Asymptomatic Dengue Infection in a Cuban Population Confirms the Protective Role of the RR Variant of the FcγRIIa Polymorphism

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Abstract. The role of human Fcγ receptors (FcγR) has been recognized considerably over the last years. These receptors vary in their affinity for IgG subclasses and the intracellular signals elicited by them. Allelic variants of FcγR genes may influence the biological phagocyte activity, accounting for an inherited pre-disposition to disease. The specific FcγRIIa (CD32) contains a polymorphic variant (H/R131) that has been associated to a reduced risk for developing dengue hemorrhagic fever (DHF). Here, we investigated the role of this polymorphism in a very well-characterized group of Cuban individuals with antecedents of DHF, dengue fever (DF), or subclinical dengue infection. The HH131 genotype was significantly associated with dengue disease, either DF (**P* = 0.016; odds ratio = 4.425; 95% confidence interval = 1.10–20.52) or DHF (*P* = 0.00018; odds ratio = 10.56; 95% confidence interval = 2.33–54.64) with respect to the subclinical infection.

INTRODUCTION

Since the 1960s, more than four million persons, mostly children, have been hospitalized, and 65,000 have died by dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). This severe syndrome is caused by any of the four dengue serotypes (DEN-1 to DEN-4). These viruses belong to the *Flaviviridae* family and are transmitted by *Aedes aegypti*, which lives in urban environments and preferentially bites humans.¹

DHF/DSS occurs more frequently in patients suffering from a secondary dengue infection with a different serotype from that of the first infection. Cross-reactive, non-neutralizing antibodies in the serum of a patient interact with the infecting virus, favoring infection of host macrophages through Fc receptors and enhancing the number of infected cells. This phenomenon is known as antibody-dependent enhancement (ADE). Despite the wide recognition of ADE, the precise nature of the virus–antibody interaction in DF and DHF/DSS cases is not properly understood.¹

Host genetic factors seem to be relevant and may predispose some individuals to DHF development. Ethnicity, some human leukocyte antigen (HLA) haplotypes,^{2–5} the genetic variants of the vitamin D receptor⁶ and also genetic variants in the CD209 promoter are associated with severity of dengue disease,⁷ supporting the involvement of genetic factors in the pathogenesis of dengue infection.

It has been suggested that Fc receptors play an important role in the immune response to many infections, including dengue.⁸ FcγRIIa (CD32), the most widely distributed of FcγR types, is expressed in polymorphic forms on most of the hematopoietic cells, providing an important link between the humoral and cellular immune systems. FcγRIIa (CD32) is a low-affinity IgG receptor capable of binding IgG1–4 immunoglobulin subtypes. A single-point mutation at position 131 of the gene, arginine (R131) or histidine (H131), encoded for

the FcγRIIa seems to be critical for the binding of human IgG subclasses in which the FcγRIIa -R/R131 genotype binds to IgG1/3 and the FcγRIIa -H/H131 genotype interacts efficiently with IgG2.^{8–10} According to this, the Fc receptor polymorphism may be relevant to FcγRIIa function, might be linked to the variability of the immune response, and therefore, may be related to dengue pathogenesis.

In contrast to the complexity of dengue transmission in Southeast Asia, dengue experience in Cuba has been more limited. After an absence of 40 years, DEN-1 was introduced in 1977 and transmitted to nearly one-half of the Cuban population. Four years later, DEN-2 (Asian origin) infected approximately 25% of the population, and a large DHF/DSS epidemic ensued. Sixteen years later, in 1997, another Asian DEN-2 virus entered the country, producing a localized epidemic in Santiago de Cuba municipality (population of 475,000). In this outbreak, DHF/DSS cases were observed in DEN-1 immune adults previously infected during the 1977 epidemic. Later, in 2001, a new serotype, DEN-3 (Asian genotype), was detected in Havana City. In this epidemic, 78 serologically confirmed DHF (all adults) and 12,811 DF cases were reported.¹¹ Finally, in 2006, the circulation of DEN-4 was reported in Havana City.¹² This epidemic was eliminated in some months.

The unique epidemiological situation of dengue in Cuba is advantageous for the development of genetic studies related to this disease.

A previous report indicated an association between the homozygosity FcγRIIa-R/R131 and reduced risk of DHF/DSS in Vietnamese children.⁶ Taking into account the Cuban epidemiological dengue situation and the possible role of FcγRIIa, we have now studied its polymorphism in Cuban adults infected by DEN-4 during the 2006 epidemic. In addition to extending our knowledge into a population with a different genetic background, we were able to compare, for the first time, the Fc polymorphism in symptomless individuals with those with a symptomatic (DF or DHF/DSS) infection.

MATERIALS AND METHODS

Samples. This study was approved by the World Health Organization (WHO) Special Program for Research and Training in Tropical Disease (TDR) and the “Pedro Kourí”

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Tropical Medicine Institute's Ethical and Scientific Committees. The subjects were contacted in 2008 using the medical records of patients hospitalized at Instituto Pedro Kourí (IPK) during the 2006 DEN-4 epidemic. A total of 97 individuals were clinically classified as DF (68 clinical cases) or DHF/DSS (29 clinical cases), according to the WHO,¹³ and dengue infection was confirmed by IgM detection in sera collected in the first 6 days of fever.¹⁴

All selected subjects were adults (67 females and 30 males) with an age range from 21 to 81 years (mean $\pm$ SD age = 46; 44 $\pm$ 13,906 years).

As part of a serological study performed during the pick of epidemic (month six after transmission detection), 500 healthy adult individuals (relatives or neighbors of confirmed dengue patients) from three selected blocks of a health area with a high incidence of the disease were tested to determine asymptomatic infection through IgM¹⁴ dengue antibody detection in sera (Pupo and others, unpublished data). No fever or any other clinical symptoms had been recorded in them during the epidemic situation. Forty-two individuals with dengue IgG titers suggestive of a secondary dengue infection ($\geq 1,280$) were included in the present research.¹⁵ These individuals were unrelated to the DF and DHF clinical cases studied in this work.

Informed consents were obtained from all individuals before recruitment.

Ethylenediaminetetraacetic acid (EDTA) blood samples (5 mL) from a total of 139 individuals were collected: 97 from healthy adult individuals with the antecedent of DF ($N = 68$) or DHF/DSS ($N = 29$) and 42 from individuals with an asymptomatic DEN-4 secondary infection (subclinical group). Genomic studies were used.

For DNA extraction, genomic DNA was extracted from the whole blood using a Qiagen DNA extraction kit, and it was stored at -20°C for further genomic analysis. To determine the polymorphism associated to FcγRIIa, the protocol by Bazilio and others¹⁶ was used. The polymerase chain reaction (PCR) was carried out to amplify the genetic region of interest using oligonucleotide primers previously published.¹⁶ Specifically, a 1-kb portion of the FcγRIIa gene, containing exon 4 and part of exon 5 separated by an intron, was amplified by PCR using sense primer P63 (5'-CAAGCCTCTGGTCAAGGTC) and antisense primer FcγRII-30 (5'-CAATGACCACAGCCACAA TC). Nested PCR was performed using the specific sense primers 494A and 494G (5'-ATTCTCCC[A/G]TTTGATC), respectively and P52 as an antisense primer (5'-GAAGA GCTGCCCATGCTG). PCR products were run on agarose gel in a DNA electrophoresis, and the allelic forms of the FcγRIIa gene of each individual were determined. The samples were tested under code.

Statistical analysis. The FcγRIIa genotypes (R/R131, H/H131, and R/H131) and the allelic frequencies were compared with χ^2 test. Two-sided $P < 0.05$ was considered to be statistically significant. Data analyses were performed by means of the SPSS software (version 11.5.1) and EpiTable Statistical Analysis package (EpiInfo, Centers for Diseases Control and Prevention, Atlanta, GA).

RESULTS

According to the study purposes, we have proceeded to analyze genotype frequency distribution in the three groups of

TABLE 1

FcγRIIa polymorphism genotype frequencies in DHF, DF, and sub-clinical cases (asymptomatic dengue infection)

Genotype	Clinical picture		
	DF	DHF	Subclinical
R/R (%)	18 (26.5%)	6 (20.7%)	16 (38.1%)
H/H (%)	17 (25.0%)	13 (44.8%)	3 (7.1%)
H/R (%)	33 (48.5%)	10 (34.5%)	23 (54.8%)
Total	68 (100%)	29 (100%)	42 (100%)

Overall $\chi^2 = 13.91$; $P = 0.008$. Comparison of H/H vs. R/R + H/R: $\chi^2 = 13.57$; $P = 0.0011$. Comparison of H/H vs. R/R + H/R between DF and subclinical cases: $\chi^2 = 5.56$; $P = 0.018$ [OR = 4.33 (1.08–20.10)]. Comparison of H/H vs. R/R + H/R between DHF and subclinical cases: $\chi^2 = 13.96$; $P = 0.00018$ [OR = 10.56 (2.33–54.64)].

selected individuals. As depicted in Table 1, the HH131 genotype was found at a significantly higher frequency ($P = 0.008$) in individuals with the antecedent of a symptomatic dengue infection: DHF (51.5%) and DF (39.4%) compared with the subclinical group (9.1%).

To ascertain the associated risk for each genetic variant, homozygote individuals for one allele were compared with the remaining individuals (heterozygote + homozygote for the other allele). Compared with the subclinical group, the HH131 genotype was associated with the development of DHF (odds ratio [OR] = 10.56; 95% confidence interval [CI] = 2.33–54.64; $P = 0.00018$), and a similar trend was observed for DF (OR = 4.33; 95% CI = 1.08–20.10; $P = 0.018$; Table 1). On the contrary, RR131 genotype was associated with protection against DHF development (OR = 0.09; $P = 0.01$).

The analysis of allelic frequencies did not show significant differences between individuals with antecedents of clinical manifestations ($\chi^2 = 0.59$; $P = 0.44$). However, when the sub-clinical group was included, differences between symptomatic and asymptomatic infections became significant ($\chi^2 = 10.92$; $P = 0.004$; Table 2). As seen in Table 2, the allele H was more frequent in DHF and DF cases with respect to the subclinical group (DHF: OR = 3.10, 95% CI = 1.46–6.62, $P = 0.001$; DF: OR = 1.9, 95% CI = 1.04–3.47, $P = 0.025$).

DISCUSSION

Cuba offers an excellent opportunity to study and possibly, to identify some of the major genetic determinants of DHF/DSS. There is overwhelming evidence that the presence of non-neutralizing dengue antibody in the individual is a prerequisite for the occurrence of DHF/DSS. Because of the outstanding record of vector control and disease monitoring, Cuba provides a natural model to investigate the implications of the genetic immunity background of the disease severity. In contrast to most tropical countries, no endemicity is observed, and all epidemics have been caused by imported dengue viruses.

TABLE 2

Distribution of FcγRIIa allelic frequency in DHF, DF, and controls (asymptomatic dengue infection)

Allele	Allelic frequency (%)		
	DF	DHF	Subclinical
H	67 (50%)	36 (62.1%)	29 (34.5%)
R	67 (50%)	22 (37.1%)	55 (65.5%)
Total	134 (100%)	58 (100%)	84 (100%)

Overall $\chi^2 = 10.92$; $P = 0.0042$. Comparison between DF and subclinical group: $\chi^2 = 5.02$; $P = 0.025$ [OR = 1.90 (1.04–3.47)]. Comparison between DHF and subclinical group: $\chi^2 = 10.49$; $P = 0.0012$ [OR = 3.10 (1.46–6.62)].

The Cuban dengue experience has generated unique research materials, including the possibility of access to immune and previously dengue-infected persons in the reported epidemics. Specifically, well-documented DF and DHF clinical records from these epidemics are available at the "Pedro Kouri" Tropical Medicine Institute, because it is possible to locate these individuals.

The results obtained in this study provide evidence for the role of FC γ RIIA polymorphism, as an inherited risk factor, in the pathogenesis of DHF/DSS in Cubans. In fact, the FC γ RIIA-H/H131 genotype was detected at an increased frequency in individuals who suffered from clinical dengue (both DF and DHF/DSS), whereas FC γ RIIA-R/R131 predominated in those individuals with an asymptomatic dengue infection.

As mentioned above, the polymorphic variability of FC γ RIIA (H/R-131) has been associated with susceptibility to different infectious diseases, whereas the polymorphic alternative associated to RR allele seems to be associated with protection against intracellular pathogen infections.¹⁷⁻¹⁹ The association of FC γ RIIA polymorphism to dengue, previously reported in Vietnamese individuals, suggests that the homozygosis for the arginine variant might be associated with a reduced risk of DHF.⁶ Our results, based on clinically and epidemiologically characterized infected dengue adults, agree with this report. Even more, we found a significantly higher expression of the FC γ RIIA RR in subjects with asymptomatic dengue infection that was not reported in the Vietnamese population. This finding strongly supports the association of the RR 131 polymorphic variant with protection against dengue.

IgG1 and IgG3 subclasses are the pre-dominant immunoglobulins during dengue infection.²⁰ It has been reported that in individuals with FC γ RIIA-R/R131 genotypes as the predominant polymorphic variants, IgG1 and IgG3 bind efficiently.^{8,21}

In addition, the signal generated after interaction between FC γ RIIA and the virus-antibody (DENV/IgG1/3) complex may be associated with an efficient phagolysosome formation²² and the elimination of the immune complex and control of viral dissemination.²³⁻²⁵

Considering the results obtained in our study and taking into account previous observations,^{8,21-25} we could hypothesize that, in individuals of FC γ RIIA-R/R131 genotype, the IgG1/3 from dengue immune complexes bind more efficiently, favoring the elimination of the infection. On the contrary, in individuals with homozygosis for FC γ RIIA-H/H131, binding of virus/antibody complexes to FC γ RIIA through IgG1/3 may be ineffective, leading to a non-efficient lysosome fusion and subsequent evasion of the proteolytic system; this favors the dissemination of the virus by ADE phenomenon.²⁵ Although this hypothesis could partially explain dengue infection outcome, recent findings reported by Bruhns and others²⁶ are in contradiction with previous observations.^{8,21} Bruhns and others²⁶ investigated the binding of polyclonal and monoclonal antibodies (IgG1-4) to FC γ R and all known polymorphic variants and established a hierarchy of affinities of human FC γ R for polyclonal IgG of all four subclasses.²⁶ Contrary to previous reports,^{8,21} they found that IgG1 binds less efficiently to FC γ RIIA-R than to FC γ RIIA-H. However, it is not possible to exclude that these recent observations could be influenced by the use of a monoclonal rather than a polyclonal antibody, because binding of human FC γ Rs could have different specificities. To test our hypothesis, further studies to determine the affinity of DENV/IgG1/3 complexes to FC γ RIIA H/R polymor-

phism variants and the possible transduction of intracellular signals favoring or not favoring ADE phenomenon are needed.

The frequency of the RR polymorphism in the population around the world varies significantly. Previous studies in the Havana City population show that the distribution on these receptors (55% RR, 32% RH, and 13% HH)²⁷ is different from the one observed in the Asian population where the RR variant (6-10%) is less common.⁶

Differences in FC γ RIIA polymorphism variants²⁸ among populations may explain, at least in part, the higher severity of the disease observed in Asia²⁹ and different disease outcome in individuals with a heterotypic dengue infection under similar epidemiological conditions. In addition, the polymorphic variability of FC γ RIIA could be associated with the higher risk for DHF in whites and in individuals with the antecedent of bronchial asthma and diabetes mellitus. Ethnicity and chronic diseases have been reported as risk factors for DHF/DSS.³⁰

To our knowledge, this is the first report on the genetic FC polymorphism in relation to the asymptomatic dengue infection in which FC γ RIIA-R/R131 genotype is associated to dengue protection. Because only DEN-4 infected individuals were tested, further studies should be conducted to test these observations against the rest of the three serotypes. In addition, more research needs to be done to clarify the role of other genetic polymorphisms in dengue infection.

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