Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

The immunoglobulin γ marker 17 allotype and KIR/HLA genes prevent the development of chronic hepatitis B in humans

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doi:10.1111/imm.13133
Received 29 July 2019; revised 18 September 2019; accepted 9 October 2019.
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Summary

Hepatitis B virus (HBV) infection causes a self-limiting disease in most individuals. However, < 10% of infected subjects develop a chronic disease. Genetic host variability of polymorphic genes at the interface of innate and acquired immunity, such as killer immunoglobulin-like receptors (KIR), their human leucocyte antigen (HLA) and IgG allotypes (GM), could explain this different clinical picture. We previously showed a protective role of the KIR2DL3 gene for the development of chronic hepatitis B (CHB), and a detrimental role of the KIR ligand groups, HLA-A-Bw4 and HLA-C2. We have expanded the previous analysis genotyping patients for GM23 and GM3/17 allotypes. The comparison of the patients with CHB with those who resolved HBV infection showed that the presence of GM17 allele virtually eliminated the risk of developing CHB (OR, 0·03; 95% CI, 0·004–0·16; $P < 0·0001$). In addition, the combination of GM17, KIR2DL3, HLA-A-Bw4 and HLA-C2 was highly sensitive to predict the outcome of HBV infection.

Keywords: hepatitis B virus; human leucocyte antigen; killer immunoglobulin-like receptor; γ marker.

Abbreviations: ADCC, antibody-dependent cell, - mediated cytotoxicity; CHB, chronic hepatitis B; Fc, fragment crystallizable; GM, γ marker; GWAS, genome-wide association studies; HBV, hepatitis B virus; HBsAg, hepatitis B surface-antigen; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HLA, human leucocyte antigens; KIR, killer immunoglobulin-like receptors; NK, natural killer; OR, odds ratio; PCR, polymerase chain reaction

Introduction

Hepatitis B virus (HBV) worldwide affects millions of people. A chronic course of HBV infection develops in less than 5% of immune-competent adults affected by acute HBV infection, while the majority of patients resolve the infection.[1] The reason for this different clinical picture is not completely known, but genetic host variability is involved.[2]

The acquired immune response is assumed to play a key role in the elimination of the virus and in disease pathogenesis. Accordingly, chronic HBV infection is characterized by an inadequate T-cell response leading to viral persistence.[3] A murine model mimicking acute HBV infection has provided clear evidence that CD8+ T-cells are the major effectors mediating HBV elimination, and that natural killer (NK) cells function as helper masters to antiviral T-cell responses.[4] The contribution of NK cells is likely to be of particular relevance also for human infection, because their frequency in the patient's liver is greatly enriched.[5] *In vitro* studies showed a possible contribution of antibody-dependent cell-mediated cytotoxicity (ADCC) in HBV infection, but the *in vivo* relevance in controlling the infection warrants additional studies.[6] In addition, antibodies to HBV could contribute to eliminate the infection and prevent HBV resurgence after the control of initial infection by cellular immune response.[7]

In the control of virus infection, polymorphic genes at the interface of innate and acquired immunity, such as killer immunoglobulin-like receptors (KIRs), mainly expressed by NK, and human leucocyte antigens (HLAs) are known to play a key role. In addition, several studies have clearly shown that immune response versus many infectious agents, vaccines and autoantigens is associated with particular immunoglobulin γ marker (GM) allotypes, encoded by autosomal codominant alleles that follow Mendelian laws of heredity on immunoglobulin heavy chain γ1, γ2 and γ3.[8–11]

Our research group has recently shown that KIR2DL3 and the KIR ligand groups HLA-A-Bw4 and HLA-C2 influence the outcome of HBV infection. In particular, we have reported a detrimental role of HLA-A-Bw4 and HLA-C2 groups, associated with the development of chronic hepatitis B (CHB), and a protective role of KIR2DL3, associated with spontaneous recovery from infection.[12] In particular, we observed that HLA-A*24 allele was overrepresented when compared with the frequency of HLA-A*24 allele in the normal reference population. These *in vivo* results confirm and extend data obtained *in vitro* with NK clones and with the binding of HLA-A*24 tetramers to KIR3DL1, strengthening the biological meaning of the observed associations.[9]

To best of our knowledge, there is no evidence for the role of GM in HBV infection.[13] In the present study we investigated the role of IgG allotypes and their interplay with KIR/HLA ligands in the control of HBV infection. Although KIR/HLA and GM are not directly related, they represent important polymorphic systems able to modulate immune response at different levels. Therefore, this study might explain to which extent the immunogenetic variability contributes to the outcome of virus infections, such as hepatitis B.

Materials and methods

Fifty-seven Caucasoid Italian patients with CHB at any stage of liver disease with or without any antiviral treatment had been recruited at the University Hospital of Palermo, Italy. As a control (resolved infection), 44 candidate blood donors screened at the Transfusion Medicine Unit of the hospital had been included. They were negative for anti-human immunodeficiency virus (HIV) I/II antibodies, anti-hepatitis C virus (HCV) antibodies, hepatitis B surface-antigen (HBsAg) and for HIV, HCV and HBV nucleic acids, but positive for anti-hepatitis B core total IgG antibodies, with or without antibodies anti-HBsAg and/or anti-hepatitis B e antigen. All subjects have donated blood samples for DNA study and have given written informed consent to the study, which was approved by Ethical Committee of Palermo University Hospital. The study was conducted in accordance with the ethical principles that have their origins in the Declaration of Helsinki. For the complete characteristics of the population under study, see the previous paper.[12]

The GM allotypes were assessed as previously described.[14] For the determination of GM3 and GM17 allotypes (A to G substitution), a direct DNA sequencing method was used. The DNA segment encoding the CH1 region of γ1 chain was amplified by polymerase chain reaction (PCR) using the following primers: 5′-CCCCTG GCACCCTCCTCCAA-3′ and 5′-GCCCTGGACTGGGGC TGCAT-3′. The purified double-stranded PCR product (364 bp) was subjected to automated DNA sequencing on an ABI PRISM 3730xl DNA Analyser. IgG2 markers GM 23− and 23+ [a G to A substitution in the fragment crystallizable (Fc) of the γ2 chain] were genotyped by a nested PCR-restriction fragment length polymorphism method. IgG3 markers GM5 and GM21 were not typed, because TaqMan genotyping assays for the IgG3 allotypes are not yet available. Because of almost absolute linkage disequilibrium at GM loci within an ethnic group, subjects positive for the IgG1 allotypes GM3 and GM17 are most likely positive for the IgG3 allotypes GM5 and GM21.[15,16] Of the 101 participants typed for KIR and their HLA ligand groups in the original study, only 88 samples were available to be typed for the GM 3/17 genotype and GM23 genotype in the present study, owing to loss of the original DNA of some individuals. Due to

technical problems, it was not possible to type for GM3/17 two cases and for GM23 two controls.

Using the PCR sequence-specific primer technique, the DNA of both groups of cases and controls had been genotyped for the presence of the three major KIR ligand groups: HLA-C1, HLA-C2 and HLA-Bw4, both HLA-B and HLA-A loci. KIR gene profiles were determined by the presence or absence of each KIR gene.[12]

For statistical analysis, we eliminated all patients with at least one or more missing records on either age/sex or the KIR/HLA system and GM allotypes. Statistical analyses performed on this data set, obtained after list-wise deletion of patients, are referred to as complete case analyses. Genotype frequencies among groups were estimated by gene counting. Crude complete case comparisons of gene frequencies were made using 2×2 contingency tables, analysed by the chi-squared test. Although KIR/HLA and GM systems are not related, considering that both of them contribute to the control of virus infection,[8–12] we performed a multiple logistic regression analysis, including both KIR/HLA system and GM allotypes, in order to assess their respective effect on HBV infection outcome. Therefore, a complete case multiple logistic regression model was considered to estimate adjusted odds ratios (ORs). The procedure started from a full model, including a set of variables as covariates encompassing GM allotypes and all KIR genes with their HLA ligands, except for those predictors having one unique value (zero-variance predictors). A stepwise procedure that compares nested models by Akaike's information criterion was carried out to explore the subset of statistically significant predictors. After estimating a preliminary model over $n = 69$ complete cases, we discarded predictors 'age' and 'GM23' in order to retain as much information as possible. In fact, both 'age' and 'GM23' were not statistically significant, with 'age' being the most important source of missingness among cases, and 'GM23' playing the same role among controls. After excluding these variables from the analysis, the final stepwise multiple logistic regression model was estimated over $n = 88$ complete cases. Three-way predictors were converted to binary variables in order to reduce the scarcity: for example, the GM17 carriers (GM17/17 plus GM3/17) were taken together. The whole analysis was performed by R 3.5.1.[17]

Results

To demonstrate the possible role of GM allotypes in the outcome of HBV infection, we compared the genotype frequencies between patients with chronic HBV infections and patients with resolved HBV infection. The genotype frequency distribution of GM allotypes is presented in Table 1. The homozygote GM17/17 haplotype was absent in subjects with CHB, and the heterozygote GM3/17 haplotype was present only in six out of 54 of these subjects (11%). The GM17 carriers (GM17/17 plus GM3/17) represented 11·1% of patients with chronic infections compared with 58·8% (20 out of 34) of subjects who resolved HBV infection (OR, 0·09, $P < 0·001$). Therefore, the GM17 allotype is highly protective, because its presence significantly reduces the risk of developing CHB. In contrast, no significant difference was found for the distribution of GM23 genotypes between the two groups of patients. Thus, this allotype has no influence on the outcome of HBV infection.

By performing a multiple logistic regression analysis (Table 2), the presence of GM17 allele practically eliminates the risk of developing CHB [adjusted OR, 0·03; 95% confidence interval (CI), 0·004–0·16; $P = 0·0001$]. The analysis also confirmed the strong association of HLA-A-Bw4 with the risk of chronic infection reported in our previous study. HLA-C2 was associated with the risk of developing CHB, as shown in the previous study, but with a marginal statistical significance, possibly because of the sample size reduction. Finally, KIR2DL3 maintained its protective role, i.e. it was associated with resolved infection.[12] We conclude that both KIR/HLA system and GM allotypes contribute to the final outcome of the infection.

The present sample size is not big enough to estimate interactions between genes that are part of a complex regulatory network, as performed in the previous paper.[12] In that paper, we demonstrated that the presence of KIR2DL3 gene is highly protective if only one of the two

Table 1. Frequency of GM allotypes in subjects with CHB and resolved HBV infection

Genotypes	Frequency chronic infection $n = 54$	Frequency resolved infection $n = 34$	Crude-OR (GM17 carriers versus GM17 non-carriers)[1]	P
GM17/17	0 (0)	3 (8·8)		
GM3/17	6 (11·1)	17 (50)		
GM3/3	48 (88·9)	14 (41·2)	0·09	< 0·001
	Frequency chronic infection $n = 56$	Frequency resolved infection $n = 32$	Crude-OR (GM23 carriers versus GM23 non-carriers)[2]	P
GM23+/+	19 (33·9)	8 (25)		
GM23+/−	26 (46·4)	18 (56·2)		
GM 23−/−	11 (19·6)	6 (18·7)	0·94	NS

[1]GM17/17 and GM3/17 (GM17 carriers) versus GM3/3 (GM17 non-carriers).
[2]GM23+/+ and GM23+/− (GM23 carriers) versus GM23−/− (GM23 non-carriers).
GM, γ marker; OR, odds ratio.

Table 2. Logistic regression model to predict CHB development in the complete case database (n = 88)

Variable	Code	Adjusted OR (95% CI)	P
GM3/17	0: 3/3	0·03 (0·004–0·16)	0·0001
	1: 3/17 + 17/17		
HLA-A-Bw4	0: Absent	7·7 (1·86–43·22)	0·009
	1: Present		
KIR2DL3	0: absent	0·02 (0·0005–0·25)	0·008
	1: present		
HLA-C2	0: absent	6·2 (0·96–52·38)	0·067
	1: present		

CI, confidence interval; GM, γ marker; HLA, human leucocyte antigen; KIR, killer immunoglobulin-like receptor; OR, odds ratio.

detrimental HLA ligand groups (HLA-A-Bw4, HLA-C2) is present. The gene is unable to confer protection when both HLA-A-Bw4 and HLA-C2 are present. Nonetheless, in this respect it is interesting to observe the combination of the predictive variables at the individual patient level. In the 14 patients who resolved HBV infection despite the absence of the GM17 protective gene, the protective KIR2DL3 gene is always present with only one of the two detrimental ligand groups in 13 out of 14 cases. The presence of both the detrimental ligand groups is observed only in one case. Then, four out of six patients with CHB despite carrying both the protective GM17 allele and the protective KIR2DL3 gene have also the detrimental combination predicting chronic infections, i.e. the presence of both the detrimental groups, HLA-A-Bw4 and HLA-C2. Only two out of six patients have the protective genotype (the protective KIR2DL3 gene and only one of the two detrimental ligand groups).

Discussion

The analysis of the KIR/HLA system and GM17 predicts the outcome of almost all the cases. This finding is of some importance because such a strong association it is not common for the majority of genetic association studies of virus infection.

A limit of the present study is that we could not perform a machine learning approach to estimate the performance of the predictive model taking into account the GM3/17 allotype. This was due to the missing data, which reduced the sample size of the study (from 101 to 88 samples). Despite the relatively small sample size, it is worth noting that the differences between the groups are remarkable and highly statistically significant. That strengths the importance of this exploratory analysis. A true predictive analysis based on machine learning is best used when the main task is to identify clinically useful patterns in large datasets,[18] hence this task is perfectly suited for future work when more data will become available.

An external validation of these data is needed to assess the generalizability of our findings to other populations, because we studied subjects of the same geographic area and ethnicity. The homogeneity of our population as well as the very high predictive capability of the KIR/HLA system and GM17 seem to suggest a possible selective pressure on the KIR/HLA and GM systems. This leads to a genotype resistant to HBV in a geographic area where the virus was endemic before vaccination.[19,20]

The GM allotypes are involved in the immunological control of virus infections, such as human cytomegalovirus (HCMV), HCV or herpes simplex virus.[16,21,22] Polygenic inheritance controls the clinical course of HBV infection.[24] Present results point out the role of GM allotypes and their cooperation with HLA/KIR genes in the elimination of the virus.

The GM allotypes can influence antibody specificity and affinity by changing the conformation of the variable region, hence modulating the kinetic competence of antigen-binding sites.[25] Therefore, allotype-mediated antibody responses against the virus may also result in different immunity to virus. However, the most important mechanism of GM gene involvement in response to HBV may refer to ADCC, i.e. the host immunosurveillance mechanism against virally infected cells. GM variation might contribute to the differences in magnitude of ADCC triggered upon ligation of Fcγ receptor to the Fc region of IgG.

Other mechanisms could involve the recognition of HBV antigens by the B-cell membrane-bound IgG receptors expressing different GM alleles, followed by processing and presentation to the peptide-binding groove of at-risk HLA alleles. GM allotypes could participate as recognition structures for the pathogenic epitopes on B-cell membranes.[11] GM allotypes could also influence the expression of idiotypes involved in immunity to the virus.[11]

Linkage disequilibrium in the GM gene complex is very strong, hence we cannot exclude that other genes in and around this complex, on chromosome 14, are responsible for the outcome of HBV infection by yet-unknown mechanisms.[26] All these possibilities are not mutually exclusive.

Finally, genome-wide association studies (GWAS) identified key genetic factors influencing the pathogenesis of HBV-related outcome. However, no study showed a role for GM allotypes.[27] However, it has to take into account that GM allotypes are not evaluated in the GWAS, because. commercial arrays do not cover this locus.[28]

Present and previous data on HCMV[21] show that genetic variations both in the innate and acquired immunity, such as NK cells and their KIR repertoire, and respectively IgG genes (GM variants), play a crucial role in the control of virus infection. Host genetic variants undoubtedly modify the clinical outcome of HBV infection. The genes have been identified as associated with

susceptibility to persistent HBV infection, disease progression and hepatocarcinogenesis by GWAS or by a candidate gene approach.[29] In particular, genetic diversity of immune response genes holds promise for explaining, in large part, the variability in outcome to viral infection amongst exposed individuals. Results presented in this paper strengthen the findings of the complexity of immunogenetic contributions to disease outcome. Understanding the various factors associated with the clinical course of HBV infection is essential for personalized treatment and surveillance of disease progression. Such studies may lead to a better identification of the pathways of immune response to viruses that should be of value in guiding efforts to prepare new anti-virus vaccines.

Acknowledgements

CCO, DDB and SR collected the data; AA, GA, MEL and JPP performed the experiments and compiled the data for the summation and analysis; MB performed statistical analysis. DDB, JPP and CCA designed the study. DDB and CC wrote the paper. All authors analysed the data, reviewed the paper, approved the final version and agreed to submit the paper. The authors thank Sarumathi Mohan for assistance in GM genotyping. Five authors of this study (DDB, AA, GA, GC and CC) have been supported by a grant from the Italian Ministry of University and Research (PRIN Prot n. 20157ATSLF 'Discovery of molecular and genetic/epigenetic signatures underlying resistance to age age-related diseases and comorbidities').

Disclosures

All authors declare that they have no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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