad day patients

Representative examples of anti-dUTPase antibody patterns observed in longitudinal samples of individual ME/CFS GDBD subjects. (A, B) Patients 029 and 109: negative for dUTPase antibodies. (C) Patient 022: positive for anti-HHV-6 and EBV encoded dUTPase antibodies; (D) Patient 023: positive for anti HHV-6, EBV and human nuclear dUTPase antibodies. (E) Patient 055: positive for anti-HHV-6 and EBV encoded dUTPase antibodies. (F-H) Patients 054, 106 and 114: positive for anti HHV-6, EBV and human nuclear dUTPase antibodies. Levels of anti-dUTPase antibodies were determined by dUTPase

protein-specific ELISA, as described in Material and Methods. Data represent the mean \pm SD of an n = 4.

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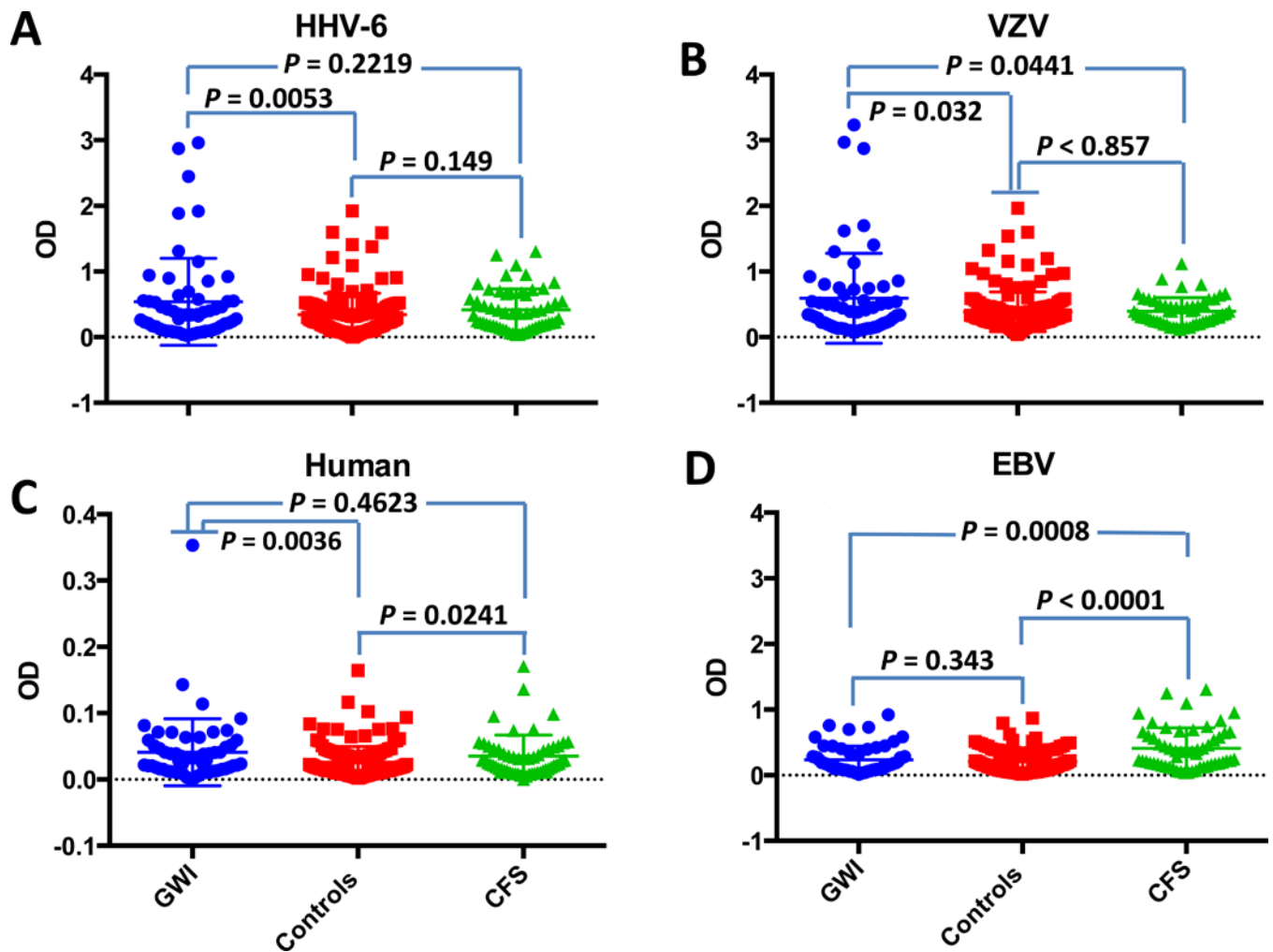


Fig. 2. Composite of the antibody response to the dUTPase encoded by EBV, HHV-6, VZV and human dUTPase in GWI and ME/CFS cohorts
 Graphs represent a composite of the anti-dUTPase antibody response of 58 GWI patients, 55 ME/CFS patients and 151 controls against (A) HHV-6 dUTPase, (B) EBV-dUTPase, (C) human nuclear dUTPase and (D) VZV dUTPase. The data represent the mean \pm SD of an n 4. A p value < 0.05 was considered significant.

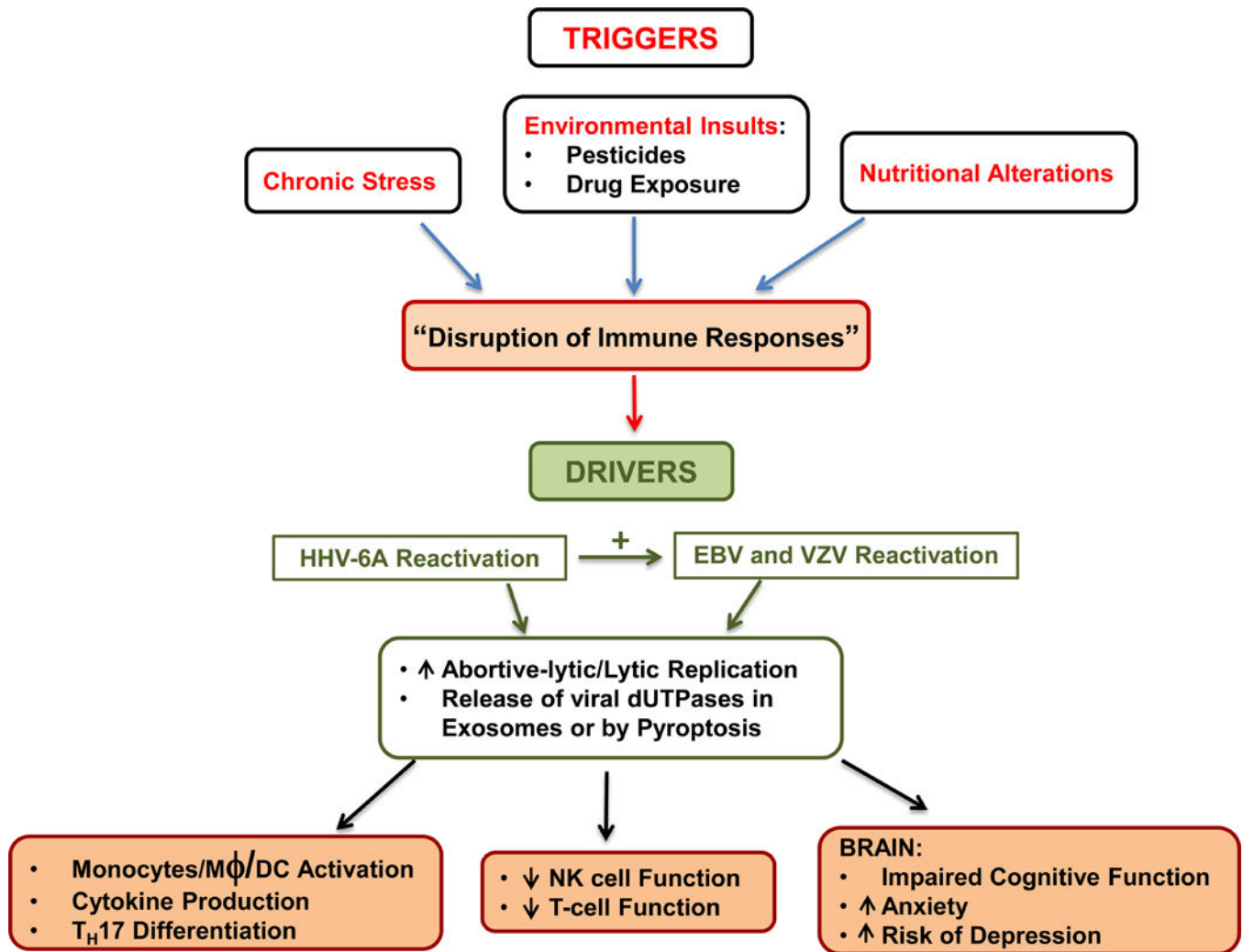


Fig. 3. Proposed mechanism(s) by which HHV-6, EBV and VZV reactivation can drive ME/CFS and GWI symptomology

Abortive/lytic reactivation of HHV-6, EBV and/or VZV results in the expression of the early gene product dUTPase and subsequent release either through pyroptosis (localized effect) or in exosomes (systemic effect). Binding of virus dUTPase to TLR2 on target cells (DCs, PBMCs, NK, T-cells) leads to NF- κ B activation, followed by induction/secretion of pro-inflammatory cytokines and the up-regulation of genes involved in inflammation, T_H17 differentiation, modulation of neurotransmitters and genes involved with brain function.

Table 1

Demographics of Study Subjects

Longitudinal: 4 samples per subject over 18 months			
	ME/CFS GDBD	N	% of Total
Gender	Male	10	14%
	Female	64	86%
	Total	74	100%
	ME/CFS GDBD	N	% of Total
Ethnicity	Asian	1	1%
	Black	4	5%
	Hispanic	14	19%
	White	51	70%
	More than One	4	5%
	Total	74	100%
Age	Average	49.18919	
	Median	49.5	
	Std. Devi	10.95405	

Single samples											
	ME/CFS	N	% of Total		GWI	N	% of Total		HC	N	% of Total
Gender	Male	20	36%	Gender	Male	50	87%	Gender	Male	79	52%
	Female	35	64%		Female	8	13%		Female	72	48%
	Total	55	100%		Total	58	100%		Total	151	100%
	ME/CFS	N	% of Total		GWI	N	% of Total		HC	N	% of Total
Ethnicity	Asian	1	2%	Ethnicity	Asian	2	4%	Ethnicity	Asian	2	1%
	Black	3	5%		Black	22	38%		Black	23	15%
	Hispanic	19	35%		Hispanic	18	31%		Hispanic	66	44%
	White	32	58%		White	16	27%		White	60	40%
	More than One	0	0%		More than One	0	0%		More than One	0	0%
	Total	55	100%		Total	58	100%		Total	151	100%
Age	Average	45.42		Age	Average	44.09		Age	Average	46.876	
	Median	44			Median	44			Median	47	
	Std. Devi	8.439			Std. Devi	5.977			Std. Devi	8.606	

Table 2

Grouping of ME/CFS Good Day Bad Day Patients based upon presence of anti-HHV-6, EBV and human dUTPase antibodies*.

dUTPase Ab Subgroups	N	Percentage (%) of total
Negative	24	32.43
HHV-6 only	2	2.70
EBV only	4	5.41
Human only	5	6.76
HHV-6 + EBV	15	20.27
HHV-6 + Human	2	2.70
EBV + Human	1	1.35
HHV-6 + EBV + Human	21	28.38

* Repetitive longitudinal serum samples (n = 4 samples/patient and a total of 74 patients) were examined for anti-HHV-6, EBV and human dUTPase antibodies by ELISA as described in Materials and Methods. Data represent the average of n = 4 measurements/sample for each recombinant protein at each time point (baseline, good day, bad day, end). A positive reaction was defined as a serum sample that led to a signal 3- times over the background OD of the control serum. A patient was considered positive for Abs to a specific dUTPase if a positive reaction occurred at any time point.

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Table 3

Grouping of GWI and ME/CFS patients based upon anti-HHV-6, EBV, VZV and Human dUTPase antibodies*.

dUTPase Ab Subgroup	GWI Cohort N (% of total)	ME/CFS Cohort N (% of total)	Control Cohort N (% of total)
Negative	29 (50)	25 (45.45)	87 (57.6)
HHV-6 only	12 (20.69)	12 (21.82)	20 (13.25)
EBV only	0 (0)	0 (0)	15 (9.93)
VZV only	0 (0)	0 (0)	2 (1.32)
Human only	0 (0)	1 (1.82)	1 (0.67)
HHV-6 + EBV	3 (5.20)	10 (18.18)	13 (8.60)
HHV-6 + VZV	1 (1.72)	0 (0)	2 (1.32)
HHV-6 + Human	2 (3.45)	1 (1.82)	0 (0)
EBV + VZV	0 (0)	0 (0)	1 (0.67)
EBV + Human	0 (0)	0 (0)	0 (0)
VZV + Human	0 (0)	0 (0)	0 (0)
HHV-6 + EBV + VZV	2 (3.45)	0 (0)	4 (2.65)
HHV-6 + EBV + Human	4 (6.90)	4 (7.27)	2 (1.32)
EBV + VZV + Human	0 (0)	0 (0)	0 (0)
HHV-6 + EBV + VZV + Human	5 (8.62)	2 (3.64)	4 (2.65)
Total	58	55	151

* A single serum sample from each patient was examined for anti-HHV-6, EBV, VZV and human dUTPase antibodies by ELISA as described in Materials and Methods. Data represent the average of n = 4 measurements/sample for each recombinant protein. A positive reaction was defined as a serum sample that led to a signal 3-times over the background OD of the control serum.