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Mechanisms of Mitochondrial Defects in Gulf War Syndrome

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14. ABSTRACT

Gulf War syndrome (GWS) is associated with increased incidences of amyotrophic lateral sclerosis, pain syndromes, muscle complaints that include fatigue and myalgias, as well as other neurological symptoms. Approximately 100,000 individuals have medical complaints consistent with GWS. Clinical manifestations are similar to those identified in Chronic Fatigue Syndrome (CFS). Mitochondrial defects are identified pathologically, metabolically, and genetically in some patients with CFS. GWS has significant evidence for mitochondrial dysfunction with abnormalities in exercise physiology, abnormalities in mitochondrial morphology, biochemical defects in mitochondrial function, abnormalities in free radical generation affecting mitochondrial integrity, gene expression in genes affecting mitochondrial function, and mtDNA mutations (inherited, somatic, and sporadic during embryogenesis). Gene expression abnormalities in CFS show abnormalities in genes that are related to mitochondrial function. Hence, investigation of mitochondrial dysfunction in GWS is a priority.

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Gulf War Syndrome, mitochondria, mitochondrial disease, chronic fatigue

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Introduction:

Gulf War syndrome (GWS) is associated with increased incidences of amyotrophic lateral sclerosis, pain syndromes, muscle complaints that include fatigue and myalgias, as well as other neurological symptoms. Approximately 100,000 individuals have medical complaints consistent with GWS. Clinical manifestations are similar to those identified in Chronic Fatigue Syndrome (CFS). Mitochondrial defects are identified pathologically, metabolically, and genetically in some patients with CFS. GWS has significant evidence for mitochondrial dysfunction with abnormalities in exercise physiology, abnormalities in mitochondrial morphology, biochemical defects in mitochondrial function, abnormalities in free radical generation affecting mitochondrial integrity, gene expression in genes affecting mitochondrial function, and mtDNA mutations (inherited, somatic, and sporadic during embryogenesis). Gene expression abnormalities in CFS show abnormalities in genes that are related to mitochondrial function. Hence, investigation of mitochondrial dysfunction in GWS is a priority.

Body:

Human Protection Approval obtained 10/13/2009. Progress on SOW tasks is summarized below.

Brief Summary of SOW Tasks:

Task 1: (Specific Aim 1)
Fifty veterans with GWS who have fatigue and myalgias are being identified through various approaches that include notification of Veterans Administration Medical Centers (VAMC) and website postings. Clinical records are requested and reviewed by the P. I. to confirm diagnosis of GWS. If the records are consistent with inclusion criteria, an appointment is made for clinical examination by the P.I., blood draw and skin biopsy. Modified criteria for chronic fatigue syndrome and fibromyalgia are used as guides for patient inclusion criteria, thus allowing comparison of the GWS patient data with CFS/fibromyalgia patient data. Based on published percentages of study groups showing evidence for mitochondrial defects, we predict that approximately 60-70% of GWS patients will harbor mitochondrial defects.

Progress:

Recruitment has been on hold while we have been applying for the no-cost extension. Once approved, it will enable us to recruit and schedule significantly more patients than we would have originally had. This will enable us to draw more meaningful conclusions from our study.

a. Website Registration
    i. The study is registered at ClinicalTrials.gov (identifier: NCT01264471; Registration date 12/20/2010)
    ii. The study is registered at the following sites
        http://www.gulfweb.org/
        http://www.ngwrc.org/
        http://www.facebook.com/#!/groups/Gulfwarvet/
        http://www.facebook.com/#!/groups/322000610955/
        http://www.immed.org/illness/gulfwar_illness_research.html
        http://cdmrp.army.mil/gwirp/default.shtml
        http://www.desert-storm.com/soldiers/gwsmap.html
        https://www.facebook.com/#!/groups/90924811301/
        https://www.facebook.com/#!/groups/149325998478946/
b. We obtained IRB approval to use an outside funding source to supply funds for travel to Atlanta for study participation. (Travel expenses are NOT charged to the DOD grant).
c. Recruitment of subjects:
    i. 26 individuals have completed clinical trial
    ii. Nine individuals have signed consent forms for the study and are being scheduled to be seen when the no-cost extension is approved
    iii. 18 individuals have had to be disqualified from participating in the study based on exclusionary factors
    iv. Three patients that were accepted into the study have changed their minds and decided not to participate at this time
    v. We will reschedule two patients that cancelled earlier appointments at the last minute once the no-cost extension is approved

Task 2: (Specific Aim 2)

Characterize mitochondrial cellular energetics in GWS patients relative to age and gender matched controls using the following approaches: (1) high resolution respirometry of intact cells [EBV transformed lymphocytes, cultured fibroblasts], (2) quantitative analysis of individual mitochondrial proteins (denatured, Western blot), (3) analysis of intact OXPHOS enzyme complexes and supercomplexes (non-denatured, Blue Native and Clear Native gels), (4) in gel enzyme activity assessment of intact OXPHOS enzyme complexes and supercomplexes (Clear Native gel, in-gel activity measurements), (5) mtDNA copy number quantitation to assess for defects in regulating mtDNA replication, and (6) cellular coenzyme Q10 quantitation (endogenous synthesis is impaired in certain types of mitochondrial dysfunction).

Progress:

1. All laboratory analyses are established in the laboratory for use with this grant. These techniques are being adapted for fibroblasts and EBV transformed lymphocytes. The status of each area of testing is outlined below.

    a. High Resolution Respirometry
        i. Fibroblast High Resolution Respirometry: 5%-95% reference intervals are validated for the parameters required for assessment of mitochondrial function. Reference ranges are established for the following parameters:

uncoupling ratio, net routine flux control ratio, respiratory control ratio, leak flux control ratio, phosphorylation respiratory control ratio. All subjects that have participated to date have been tested and analyzed. One patient's cells failed to grow in culture and we were unable to analyze his sample. Of the participants that have participated, 32% (8/25) have abnormal or equivocal respirometry results. This percentage is comparable to the numbers that we see in patients with chronic fatigue syndrome/myalgia (47% = 35/74).

ii. High Resolution Respirometry on EBV transformed cell lines: 5%-95% reference intervals are validated for the parameters required for assessment of mitochondrial function. Reference ranges are established for the following parameters: uncoupling ratio, net routine flux control ratio, respiratory control ratio, leak flux control ratio, phosphorylation respiratory control ratio. Sample testing of all subjects to date has been completed and analyzed. These data are similar, but not identical, to the results seen in the fibroblast respirometry. We will be able to better comment on this when more subjects have been analyzed.

b. **Western Blot** (denatured oxidative phosphorylation subunit) Quantitative analysis of individual mitochondrial proteins. The technique has been established and validated for muscle, fibroblasts, and EBV transformed lymphocytes. During the course of this study, we have developed more accurate approaches based on modification of the mitochondrial isolation procedure (immunocapture of mitochondria). After considerable adjustment of technique, we have very comparable results between a muscle mitochondria and fibroblast mitochondria. A comparison is shown in Figure 1. Sample testing for all study participants to date is complete and data will be analyzed as a whole when the study nears its end.

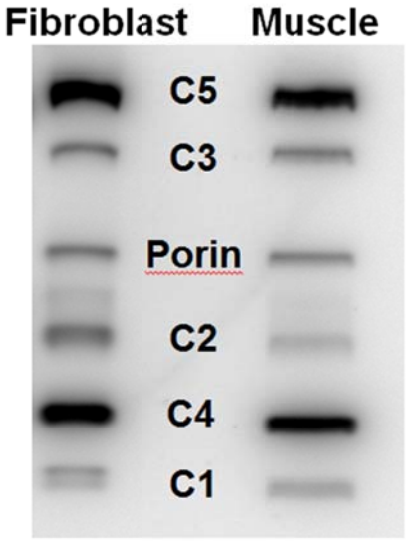

Figure 1. Representative Western Blot of selected mtDNA or nDNA coded subunits from Complexes I-V. Subunits tested: C1=ND6 subunit, Complex I, mtDNA coded; C2=30kDa subunit of Complex II, nuclear DNA coded; C3=core 2 subunit of Complex III, nuclear DNA coded; C4=subunit II of Complex IV, mtDNA coded; C5= F1 Alpha subunit of Complex V; nuclear DNA coded. The outer mitochondrial membrane porin is included for normalization of mitochondrial proteins.

c. **Blue Native and Clear Native Analyses** (non-denatured analysis of supercomplex formation and monomeric oxidative phosphorylation enzyme assembly). These approaches assess supercomplex formation and monomeric oxidative phosphorylation enzyme assembly. The process has been validated for skeletal muscle and EBV transformed lymphocytes. Despite initial data in fibroblasts indicating low mitochondrial protein concentration, we have developed the use of a glycolysis inhibition media during cell culture in order to upregulate these proteins and enhance visualization of the supercomplex formation and monomeric oxidative phosphorylation enzyme assembly on gels. Sample testing for all study participants to date is complete and data will be analyzed as a whole when the study nears its end.
d. **Clear Native Oxidative Phosphorylation Enzyme activity**: This technique assesses activity of individual oxidative phosphorylation enzymes. Intact oxidative phosphorylation enzymes are isolated by gel electrophoresis and the activity assessed (as isolated enzymes). Sample testing for all study participants to date is complete and data will be analyzed as a whole when the study nears its end.

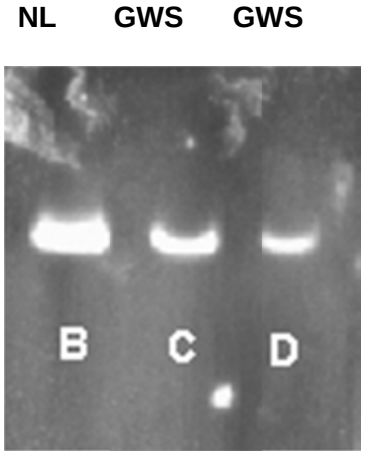

Figure 2. Clear Native oxidative phosphorylation enzyme activity analysis in fibroblasts. One control sample (B) and two GWS samples (C and D) are shown. Complex V ATPase activity appears compromised in the two GWS samples. NL = control

e. **mtDNA copy number analysis**: This technique is well validated for muscle. During this study, we have established reference ranges in uncultured skin cells, fibroblasts, and EBV transformed cell lines. Sample testing for all study participants to date is complete. These limited data show that 22% of GWS subjects (5/23) have mtDNA copy numbers below the 5% reference interval in at least one tissue type. As we evaluate the data from a larger number of subjects, we will be able to better determine the significance of this finding.

f. **Cellular Coenzyme Q10 (CoQ10) quantitation in fibroblasts and EBV transformed lymphocytes:** During the course of this study our laboratory has established reference ranges for these assays. The variability of CoQ10 levels in uncultured skin cells made reference ranges difficult to interpret and these samples are not felt to be helpful in the assessment of GWS. The measurement of CoQ10 levels in CULTURED skin cells is proceeding. The data on all subjects to date show that 33% (7/21) of the GWS subjects have CoQ10 levels below the 5% reference interval. We will be able to better comment on this when more subjects have been analyzed.

Task 3: (Specific Aim 3)
Assess the mitochondrial DNA (mtDNA) from each patient with GWS for mtDNA mutations by whole genome sequencing of leukocyte and skin cell mtDNA. Based on the findings from Specific Aim II, selected nuclear coded OXPHOS genes will be sequenced to assess for mutations that increase susceptibility to GWS.

Progress:

1. Sequencing and analysis of fibroblast and leukocyte mtDNA from all participants to date is complete. As is commonly seen in patients with chronic fatigue syndrome/fibromyalgia, many rare or novel variants that have characteristics of pathogenic mutations have been detected in several of the GWS participants. These variants are summarized in Table 2. Interestingly, a few of the variants have been detected in only one tissue type (i.e., detected in fibroblasts and not in leukocytes or visa-versa) and several were confirmed to be heteroplasmic by a restriction fragment length polymorphism (RFLP) approach. Both of these properties can be characteristic of pathogenic mutations.
2. We have developed a Next Generation sequencing (NGS) approach to assess over 650 key genes of cellular energetics. Participant samples have been banked and selected genes from appropriate participants (based on the findings from Aim II) will be will be analyzed.

Table 2. mtDNA variants of possible significance in study participants

Participant	Variant	Gene	Frequency (#/2704)	Homology	Additional Comments
GWS01	5814 T>C	tRNA Cysteine	10 (0.37%)	moderately conserved	This variant has been associated with mitochondrial encephalopathy in the following publications (Manfredi, G.,et al (1996). Human Mutation 7 (2): 158-163; Neuromuscul Disord 1997;7(3):156-9).
GWS01	7804 A>G, p.Leu73Leu	COX2	1 (0.04%)	N/A	This variant appears to be heteroplasmic in both leukocytes and fibroblasts. Mutations that do not alter an amino acid may be difficult to assess since they may produce abnormalities in structures NOT assessed by conventional analysis paradigms (e.g. mRNA expression and processing) (Science 2006:314 (5807):1930-1933).
GWS01	13924 C>T, p.Pro530Ser	ND5	4 (0.15%)	highly conserved	PolyPhen-2 predicts this variant to probably be damaging to the ND5 polypeptide structure and/or function.
GWS02	10704 G>A, p.Val79Ile	ND4L	0 (0%)	highly conserved	none
GWS04	3865 A>G, p.Ile187Val	ND1	1 (0.04%)	highly conserved	none
GWS04	2623 A>G (heteroplasmic)	16s rRNA	0 (0%)	highly conserved	This variant is heteroplasmic in fibroblasts ONLY (34% mutant mtDNA, 66% normal mtDNA). It was not detected in leukocytes.
GWS04	11819 C>T, p.Leu354Leu	ND4	0 (0%)	N/A	This variant appears to be heteroplasmic in leukocytes ONLY. It was not detected in the fibroblast sample. Mutations that do not alter an amino acid may be difficult to assess since they may produce abnormalities in structures NOT assessed by conventional analysis paradigms (e.g. mRNA expression and processing) (Science 2006:314 (5807):1930-1933).
GWS05	9525 G>A, p.Ala107Thr	COX3	1 (0.04%)	poorly conserved	Both SIFT and PolyPhen-2 predict this variant to possibly be damaging to the COX3 polypeptide structure and/or function.
GWS05	9804 G>A, p.Ala200Thr	COX3	8 (0.30%)	highly conserved	This variant was originally identified in patients with Leber hereditary optic neuropathy (LHON) (at a higher frequency than controls; Biochemical and Biophysical Research Communications. 196 (2): 810-815; 1993). The role of this mutation in producing a disease is controversial (J Med Genet 2002;39:162–169; Hum Mutat. 2009 Jun;30(6):891-8).
GWS06	2220 A>G	16s rRNA	3 (0.11%)	poorly conserved	none
GWS07	13468 C>A, p.Leu378Met	ND5	0 (0%)	highly conserved	Both SIFT and PolyPhen-2 predict this variant to be damaging to the ND5 polypeptide structure and/or function.
GWS09	2636 G>A	16s rRNA	0 (0%)	strictly conserved	This variant is heteroplasmic in fibroblasts ONLY (49% mutant mtDNA, 51% normal mtDNA). It was not detected in leukocytes.

Table 2. mtDNA variants of possible significance in study participants (continued)

Participant	Variant	Gene	Frequency (#/2704)	Homology	Additional Comments
GWS15	2557 C>T	16s rRNA	0 (0%)	poorly conserved	none
GWS15	14831 G>A, p.Ala29Thr	CYTB	5 (0.18%)	poorly conserved	This variant has been reported in association with LHON (Biochemical and Biophysical Research Communications (2002) 295 (2): 342-347), however, more recent data supports its status as a neutral polymorphism (American Journal of Human Genetics (2006) 78 (3): 487-497; Molecular Vision (2007) 13: 1516-1528; Human Mutation (Online) (2009) 30 (6): 891-898).
GWS16	914 A>G (heteroplasmic)	12s rRNA	0 (0%)	highly conserved	This variant is heteroplasmic in fibroblasts ONLY (51% mutant mtDNA, 49% normal mtDNA. It was not detected in leukocytes.
GWS16	7962 T>C, p.Leu126Ser	COX2	0 (0%)	poorly conserved	This variant is predicted by PolyPhen-2 to be damaging to the COX2 polypeptide structure and/or function.
GWS16	12788 C>T, p.Ser151Phe (heteroplasmic)	ND5	0 (0%)	strictly conserved	This variant is heteroplasmic in fibroblasts ONLY (28% muatant mtDNA, 72% normal mtDNA). It was not detected in leukocytes. Both SIFT and PolyPhen-2 predict this variant to be damaging to the ND5 polypeptide structure and/or function.
GWS16	12789 C>T, p.Ser151Phe (heteroplasmic)	ND5	0 (0%)	N/A	This variant is heteroplasmic in fibroblasts ONLY (28% muatant mtDNA, 72% normal mtDNA). It was not detected in the leukocyte sample. Through RFLP analysis, it was determined that the mtDNAs with the 12788C>T variant also harbored this variant. Thus, the consequences of this change are the same as the 12788 change Ser151Phe.
GWS16	14607 G>A, p.Pro23Ser (heteroplasmic)	ND6	0 (0%)	strictly conserved	This variant is heteroplasmic in fibroblasts ONLY (46% mutant mtDNA, 54% normal mtDNA). It was not detected in leukocytes. Both SIFT and PolyPhen-2 predict this variant to be damaging to the ND6 polypeptide structure and/or function.
GWS19	14198 G>A, p.Thr159Met	ND6	2 (0.07%)	poorly conserved	none
GWS22	1792 G>A (appears heteroplasmic)	16s rRNA	0 (0%)	strictly conserved	This variant appears to be present in fibroblasts ONLY and appears to be heteroplasmic. RFLP analysis is pending.
GWS22	4674 A>G, p.Ile69Val (appears heteroplasmic)	ND2	0 (0%)	poorly conserved	This variant appears to be present in fibroblasts ONLY and appears to be heteroplasmic. RFLP analysis is pending.
GWS22	10596 A>G, p.Met43Val (appears heteroplasmic)	ND4L	0 (0%)	poorly conserved	This variant appears to be present in fibroblasts ONLY and appears to be heteroplasmic. RFLP analysis is pending. SIFT predicts this variant to be damaging to the ND4L polypeptide structure and/or function.
GWS22	13327A>G, p.Thr331Ala (appears heteroplasmic)	ND5	0 (0%)	highly conserved	This variant appears to be present in fibroblasts ONLY and appears to be heteroplasmic. RFLP analysis is pending. Both SIFT and PolyPhen-2 predict this variant to be damaging to the ND5 polypeptide structure and/or function.
GWS24	2623 A>G (appears heteroplasmic)	16s rRNA	0 (0%)	highly conserved	This variant appears to be present in fibroblasts ONLY and appears to be heteroplasmic. RFLP analysis is pending.
GWS24	10014 G>A (appears heteroplasmic)	tRNA Glycine	0 (0%)	poorly conserved	This variant appears to be present in fibroblasts ONLY and appears to be heteroplasmic. RFLP analysis is pending.
GWS24	12128 T>G (appears heteroplasmic)	ND4	0 (0%)	poorly conserved	This variant appears to be present in fibroblasts ONLY and appears to be heteroplasmic. RFLP analysis is pending.
GWS26	15947 A>G	tRNA Threonine	1 (0.04%)	poorly conserved	none

Key Research Accomplishments:

PLEASE NOTE: We have applied for a second no-cost extension for this grant that is currently moving through the approval process. This extension will allow us to recruit a greater number of subjects so that we can draw more meaningful conclusions from our data. Therefore, the data presented in this report are preliminary and are NOT the final analyses. We will submit a Final Addendum to this report when the no-cost extension runs out. If, for any reason, we are not granted the no-cost extension, we will immediately submit a Final Addendum detailing the conclusions that we are able to draw from the analyses that have been completed on the study subjects to date.

1. A majority of the data collected for this study will be best analyzed in the context of greater numbers of participants. A second no-cost extension will enable to recruit more participants and most meaningful analysis for the Gulf War Syndrome subjects will take place in the next few months. Comparison of the Gulf War Syndrome data with appropriate normal controls and disease groups is essential for interpretation of the data. We have analyzed data from patients with known pathogenic mutations affecting oxidative phosphorylation as well as patients with chronic fatigue/fibromyalgia diagnoses. A comparison of data from these groups with the Gulf War Syndrome patients could be insightful.
    a. We have made considerable progress in characterizing the fatigue-myalgia population that is being used for comparison with GWS. We have performed detailed characterization of approximately 110 individuals with fatigue-myalgia. The characterization is clinical, metabolic, biochemical, and genetic.
2. The data that we have generated to date suggest that patients with GWS have a similar cellular energetics profile as those patients with chronic fatigue/fibromyalgia who harbor oxidative phosphorylation defects. As we analyze these data, there appears to be little difference in the fatigue-myalgia patients and the GWS patients.
3. This observation is very important since it implies that these patients with GWS are at increased risk for developing cerebral folate defects as well as small fiber neuropathies. Cerebral folate defects are treatable metabolic defects.

Progress

An essential aspect of the Gulf War Syndrome patient analysis is comparison of data with patients who harbor known mitochondrial mutations (nuclear DNA or mtDNA) as well as patients who have chronic fatigue or fibromyalgia. We are in the process of writing three manuscripts that will reference this grant. Some of the more recent data has led to expansion of the second manuscript to include mutations other than Complex V (ATP synthase). The topics of each manuscript are summarized below.

1. The first manuscript focuses on the characteristics of patients who harbor mutations in SURF1 (an assembly factor for Complex IV). This paper is directly relevant to this grant because it further defines how the types of changes that are observed in patients with OXPHOS defects are caused by various mechanisms.

2. The second manuscript is a detailed assessment of the supercomplexes in patients who harbor mutations in both nuclear and mitochondrial DNA. This paper is directly relevant to this grant because it further defines how the types of changes that are observed in patients with OXPHOS defects by various mechanisms affect these techniques. Both of these papers are essential to assessing the complex data sets anticipated in the Gulf War Syndrome patients.
3. The third manuscript describes over 100 patients diagnosed with chronic fatigue and myalgia by the techniques used in this grant. This group is essential to characterize and compare with the Gulf War Syndrome patients as discussed above.

Reportable Outcomes:

1. We are continuing work on the previously mentioned manuscripts which will reference this grant.
2. While more data are required on GWS patients, the results to date are consistent with the hypothesis in the grant proposal linking GWS with mitochondrial defects.

Conclusion:

We have applied for a second no-cost extension for this grant that is currently moving through the approval process. This extension will allow us to recruit a greater number of subjects so that we can draw more meaningful conclusions from our data. Therefore, the data presented in this report are preliminary and are NOT the final analyses. We will submit a Final Addendum to this report when the no-cost extension ends. If, for any reason, we are not granted the no-cost extension, we will immediately submit a Final Addendum detailing the conclusions that we are able to draw from the analyses that have been completed on the study subjects to date.

References:

None at this time.

Appendices:

No additional information to submit at this time.