Association between human leukocyte antigens (HLAs) and human neutrophil antigens (HNAs) and autoimmune neutropenia of infancy in Danish patients.

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Running title: HLA and HNA genotypes and association to AIN

Keywords: HLA, HNA, genotype, autoimmune, neutropenia

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Abstract

BACKGROUND: Autoimmune neutropenia of infancy (AIN) is a frequent cause of neutropenia in children. The disease is caused by antibodies recognizing membrane antigens of neutrophils, located on immunoglobulin G (IgG) Fc receptor type 3b (FcγIIIb receptor). In this study, we investigated the possible association of human neutrophil antigens (HNA), human leukocyte antigen (HLA)-DR and HLA-DQ alleles with AIN and the association of these genotypes with the presence of anti-HNA-1a autoantibodies. METHODS: Eighty AIN cases with a median age of 13.5 months were included in this study. Controls were healthy unrelated Danish blood donors. Anti-HNA-1a autoantibodies were detected using a flow cytometric granulocyte immunofluorescence test (Flow-GIFT). Molecular determination of HNA genotypes was determined using real-time polymerase chain reaction (q-PCR). High-resolution HLA-DR and HLA-DQB1 were determined by next-generation sequencing. RESULTS: Antibodies against HNA-1a were detected in 51% (n=41) of AIN patients, and anti-HNA-1b was detected in 3% (n=2) of cases. *FCGR3B*01+,*02-,*03-* was more common (odds ratio, 6.70; $p < 0.0001$), and *FCGR3B*01-,*02+,*03-* was less common (odds ratio, 0.30; $p < 0.0001$) among AIN cases. HNA-1a antibodies were significantly more frequent among AIN cases with the *FCGR3B*01+,*02-,*03*-genotype (odds ratio, 3.86; $p < 0.007$). The HLA-DR*14 and HLA-DQB1*05:03 alleles were significantly more common (odds ratio, 7.44; $p < 0.0001$ and odds ratio, 2.50; $p < 0.0001$, respectively) in AIN patients. CONCLUSION: The HLA haplotype HLA-DR*14- DQB1*05:03 is associated with Danish AIN cases. Among Danish AIN patients, anti-HNA-1a is the most common autoantibody, and the antibody is more common in cases with the *FCGR3B*01-,*02+,*03-* genotype.

Abbreviations

AIN = Autoimmune neutropenia of infancy, CI = 95% confidence interval, GIFT = Granulocyte immunofluorescence test , HNA = Human neutrophil antigen, , OR =Odds ratio, p^c = Corrected p value, rs = RefSNP number.

Introduction

Primary autoimmune neutropenia of infancy (AIN) is the most frequent cause of severe neutropenia in children. The disease is caused by autoantibodies directed against membrane proteins on neutrophil granulocytes. The antibodies are directed against human neutrophil antigen (HNA) antigens, most frequently epitopes on the Fc fragment of IgG low affinity IIIb receptor (FcγIIIb) (CD16b) (1,2). The HNA nomenclature defines six neutrophil antigens that arise from single-nucleotide polymorphisms. There are three alleles of the *FCGR3B* locus: *FCGR3B*01* (HNA-1a), *FCGR3B*02* (HNA-1b, 1d), and *FCGR3B*03* (HNA-1c). The HNA-3, HNA-4 and HNA-5 are biallelic antigens encoded by the choline transporter-like protein-2 gene (*SLC44A2*), the integrin alpha M gene (*ITGAM*) and the integrin alpha L (*ITGAL*) gene, respectively. The complete genetic background for the HNA-2 genotype remains to be elucidated(1). The disease is often self-limiting, and most patients are in complete remission after 2-3 years. The over-all risk of severe invasive bacterial and fungal infections is relatively low; however, the clinical impact of neutropenia is directly proportional to the degree of neutropenia and is usually mild when the neutrophil count is between 1000 and 1500/µl, moderate when the neutrophil count is between 500 and 1000/µl, and severe when less than 500/µl. It has been suggested that AIN is triggered by a viral or bacterial infection; however, no evidence for this theory exists(2). The presentation of allo- and autoantigens to the immune system relies on the highly polymorphic human leukocyte antigens (HLAs). Specific HLA types are strongly associated with the risk of autoimmune diseases(3). This association is often disease-specific and may represent the superiority of these HLA proteins in binding antigens with autoantigenic potential. Associations with certain HLA-DR genotypes (4) and the HLA-DRB1*14 and DQB1*0503 genotypes (5) have previously been suggested in AIN. In the present study, we investigated the association between the HLA–DRB1 and –DQB1 and HNA-1, -3, -4 and -5 genotypes and the risk of AIN in a cohort of 80 Danish AIN patients. To obtain better insight into the relationship between AIN and HNA antibodies, we investigated the association between HNA genotypes and anti-HNA-1a antibodies, since it has previously been shown that primary AIN is more frequent in individuals with the *FCGR3B*01* (HNA-1a) allele(6).

Methods

Study Cohort

Eighty AIN patients diagnosed between 2005-2015 at the Department of Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark, were included in the study. Inclusion criteria were the presence of chronic neutropenia defined as absolute neutrophil count (ANC) < 1.5x109/l in two repeated tests, age ≤ 5 years and the presence of anti-neutrophil antibodies in the flow cytometric indirect granulocyte immunofluorescence test (Flow-GIFT). The exclusion criteria were inborn neutropenia, neutropenia related to inborn syndromes and hematological malignancies. Genetic material for HNA and HLA genotyping was

available from all patients and consent for study participation was obtained from the legal guardian. Antibody specificity was determined from the panel reactivity in the Flow-GIFT. The control group consisted of healthy Danish blood donors form the Aalborg University Hospital blood bank, Aalborg, Denmark. A group of 200 randomly selected Danish blood donors were tested for the HNA-1, -3, -4 and HNA-5 genotypes. The control group for HLA genotyping consisted of 298 randomly selected bone marrow donors from the Danish bone marrow registry. Both control groups consisted primarily of Caucasians. All samples were handled anonymously. The study was approved by the local ethics committee

Samples

Blood samples consisted of EDTA-stabilized whole blood for genotyping and blood serum for Flow-GIFT.

DNA preparation

DNA was extracted from peripheral blood using the Maxwell16 Blood DNA Kit on the Maxwell16 Instrument (Promega Corporation, Madison, WI, USA).

HNA genotyping

Real-time polymerase chain reaction using TaqMan probes was used for the genotyping of eight single-nucleotide polymorphisms in the HNA-1 (*FCGR3B*) (rs447536. rs448740, rs52820103, rs428888 and rs2290834), HNA-3 (*SLC44A2*) (rs2288904), HNA-4 (*ITGAM*) (rs1143679) and HNA-5 (*ITGAL*) (rs2230433) loci as previously described(7).

High-resolution HLA-DRB1 and HLA-DQB1 genotyping

High-resolution HLA- typing was performed by next-generation sequencing -based HLA typing using the Illumina MiSeq platform at Histogenetics (NY, USA). HLA typing with 2x high resolution at the 4-digit allelic level without ambiguities for HLA class I and II genotyping was performed. Exons 2 and 3 were sequenced for HLA class I and II alleles.

Flow-GIFT analysis

Briefly, EDTA-stabilized whole blood from HNA phenotyped donors was washed three times in PBS with 5% fetal calf serum. Packed cells were incubated for 30 minutes with patient sera at 37 degrees Celsius, washed twice and blocked with heat inactivated rabbit serum. After 30 minutes of incubation with polyclonal rabbit anti-human IgM and IgG antibodies (DAKO, Denmark) erythrocytes were lysed and the cells were fixed. 100.000 cells were analyzed. The samples were analyzed using a BD FACS Canto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Flow-GIFT was considered positive if mean fluorescent intensity (MFI) ≥2 times the negative control using AB serum. The analysis was performed as part of routine clinical practice in an International Society of Blood Transfusion (ISBT) reference laboratory.

The analysis is ISO accredited (DANAK, Denmark) and validated continuously in the IGIW and INSTAND programs.

Statistical analysis

STATA software (StataCorp, USA) and Excel (Microsoft, USA) were used for statistical analysis. The allele frequencies were calculated by direct counting. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated, and differences between populations were tested using Fischer's exact test. The Bonferroni corrected significance level was used in all analyses.

Results

Baseline characteristics

We included 80 children with newly diagnosed AIN with a median age at diagnosis of 13.5 months (5 – 40 months).

HNA Antibodies

Forty-three samples contained antibodies with conclusive anti-HNA specificity. Forty-one samples (51%) had anti-HNA-1a specificity, and 2 samples (3%) had anti-HNA-1b specificity. Thirty-seven samples (46%) revealed an inconclusive pattern of reactivity.

HNA genotypes

The presence of *FCGR3B*01+,*02-,*03-* (OR= 6.70 (3.70-12.10)) was significantly higher in the AIN patient group compared to healthy controls, whereas the presence of *FCGR3B*01-,*02+,*03-* (OR= 0.30 (0.14-0.51)) was decreased in the AIN group compared to healthy controls. No other HNA genotypes were significantly associated with AIN (see Table 1).

HLA class II genotypes

The distributions of all HLA-DRB1 and HLA-DQB1 alleles are reported in Supplementary Tables S1 and S2. HLA-DRB1*14 (OR=7.44 (2.83-19.30) and HLA-DQB1*05.03 (OR=2.50 (1.64-3.80)) were significantly associated with AIN, ss was the DRB1*14/DQB1*0503 haplotype (OR=7.44 (2.83-19.30)) (see Table 2).

HNA genotypes and anti-HNA-1a association

The presence of anti-HNA-1a-specific antibodies was strongly associated with the presence of the *FCGR3B*01+,*02-,*03-* genotype (OR=6.70 (3.70-12.10)) and the absence of the *FCGR3B*01-,*02+,*03-* genotype (OR=0.30 (0.14-0.51)).

HLA alleles and anti-HNA-1a association

We observed an association between the presence of the HLA-DQ05.03 genotype and HNA-1a-specific antibodies (OR=2.56 (1.24-5.24)). The HLA-DRB1*14/DQB1*05.03 haplotype was not associated with antibody specificity (see Tables S3 and S4).

Discussion

In the present study we investigate the possible association between HLA and HNA genotypes and AIN. Immune Thombocytopenia (ITP), a disease that have some similarity to AIN with respect to the antibody mediated destruction of one cell lineage, have been studied more intensively for genetic and environmental risk factors. In ITP, certain HLA-DR, -DQ and cytokine genotypes have been investigated for association with the disease(8–10) and infections such as Cytomegalovirus, *helicobacter pylori* and Hepatitis-C seems to be possible environmental risk factors, especially in patients with certain genotypes(11). The mechanism responsible could be molecular mimicry initiating the activation of autoreactive B-cells and CD4+ T-cells. Since auto-antibody production continues after the initiating environmental trigger is eliminated, epitope spreading is proposed as a underlying mechanism(12,13). In the case of AIN, we do not yet have the same number of studies to propose a genetic or environmental trigger. The findings in ITP however suggests that these mechanisms should be explored further in AIN. The Fc gamma receptor 3b is polymorphic and encodes HNA-1 phenotypes known to be associated with AIN. Bruin et al. suggested an association between the HNA-1a homozygous state encoded by the *FCGR3B*01+,*02-,*03-* genotype and the risk of AIN (6). Audrain et al. reported similar findings in a French AIN cohort(14). Here, we confirmed these findings and discovered that the presence of this *FCGR3B* variant appears to be responsible for all our cases of anti-HNA-1a-positive AIN. We therefore suggest that the Fc gamma receptor 3b receptor could play an important role as an autoantigen in this disease. Since neutrophils are an essential part of the inflammatory process, we speculated whether the combination of certain neutrophil antigens, viral antigens and presentation through some HLA types might explain some cases of this disease. Autoimmunity is generally associated with specific HLA genotypes (15), and two former small studies have explored the association between the HLA system and the risk of AIN. Bux et al. investigated (16) 26 children using serological HLA typing methods and found an association between AIN and the HLA-DR2 phenotype (4). This finding could not be reproduced by Wang et al., who genotyped 55 AIN patients and found a strong association with the HLA-DQ*05:03 genotype in Taiwanese children (5). Surprisingly, we discovered the exact same association in our cohort of Danish Caucasian patients, suggesting that the trigger of this autoimmune disease might be the same in these genetically distant populations. This seems to be unrelated to the different allele frequencies of the *FCGR3B* gene between these populations(17). Different HLA epitopes binds allo and-autoantigens differently(3) and The DQB1*05.03 allele encodes a negatively charged aspartic acid at amino acid position 57 of the b chain, replacing positively charged amino acids such as valine, serine, or alanine. The peptide binding motif is changed compared to different HLA-DQ*05 genotypes. We therefore suggest that if cross reactivity towards a viral antigen(18) is the trigger of AIN, it seems to be an agent affecting Asian and European

populations in the same manner. When we looked at the association with HLA genotypes and the specificity of the autoantibodies, we did not find an association (Table S3). Antibody specificity in AIN can change from a Fc gamma receptor 3b reactive antibody to anti-HNA-1a specificity during the disease. This finding might suggest that another autoantigen triggers the disease and the anti-HNA-1a specificity in some cases is caused by epitope spreading. The HLA-DQ genotypes have also been associated with immunization towards food antigens (19), and it is established that food allergens can lead to general allergic reactions such as urticaria and anaphylaxis. Since food allergens are introduced at the same age as we usually observe immune cytopenia, and we believe this possible mechanism of cross reactivity needs to be investigated in further studies. Since we are now including patients prospectively for the Danish AIN cohort, we hope to update these data and hopefully add further information. We also did not investigate the binding of antigens to the HLA epitopes associated with AIN and this remains a topic for further studies.
In conclusion, we report an association between certain HNA and HLA antigens and AIN. We support the findings from smaller studies and suggest that antigen presentation from HLA-DQ phenotypes might be important in solving the etiology of AIN.

Author Contributions

KRN and RS were involved in all aspects of the study conception, design, analysis, interpretation and report generation and provided approval of the final version of the submitted manuscript. JB, TMH and AF were involved in data acquisition, analysis and report drafting and provided approval of the final version of the submitted manuscript.

Dislosure of Conflicts of interests

On behalf of all authors, I hereby declare no conflicts of interest.

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