HLA-DR Antigen Frequencies in Mexican Patients With Dengue Virus Infection: HLA-DR4 as a Possible Genetic Resistance Factor for Dengue Hemorrhagic Fever

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ABSTRACT: The human leukocyte antigen DRB1 locus (HLA-DRB1) was typed in genomic DNA extracted from whole blood samples of 34 Mexican dengue hemorrhagic fever (DHF) patients and 47 dengue fever (DF) patients, by polymerase chain reaction–sequence-specific oligonucleotide reverse dot blot. HLA-DRB1*04 was negatively associated with risk of DHF (OR 0.31, 95% CI 0.11–0.85). HLA-DR4 homozygous individuals were 11.6 times less likely to develop DHF in comparison to DR4 negative persons (OR 0.08, 95% CI 0.01–0.75). After adjusting for gender and infection type by logistic regression, DR4 positive individuals were 3.6 times less likely to develop DHF than DR4 negative persons (OR 0.28, 95% CI 0.12–0.66). A secondary dengue virus infection was also positively linked with DHF risk (OR 2.89, 95% CI 0.92–9.07). This data suggests that genes of the major histocompatibility complex play a major role in the susceptibility and/or resistance to develop DHF. In Mexicans, HLA-DR4 may be a genetic factor that is protective against DHF. Because HLA-DR4 has been positively selected in Latin American populations, these results may apply also to other similar ethnic groups, particularly those with high percentages of admixture with indigenous Amerindian genes. Human Immunology 63, 1039 –1044 (2002). © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: dengue hemorrhagic fever; human leukocyte antigens

ABBREVIATIONS

DF dengue fever
DHF dengue hemorrhagic fever
HLA human leukocyte antigens
MHC major histocompatibility complex

INTRODUCTION

Dengue virus infections are a serious cause of morbidity and mortality in many parts of the world [1]. There are an estimated 50 to 100 million cases of dengue fever (DF) and 250,000–500,000 cases of dengue hemorrhagic fever (DHF) annually worldwide [2].

Persons with symptomatic dengue generally develop an undifferentiated fever or classic DF, characterized by fever, headache, joint and muscle pain, and a maculopapular rash. Mild hemorrhage and a low platelet count
may occur [2]. DHF, the more severe form of dengue infection, occurs in a minority of patients and is defined by fever, hemorrhage, thrombocytopenia, and plasma leakage. The latter is attributed to increased vascular permeability, and manifested by elevated hematocrit values, hypoproteinemia, or effusions into serous cavities [2]. The exact pathophysiologic mechanisms behind this phenomenon are obscure. An inappropriate immune response to dengue virus is at least partly involved in this process [3]. Several studies have reported significant differences in the levels of cytokines between DHF and dengue fever patients, such as TNFα, IL-2, IL-6, and INFγ and soluble immune factors like the complement molecules C3a and C5a [4–6]. CD4⁺ and CD8⁺ lymphocytes are also highly activated in DHF, which is revealed by elevated levels of the soluble CD4⁺ and CD8⁺ receptors (sCD4 and sCD8) in DHF patients [4]. The epidemiologic observation that DHF occurs predominantly in persons experiencing a secondary heterotypic dengue infection leads to the antibody-dependent enhancement theory [7]. According to this hypothesis, pre-existing non-neutralizing antibodies present during a sequential heterotypic infection bind the virus and facilitate its entry and replication in cells of the monocyte/macrophage lineage [7]. However, other studies suggest that the virulence of the infecting serotype may be important in triggering the immunopathogenic mechanisms of DHF [8].

Dengue fever re-emerged in Mexico in the late 1970s and there was a marked transmission of serotypes 1, 2, and 4 during the 1980s. A seroprevalence study done in 1986 revealed a high prevalence of antibodies to dengue virus in several states [9, 10]. Dengue virus serotype 3 was introduced in 1995 and rapidly disseminated throughout the country [11, 12]. The high seroprevalence together with the co-circulation of multiple serotypes suggested that Mexico is in risk for a major DHF epidemic. The first cases of DHF in Mexico occurred in 1994–1995, and since then there has been an irregular epidemic activity in several parts of the country [10]. The State of Morelos, one of the endemic areas of Mexico, experienced its first significant DHF outbreak in 1998 in which 1758 DF and 41 DHF cases were reported [13], and virological studies demonstrated the circulation of all four serotypes that year.

The emergence of DHF is determined by both viral and host susceptibility or resistance factors. DHF tends to emerge in areas where multiple serotypes are simultaneously circulating. This hyperendemcity may lead to high secondary infection rates and the emergence of more virulent strains of the virus. However, in endemic areas, only a relatively small proportion of persons with sequential infections develops DHF [14]. This might suggest that there are host resistance factors operating in infected individuals. Such resistance or susceptibility genes could be found within the human leukocyte antigen complex (HLA), which mainly encode for major histocompatibility complex (MHC) class I and II molecules [15, 16]. HLA antigens participate in the induction of immune response by presenting peptides to CD4⁺ and CD8⁺ lymphocytes [15, 16].

Earlier studies in Thailand and Cuba found that certain class I alleles of the HLA complex are associated with resistance or susceptibility to DHF [17–19]. Class II HLA alleles, however, have not been investigated for their potential association with DHF. The class II locus, DRB1, is one of the most polymorphic HLA loci and has been implicated in the pathogenesis of several diseases [20, 21].

The aim of this study was to determine whether HLA-DRB1 alleles were associated with dengue hemorrhagic fever in Mexico.

**MATERIALS AND METHODS**

**Patient Population**

Thirty-four people with DHF and 47 with DF were selected from a database of patients with confirmed dengue infections diagnosed during the period January 1997 to December 1999 in the state of Morelos, Mexico. The database included relevant clinical, epidemiologic, and laboratory information. All study participants were Mexican Mestizo patients, residents of Morelos, and were managed as inpatients or ambulatory patients at collaborating hospitals. Each individual was asked about their birthplace as well as that of their parents and maternal and paternal grandparents. We considered patients Mexican Mestizos only if those individuals who for two generations, including their own, had been born in Mexico. A Mexican Mestizo is defined as someone born in Mexico who is a descendant of the original autochthonous inhabitants of the region and of individuals, mainly Spaniards, of Caucasian and/or black origin, who came to America during the 16th century. Their case histories were available for review. These patients were contacted during the period March 2001 to May 2001, and invited to participate in the present study. Informed consent was obtained for blood sample. The Ethics, Biosecurity, and Research Committees of the National Institute of Public Health approved the research protocol.

**Sample Collection and Processing**

The clinical diagnosis of DHF was reconfirmed retrospectively by review of case histories by the researchers involved in the study, in accordance with the WHO criteria, which includes fever, thrombocytopenia, hemorrhage, and evidence of plasma leakage. Dengue virus infection had been confirmed for each
was done by polymerase chain reaction (RT-PCR) [24]. A primary dengue virus infection was defined as the presence of IgM antibodies against dengue virus in acute or convalescent phase serum samples, whereas both IgM and IgG antibodies in serum were indicative of a secondary infection. Viral isolation or RT-PCR was carried out on blood samples taken during the first week of illness.

For HLA typing, a 5-ml blood sample was taken from each patient in sterile vacutainer tubes containing EDTA. Samples were stored at room temperature until DNA extraction.

**DNA Extraction**

Genomic DNA was extracted from whole blood samples according to the procedure described by Davis *et al.* [25].

**HLA Typing**

Generic HLA typing was done by polymerase chain reaction–sequence-specific oligonucleotide (PCR-SSO) reverse dot blot using the Dynal RELI SSO HLA-DRB test kits (Hoffmann-La Roche Ltd. and Roche Molecular Systems, Inc., Alameda, CA, USA) as described by Bignon and Fernandez-Viña [26]. HLA-DRB1*04 typing was done by polymerase chain reaction–sequence-specific oligonucleotide probe (PCR-SSOP) using protocols of the 12th International Histocompatibility Workshops. Samples exhibiting unclear SSOP reactivity patterns, were cloned and sequenced in a Perkin-Elmer 310 automated DNA sequencer (Foster City, CA, USA).

**Statistical Analysis**

Gene frequencies were calculated for DHF and DF cases and a two-tailed Fisher’s exact test was used to determine differences in frequencies between these two groups. The *p* values were corrected according to the number of tested specificities and the number of performed comparisons. The significance level was set arbitrarily at 0.05. The gene frequencies of DF patients without hemorrhagic manifestations or with unusual hemorrhage manifestations were compared with DHF patients. Odds ratio (OR) with corresponding 95% confidence intervals (95% CI) were calculated to measure the strength of association between HLA-DRB1 alleles and DHF.

Statistical associations between DHF and type of infection (primary or secondary), gender, and age were also determined. Infection type and gender were dichotomized and ORs with corresponding 95% CIs were calculated. Age was analyzed as a continuous variable and a *t*-test for independent samples was used to compare the mean ages of DHF and DF patients. Finally, unconditional logistic regression was performed in which only significant variables were introduced into the regression model. Data analysis was performed using the SPSS statistical package for Windows (Chicago, IL, USA).

**RESULTS**

Fifty percent (*n* = 17) of DHF cases and 63.8% (*n* = 30) of DF patients were females. This difference in proportions of females among DHF and DF patients was not statistically significant (Chi-square two-tailed *p* value 0.213). The mean age of DHF patients was 27.7 years old (range 12–49 years old), whereas that of DF patients was 28.6 years old (range 4–58 years old), a difference not statistically relevant (two-tailed *p* for Student’s *t*-test 0.728).

Clinical and laboratory data for both DHF and DF patients are illustrated in Table 1. All DHF cases fulfilled the diagnostic criteria recommended by the WHO. An increase hematocrit >20% for age, sex, and geographical altitude, was the main manifestation of increased vascular permeability and capillary leakage. Plasma leakage into serous cavities was infrequent because only one patient had demonstrable ascites.

Hemorrhagic manifestations were mild; petequias, bleeding from the nose and gum, and a positive tourniquet test were the most frequent, although some patients had upper gastrointestinal bleeding (data not shown). Thirty-three DHF patients were classified as Grade II DHF according to the WHO criteria. One patient had a

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**TABLE 1 Clinical manifestations of DHF and DF patients**

<table>
<thead>
<tr>
<th>Manifestation</th>
<th>DHF</th>
<th>DF</th>
<th>Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>34</td>
<td>47</td>
<td>100</td>
</tr>
<tr>
<td>Myalgia</td>
<td>34</td>
<td>47</td>
<td>100</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>34</td>
<td>29</td>
<td>61.7</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>34</td>
<td>27</td>
<td>57.4</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>31</td>
<td>45</td>
<td>95.7</td>
</tr>
<tr>
<td>Headache</td>
<td>31</td>
<td>46</td>
<td>97.9</td>
</tr>
<tr>
<td>Retro-orbital pain</td>
<td>28</td>
<td>38</td>
<td>80.9</td>
</tr>
<tr>
<td>Secondary infection</td>
<td>24</td>
<td>18</td>
<td>58.1</td>
</tr>
<tr>
<td>Rash</td>
<td>25</td>
<td>31</td>
<td>66</td>
</tr>
<tr>
<td>Positive tourniquet test</td>
<td>11</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Increase hematocrit</td>
<td>22</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>18</td>
<td>20</td>
<td>42.6</td>
</tr>
<tr>
<td>Hypoproteinemias</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Primary infection</td>
<td>6</td>
<td>13</td>
<td>41.9</td>
</tr>
<tr>
<td>Ascites</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Chi-square test; b 30 DHF and 31 DF patients confirmed serologically; *t* test performed for 16 DHF and 25 DF patients.*
TABLE 2  Distribution and association of HLA-DRB1 alleles in the study participants

<table>
<thead>
<tr>
<th>Allele</th>
<th>DHF (n = 34)</th>
<th>Controls (n = 99)</th>
<th>DF (n = 47)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>af</td>
<td>Number</td>
</tr>
<tr>
<td>DR8</td>
<td>15</td>
<td>0.441</td>
<td>16</td>
</tr>
<tr>
<td>DR4</td>
<td>12</td>
<td>0.352*</td>
<td>30</td>
</tr>
<tr>
<td>DR2</td>
<td>9</td>
<td>0.264</td>
<td>11</td>
</tr>
<tr>
<td>DR13</td>
<td>6</td>
<td>0.176</td>
<td>6</td>
</tr>
<tr>
<td>DR14</td>
<td>5</td>
<td>0.147</td>
<td>4</td>
</tr>
<tr>
<td>DR7</td>
<td>4</td>
<td>0.117</td>
<td>6</td>
</tr>
<tr>
<td>DR11</td>
<td>4</td>
<td>0.117</td>
<td>1</td>
</tr>
<tr>
<td>DR1</td>
<td>2</td>
<td>0.058</td>
<td>5</td>
</tr>
<tr>
<td>DR3</td>
<td>2</td>
<td>0.058</td>
<td>2</td>
</tr>
<tr>
<td>DR9</td>
<td>2</td>
<td>0.058</td>
<td>0</td>
</tr>
<tr>
<td>DR12</td>
<td>2</td>
<td>0.058</td>
<td>0</td>
</tr>
<tr>
<td>DR10</td>
<td>1</td>
<td>0.029</td>
<td>1</td>
</tr>
</tbody>
</table>

* Decreased in DHF when compared with DF (p = 0.011, p = NS, OR = 0.31, 95% CI = 0.11–0.85).
† Decreased in DF patients when compared with healthy controls (p = 0.003, p = 0.036, OR = 0.09, 95% CI = 0.0–0.64).

Abbreviations: af = antigen frequency; DHF = dengue hemorrhagic fever; DSS = dengue shock syndrome; DR = human leukocyte antigen; NS = not significant; OR = odds ratio; p = corrected p value; 95% CI = 95% confidence interval.

Positive tourniquet test, as the only hemorrhagic finding, and was therefore classified as DHF Grade I. Sixty-two percent of DF patients had some form of hemorrhage and 57.4% presented thrombocytopenia. None of DF patients who had both hemorrhage and a low platelet count had suggestive clinical data of hemococoncentration and plasma leakage, and therefore were not classified as DHF.

Thirty DHF and 31 DF patients had dengue infection confirmed serologically; 4 DHF and 16 DF seronegative patients were confirmed by virus isolation or by virus RNA amplification (RT-PCR). Dengue 2 and 3 were the most commonly identified serotypes in both DF and DHF patients. Eighty percent of DHF and 58.1% of DF patients with positive serologic tests had secondary infections as evidenced by the presence of both IgM and IgG against dengue virus. When the OR was calculated for this variable, persons with a secondary dengue virus infection were almost three times more likely to have DHF as compared with individuals with a primary infection (OR 2.89, 95% CI 0.92–9.07).

Table 2 illustrates the distribution of HLA-DRB1 antigen frequencies in the study participants. HLA-DRB1*08 was the most frequent antigen in DHF patients, whereas DRB1*04 was more frequent in DF patients. The antigen frequency of DRB1*04 was moderately decreased in DHF patients when compared with DF patients (p = 0.011, p = NS, OR = 0.31, 95% CI = 0.11–0.85). DRB1*04 subtyping analysis revealed similar distribution in DHF and DF patients without significant differences. Comparison with healthy controls demonstrated an increased antigen frequency of DR4 and a decreased antigen frequency of DR11 in DF patients (p = 0.049, p = NS, OR = 2.03, 95% CI = 0.94–4.43 and p = 0.003, p = 0.036, OR = 0.09, 95% CI = 0.0–0.64, respectively). DRB1*04 positive individuals were three times less likely to develop DHF in comparison with individuals DRB1*04 negative.

The antigen frequencies of DRB1 revealed similar patterns in the DF groups without hemorrhagic manifestations and those with unusual hemorrhage. Only a moderate increased antigen frequency of HLA-DRB1*04 was observed in DHF patients when compared with DF patients (data not shown).

Ten individuals were DRB1*04 homozygous, 9 had DF and 1 had DHF. Patients homozygous for DRB1*04 were 11.6 times less likely to develop DHF than patients DRB1*04 negative (p value for two-tailed Fisher’s exact test 0.012, OR 0.086, 95% CI 0.010–0.745). This association was also seen when patients in DF subgroups were compared with DHF patients (p value for two-tailed Fisher’s exact test 0.043, OR 0.076, 95% CI 0.006–0.889 for DF patients without hemorrhage and p = 0.031, OR 0.091, 95% CI 0.01–0.846 for DF patients with unusual hemorrhage).

To control for possible confounding due to age, gender or infection type, a logistic regression model was constructed. After adjusting for age, gender, and infection type, DRB1*04 positive persons were 3.6 times less likely to have DHF compared with individuals DRB1*04 negative (OR 0.28, 95% CI 0.12–0.66).

Several other alleles characterized positive associations with DHF but were not statistically relevant.

DISCUSSION

In this study we found, albeit with a relatively small sample, that the frequency of HLA-DRB1*04 was lower in persons who had DHF than in DF patients and that this allele was negatively associated with DHF risk. This protective association was also observed when DF patients (without hemorrhage or with unusual hemorrhage) were compared with DHF individuals. Persons homozygous for DRB1*04 were also much less likely to develop DHF than persons DRB1*04 negative. Earlier studies have reported that this allele is the most frequent DRB1 allele in the Mexican Mestizo population (gene frequency [gf] = 0.237) [27, 28].

Studies in Thailand and Cuba reported associations between certain class I HLA alleles and DHF. The Thai study found that HLA-B13 was negatively associated with dengue shock syndrome (DSS), whereas HLA-A2 and B-blank were risk factors for DHF [17]. Cuban studies reported that HLA-B14 and -A29 are negatively
correlated with DHF risk and HLA-A1 and B-blank were positively associated with DHF/DSS [18, 19].

In this study we used molecular methods to type the HLA-DRB1 locus, one of the most polymorphic loci of the HLA complex, and found that HLA-DRB1*04 is negatively associated with the risk of DHF. Although several other HLA alleles reported positive associations with DHF, the associations observed were not statistically significant. These observations, however, suggest the need to conduct larger studies of the DRB1 locus to clarify whether these alleles are in fact risk factors for DHF. This study analyzed the DRB1 locus in DHF cases occurring in Mexico.

The pathophysiology of DHF is unclear [3]. Evidence to date suggests that the disease is, at least in part, mediated by an inappropriate immunologic response to the virus. The envelope protein (E) of the virus is its dominant antigen and is responsible for viral entry into target cells. This protein also induces protective immunity, but may also stimulate cross reactive antibodies and target cells. This protein also induces protective immunity. These observations, however, suggest the need to conduct larger studies of the DRB1 locus to clarify whether these alleles are in fact risk factors for DHF. This study analyzed the DRB1 locus in DHF cases occurring in Mexico.

The clinical manifestations of DHF and DF were similar to that reported by other studies. Hemorrhage and thrombocytopenia were however more common in our DF patients than have been previously reported. It is quite possible that some of the DF patients with both thrombocytopenia and hemorrhage were potential DHF cases, but due to the absence of clinical data supportive of plasma leakage, it was not possible to classify them as such.

In this study we also confirmed that a secondary heterotypic dengue virus infection was associated with risk of DHF. Interestingly, the majority of DF patients also had secondary infections. Consequently, a secondary infection per se is probably not a risk factor for the hemorrhagic disease, but is rather a surrogate for the presence of non-neutralizing enhancing antibodies; however, another biological factors cannot be excluded.

Males and females were equally affected by DHF and the mean ages of individual with diagnoses of DHF and DF were similar (27.7 and 28.6 years old, respectively). Various studies have reported that the age and sex distribution of DHF in the Americas does not follow the pattern observed in South East Asian countries [29]. DHF in South East Asia is primarily a childhood disease and females are at greater risk [29]. By contrast, in countries of the Americas DHF occurs in all age groups, but adults are principally affected and there is no predisposition of a particular gender. Our clinical and laboratory findings concur with earlier reports in Mexico and other Latin American countries [10, 29].

Sufficient data on dengue serotypes isolated from DHF and DF patients was not available and hence we did not take this factor into consideration for the analysis, however dengue 2 and 3 were the most common identified serotypes in both DF and DHF patients.

In conclusion, we showed that HLA-DRB1*04 is an important resistance allele to DHF in the studied population. This allele is also one of the most frequent in the Mexican Mestizo and Amerindian populations of the Americas [30]. If these findings are corroborated in other studies, HLA-DRB1*04 may indeed be an important resistance genetic factor for DHF in Mestizo populations of the Americas. It is left to be seen how the DRB1 gene participates in the protection of individuals from DHF.

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