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Research Paper

Reduced Human Leukocyte Antigen (HLA) Protection in Gulf War Illness (GWI)

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ABSTRACT

Background: Gulf War Illness (GWI) is a disease of unknown etiology with symptoms suggesting the involvement of an immune process. Here we tested the hypothesis that Human Leukocyte Antigen (HLA) composition might differ between veterans with and without GWI.
Methods: We identified 144 unique alleles of Class I and II HLA genes in 82 veterans (66 with and 16 without GWI). We tested the hypothesis that a subset of HLA alleles may classify veterans in their respective group using a stepwise linear discriminant analysis. In addition, each participant rated symptom severity in 6 domains according to established GWI criteria, and an overall symptom severity was calculated.
Findings: We found 6 Class II alleles that classified participants 84.1% correctly (13/16 control and 56/66 GWI). The number of copies of the 6 alleles was significantly higher in the control group, suggesting a protective role. This was supported by a significant negative dependence of overall symptom severity on the number of allele copies, such that symptom severity was lower in participants with larger numbers of allele copies.
Interpretation: These results indicate a reduced HLA protection (i.e. genetic susceptibility) in veterans with GWI.
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1. Introduction

Shortly after the Gulf War (1990–91), veterans started to report a variety of health problems that began during, or soon after returning from, deployment, prompting investigation into the epidemiology and etiology of the complaints. Those investigations revealed that diffuse symptoms such as fatigue, musculoskeletal pain, mood and neurocognitive complaints, gastrointestinal problems, and rashes were most commonly reported. The constellation of symptoms, now commonly referred to as Gulf War Illness (GWI), has affected a substantial number of Gulf War veterans (Fukuda et al., 1998; Institute of Medicine, 2006, 2010; Kang et al., 2009; Steele, 2000). Several population-based studies have demonstrated that these symptoms occur at significantly higher rates in deployed Gulf War veterans relative to their nondeployed peers and other veterans (Fukuda et al., 1998; Kelsall et al., 2009; Unwin et al., 1999), raising the issue about possible in-theater exposures and stress as contributing factors. However, these symptoms are also present in non-deployed military personnel (Steele, 2000), leading some to suspect other causes, including reactions to vaccine adjuvants (Israeli, 2012; Toubi, 2012). In summary, GWI is now a recognized constellation of symptoms of unclear etiology, also co-occurring with psychiatric disorders.

To date, the pathophysiology of GWI remains poorly understood. The overlap of GWI symptoms with symptoms of autoimmune disorders (e.g., chronic fatigue), together with evidence of abnormal immune activation following exercise challenge (Broderick et al., 2012, 2013), raise the possibility that GWI reflects an abnormal immune process (Broderick et al., 2012, 2013; Hotopf et al., 2000; Israeli, 2012; Moss, 2013; Toubi, 2012). This probable involvement of altered immune mechanisms prompted our investigation of Human Leukocyte Antigen (HLA) in GWI.

HLA genes are located in the Major Histocompatibility Complex (MHC) of chromosome 6 and play a central role in immune recognition (Meuer et al., 1982). Most investigations of association of HLA to various

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diseases have focused on evaluating HLA allele frequencies in diseases of interest, as compared to the general, healthy population. Such studies have demonstrated HLA involvement with cancer, autoimmune, and infectious diseases (Rioux et al., 2009; Trowsdale and Knight, 2013). HLA Class I proteins (HLA-A, B, C) are expressed on all nucleated cells and present peptides from endogenous proteins to cytotoxic T lymphocytes engaged in immune surveillance. HLA Class II proteins (HLA-DRB1, DRB3/4/5, DQB1, DPB1) are expressed on antigen-presenting cells and present peptides derived from exogenous proteins to CD4+ helper T cells (Reche and Reinherz, 2003). A previous study of Gulf War syndrome in 27 veterans found that HLA DRB1*15 was more prevalent in cases than controls with an odds ratio of 1.66, although this association was not statistically significant (O'Bryan et al., 2003).

Here we tested the hypothesis that HLA may be a contributing factor in developing GWI by performing a stepwise linear discriminant analysis which assessed how well the number of copies of HLA alleles could classify participants in their respective group.

2. Materials and Methods

2.1. Study Participants

Veterans Affairs (VA) medical records were reviewed to identify potential participants. Participants were recruited if they had completed a Gulf War physical or if they had served during the Persian Gulf War. Exclusionary criteria included central nervous system disorders (e.g. Parkinson's disease, dementia, cerebral vascular accidents, a history of traumatic brain injury, etc.), lifetime psychotic or bipolar diagnoses, and current drug dependence. Veterans who might have had difficulty understanding the protocol were also excluded from recruitment. Participants provided written informed consent prior to participation and were compensated for their time. The study protocol was approved by the Institutional Review Board at the Minneapolis VA Health Care System.

We studied a total of 82 participants, 16 controls (15 men, 1 woman; age range 43–71 years; 54.9 ± 10.2 years, mean ± SD) and 66 GWI (64 men, 2 women; age range 39–76 years; 50.6 ± 7.9 years). The mean age did not differ significantly between the 2 groups ($P = 0.13$, t-test). Participants completed a symptom presence/severity questionnaire developed for use in Kansas Gulf War veterans (Steele, 2000) that evaluates a range of symptoms associated with GWI and permits determination of case status according to either the Centers for Disease Control and Prevention (CDC) criteria (Fukuda et al., 1998) or the Kansas GWI case definition (Steele, 2000). Participants meeting either set of Gulf War case definition criteria were included in the study. All participants had deployed during the Gulf War and were free of any autoimmune disease.

The questionnaire asks participants to indicate if they have had a persistent problem over the last 6 months with various symptoms from the following six domains: fatigue, pain, neurological-cognitive-mood, skin, respiratory, and gastrointestinal. For each symptom rated as present, participants are asked to rate the severity of the symptom as mild, moderate, or severe, and to indicate whether the symptom first became problematic before, during or after deployment to the Gulf. Only symptoms that began during or after Gulf War service are counted toward diagnosis. There were six symptom domains: fatigue, pain, neurological-cognitive-mood, skin, gastrointestinal, and respiratory (Steele, 2000). Individual symptom severity was reported in a scale from 0 to 3. For each participant, an average score per domain was calculated, and a grand average across domains was used in the regression analysis below.

2.2. HLA Genotyping

DNA isolation was carried out from 3 ml of whole blood drawn in EDTA tubes, using a commercially available kit (ArchivePure cat. 2,300,730) from 5Prime (distributed by Fisher Scientific or VWR) with an expected yield of 50-150 μg of DNA. The purified DNA samples were sent to Histogenetics (http://www.histogenetics.com/) for high-resolution HLA Sequence-based Typing (SBT; details are given in https://bioinformatics.bethematchclinical.org/HLA-Resources/HLA-Typing/High-Resolution-Typing-Procedures/ and https://bioinformatics.bethematchclinical.org/WorkArea/DownloadAsset.aspx?id=6482). Their sequencing DNA templates are produced by locus- and group-specific amplifications that include exon 2 and 3 for class I (A, B, C) and exon 2 for class II (DRB1, DRB3/4/5, DQB1, and DPB1) and reported as Antigen Recognition Site (ARS) alleles as per ASHI recommendation (Cano et al., 2007).

2.3. Data Analysis

Standard statistical tests were used to analyze the data using the IBM-SPSS statistical package (version 23) and ad hoc Fortran computer programs employing the International Mathematics and Statistics Library (IMSL; Rogue Wave Software, Louisville, CO, USA) statistical and mathematical libraries. We performed a stepwise linear discriminant analysis to identify those alleles that would correctly classify participants to their respective groups (control, GWI) using the SPSS statistical package above. In that analysis, group membership was the classification variable and the numbers of copies for each allele for each participant were the predictor variables ($N = 144$ alleles). This analysis identified 6 alleles that, as a group, classified correctly 84.1% of the participants (see below). We validated the robustness of this allele set by calculating the classification rate using the leave-one-out method in the original sample and by performing an extensive analysis based on the bootstrap (Efron and Tibshirani, 1993), as follows. Each participant was classified 10,000 times using bootstrap samples of participants and associated data from the 6 alleles above as predictors. Specifically, each bootstrap sample was generated by random sampling with replacement from each one of the 2 groups (control and GWI) to a total of $N = 200$ participant values per group (in separate additional analysis, bootstrap samples of $N = 100$ participants were used). We then performed a linear discriminant classification analysis to classify the given participant to one of the 2 groups, based on the 2 bootstrapped participant samples. Finally, we calculated the correct classification rate for each group. The quality and strength of classification was assessed using standard methods, including chi-square analysis, calculating confidence intervals on the sensitivity and specificity using the binomial theorem and Wilson's conservative test (Wilson, 1927), and calculating the receiver operating characteristic (ROC) curve and its associated statistics.

A different issue concerns the possible effect of unequal group sizes on the results of the discriminant analysis (Sanchez, 1974). Ideally, the group sizes should be equal, or as closely equal as possible, because, then, the null hypothesis for chance classification is the same or very similar across groups. To remedy this sample inequality, Sanchez (1974) proposed the following procedure aimed to perform the discriminant analysis using the same (or very similar) sample sizes. Let $G1$ and $G2$ be two groups to be discriminated with sample sizes m and km, respectively. The standard discriminant analysis would be somewhat hampered by the unequal sample sizes, $km > m$. To remedy this, Sanchez (1974) proposed that, instead of a single discriminant analysis, k such analyses be performed, where one group is always the same $N(G1) = m$, whereas the other group is a random subsample (without replacement) from $G2$, of size $N(G2) = \frac{km}{k} = m$, i.e. the same to that of $G1$, with equal chance probabilities of 0.5. The final classification rate is the average of the k classification outcomes. We applied this approach to our data as follows. The sample size of the control group was 16, and that of the GWI was 66. Since 66 cannot be divided exactly by 16, we performed the following steps.