

Increased frequency of HLA-DR2 in patients with paroxysmal nocturnal hemoglobinuria and the PNH/aplastic anemia syndrome

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Many autoimmune diseases are associated with HLA alleles, and such a relationship also has been reported for aplastic anemia (AA). AA and paroxysmal nocturnal hemoglobinuria (PNH) are related clinically, and glycoposphoinositol (GPI)-anchored protein (AP)-deficient cells can be found in many patients with AA. The hypothesis was considered that expansion of a PNH clone may be a marker of immune-mediated disease and its association with HLA alleles was examined. The study involved patients with a primary diagnosis of AA, patients with myelodysplastic syndrome (MDS), and pa-

tients with primary PNH. Tests of proportions were used to compare allelic frequencies. For patients with a PNH clone (defined by the presence of GPI-AP-deficient granulocytes), regardless of clinical manifestations, there was a higher than normal incidence of HLA-DR2 (58% versus 28%; $z = 4.05$). The increased presence of HLA-DR2 was found in all frankly hemolytic PNH and in PNH associated with bone marrow failure (AA/PNH and MDS/PNH). HLA-DR2 was more frequent in AA/PNH (56%) than in AA without a PNH clone (37%; $z = 3.36$). Analysis of a second cohort of patients with bone mar-

row failure treated with immunosuppression showed that HLA-DR2 was associated with a hematologic response (50% of responders versus 34% of nonresponders; $z = 2.69$). Both the presence of HLA-DR2 and the PNH clone were independent predictors of response but the size of PNH clone did not correlate with improvement in blood count. The results suggest that clonal expansion of GPI-AP-deficient cells is linked to HLA and likely related to an immune mechanism. (Blood. 2001;98:3513-3519)

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Introduction

Idiopathic aplastic anemia (AA) shows striking similarities to diseases of other organ systems with classically established autoimmune pathophysiology: AA frequently affects younger individuals, there is laboratory evidence for immune activation and destruction of the target organ by autoreactive T cells,¹ and patients often respond to immunosuppressive therapies.² Many autoimmune diseases have clear associations with particular HLA alleles: ankylosing spondylitis with HLA-B27 is the classical example, but similar relationships have been found in psoriasis (HLA-B13, HLA-B17-Cw6), rheumatoid arthritis (HLA-DR1 and HLA-DR4), juvenile diabetes mellitus (HLA-B8, HLA-B15, and HLA-B54), and multiple sclerosis (HLA-B7 and HLA-DR2). In hematology, agranulocytosis secondary to clozapine (HLA-B38, HLA-DR4, and DQw3) or methimazole (HLA-DR8) and mixed cryoglobulinemia in hepatitis C virus-infected patients (HLA-B8 and HLA-DR3) exemplify links between immunogenetics and pathophysiology.³⁻⁶ Fundamentally, the presence of particular HLA alleles likely allows for preferential presentation of particular peptides, perhaps derived from autoantigens, to specific T cells, driving the autoimmune process.

In AA, HLA associations have also been examined.⁷⁻¹⁹ An increased frequency of HLA-DR2 has been reported in some but not all studies.^{9-13,16} An association with class I alleles (especially HLA-A2) has been inconsistently identified.^{7,10,13-15} The presence of HLA-DR15 (split of DR2) was associated with good response to cyclosporine therapy, suggesting that this allele may be a specific

predisposing factor for the development of immune-mediated bone marrow failure.¹⁷⁻¹⁹

Clinically, AA overlaps with other bone marrow failure syndromes, especially paroxysmal nocturnal hemoglobinuria (PNH) and hypocellular myelodysplastic syndrome (MDS), and both syndromes have appeared to evolve from initially typical AA. The peculiar association of AA and PNH has been a subject of some scientific interest.^{20,21} The introduction of flow cytometric tests for the detection of glycoposphatidylinositol (GPI)-anchored protein (AP)-deficient cells has led to realization that a PNH clone can be present in 25% to 45% of AA patients at presentation, arguing against PNH as a late clonal complication of marrow failure.^{22,23} Expansion of an abnormal hematopoietic cell clone might be facilitated by an extrinsic growth advantage for PNH cells, as under conditions of autoimmune attack directed against normal marrow target cells.²⁰ T-cell receptor V β -chain skewing suggesting a T-cell-mediated process leading to suppression of hematopoietic function has been described in PNH²⁴ as well as in AA.²⁵

In contrast to the diagnosis of PNH by clinical symptoms and signs combined with the insensitive Ham test, flow cytometric phenotyping of GPI-anchored molecules on the surface of leukocytes allows a much more exact description of syndromes associated with PNH. Using this method, detection of numerically very few GPI-anchored protein-deficient cells can serve as marker of a distinct pathophysiology operating in some bone marrow failure syndromes.²² Based on the hypothesis that PNH requires for its

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evolution an immune-mediated process to provide a relative growth advantage for the GPI-anchored protein-deficient clone, we tested for an association of an expanded PNH clone with HLA class I and II alleles. This analysis was performed in patients with classical hemolytic PNH, AA, MDS, as well as in overlapping combinations of these clinical entities.

Patients, materials, and methods

Patients

Patients were studied at the Hematology Branch of the National Heart, Lung and Blood Institute (Bethesda, MD) during diagnostic evaluation for bone marrow failure. Informed consent for peripheral blood (PB) sample collection was obtained according to protocols approved by the Institutional Review Board of the National Heart, Lung and Blood Institute. Two databases were used. The first consisted of 415 patients for whom HLA typing was available: 260 patients had AA, 139 had MDS, and 16 had primary hemolytic PNH. The second database consisted of 308 patients with bone marrow failure (AA, MDS) who had received in the past treatment with intensive immunosuppression (see below). A total of 251 patients were in both data sets. In most of the patients, HLA alleles were analyzed using molecular techniques. For some patients only class I typing was performed.

Diagnostic criteria and definitions

The diagnosis of AA was established by bone marrow biopsy and PB counts according to the criteria of the International Study of Aplastic Anemia and Agranulocytosis; severity was classified by the criteria by Camitta and colleagues.²⁶ For the diagnosis of severe AA (sAA), in addition to hypocellular bone marrow without evidence for karyotypic abnormalities or morphologic dysplasia, patients had to fulfill 2 of 3 PB criteria: absolute neutrophil count (ANC) less than 500/ μ L, absolute reticulocyte count (ARC) less than 60 000/ μ L, and platelet count less than 20 000/ μ L of blood. Patients with hypocellular bone marrow without evidence of dysplasia and with normal karyotype, whose pancytopenia did not fulfill the above-mentioned severity criteria but with 2 of 3 PB parameters lower than ANC less than 1200, platelet count less than 60 000/ μ L, and hemoglobin less than 8 g/dL, were classified as having moderate AA (mAA). Both sAA and mAA were included in this study and categorized as AA. "Primary" hemolytic PNH was diagnosed by the clinical history of hemolysis and the presence of a GPI-AP-deficient clone in PB, hypercellular bone marrow with normal karyotype, absence of overt dysplasia, and a platelet count more than 60 000/ μ L, ANC more than 1200/ μ L, and reticulocyte count more than 100 000/ μ L (Table 1). The diagnosis of MDS was established based on characteristic morphologic abnormalities in the bone marrow or the presence of cytogenetic abnormalities and peripheral cytopenia; all patients were subclassified according to French-American-British nomenclature.²⁷ MDS was also categorized based on the cellularity, with hypocellular MDS diagnosed if the overall biopsy cellularity was less than 25%. All bone marrow biopsies and aspirates were evaluated for morphology by an independent hematopathologist not associated with the study. For the purpose of this study, patients with a PNH clone and otherwise fulfilling criteria of sAA or mAA were classified as having AA/PNH syndrome and those with myelodysplasia and a PNH clone as MDS/PNH syndrome; for the diagnosis of AA/PNH and MDS/PNH syndrome, evidence for the presence of GPI-AP-deficient clone (see below) and/or positivity in the Ham test was required (Table 1). For the patients treated with immunosuppression, regimens consisted of antithymocyte globulin (ATG) alone, ATG plus cyclosporin A (CsA), ATG plus CsA plus mycophenolate mofetil (MMF) acid, or cyclophosphamide plus CsA. In this study, response to the therapy was determined 6 months after treatment and defined as transfusion independence for red cell and platelets and improvement in counts such that patients no longer fulfilled severity criteria.²⁸

As a control for HLA-A and HLA-B phenotype, historical data were derived from reference information provided by the National Marrow

Table 1. Diagnostic criteria and definitions

Main clinical diagnosis	Clinical subgroups based on past medical history	N
AA	AA without PNH clone	212
	AA with PNH clone	48
MDS (including MDS that evolved from AA)	MDS without PNH clone	110
	MDS with PNH clone	29
Patients with an expanded PNH clone (includes all categories of bone marrow failure syndrome) at any time during the course of their disease	Primary hemolytic PNH	16
	AA/PNH syndrome, current or past diagnosis of AA with positive PNH test	48
	MDS/PNH syndrome underlying diagnosis of MDS and positive PNH test	29
	Hemolytic form of PNH	16
Hemolytic form of PNH	Primary hemolytic PNH, no evidence of bone marrow failure	19
	Hemolytic PNH evolved from initial AA	11
	Hemolytic PNH in context of MDS (dysplasia, karyotypic abnormalities)	11

HLA-Cw and class II typing was not available for all patients.

Donor Program Registry.²⁹ For Cw genotyping, normal values were obtained by screening control groups of blood donors and healthy volunteers, performed by the Clinical Center Department of Transfusion Medicine. HLA-Cw typing was not done for all patients. As control for class II phenotyping, historical data were derived from 678 healthy donors.³⁰ Ethnic differences between test groups were adjusted based on the method described below (see "Statistical analysis"). Within the AA group, 172 patients were white, 29 African Americans, 24 of Asian origin, 26 of Hispanic origin, and 9 of unknown ethnicity. For MDS, 115 patients were white, 9 African American, 4 Asian American, and 11 of Hispanic origin. Within the pure hemolytic PNH group, the composition was 12 whites, 2 African Americans, 1 Asian, and 1 Hispanic.

HLA typing

Molecular typing was used for the determination of HLA genotypes. Because many alleles were not included in the older high-resolution typing, inclusion of high-resolution typing (4 digits for each allele) reported at earlier dates may result in inaccurate determination of the allelic frequency. In addition, the allelic frequency in control populations has not been established, precluding reliable comparisons. Therefore, for consistency we applied low-resolution HLA-typing results for class I (A, B, Cw) and class II (DR, DQ) testing using sequence-specific primer amplification (SSP), a form of polymerase chain reaction (PCR). For typing, genomic DNA was derived from peripheral blood; DNA was resuspended in Tris buffer and the concentration was measured using a Gene Quant II spectrophotometer. The positive control primers amplify a 796-base pair (bp) fragment from the third intron of HLA-DRB1, or a 796-bp fragment of the DP α and ϵ region. The final PCR product was visualized on a 1.5% agarose gel stained with ethidium bromide, and the presence or absence of appropriately sized bands was assessed. The results of molecular typing were converted to the serologically established HLA phenotypic equivalents. For example, HLA-DRB1*15 and -DRB1*16 were analyzed as HLA-DR2.

Flow cytometric analysis

Due to the high sensitivity and lack of confounding by transfusions, we used GPI-AP expression on granulocytes as a diagnostic test for the

presence of the PNH clone.²² Furthermore, flow cytometry of GPI-AP-deficient granulocytes and monocytes was shown to be superior to phenotyping of erythrocytes in detection and quantitation of the PNH clone.³¹ Analysis of granulocyte GPI-AP expression was performed using CD66b-fluorescein isothiocyanate (FITC; clone 80H3 mouse IgG1 [Immunotech, Marseilles, France]) and CD16-PECy5 (clone 2G8, mouse IgG1 [Caltag, Burlingame, CA]), with CD15-phycoerythrin (PE)-conjugated (clone 80H5 mouse IgM [Immunotech]) as a non-GPI-anchored marker to positively identify neutrophils. The isotypic controls for granulocyte staining consisted of mouse IgG-FITC (clone X40 [Becton Dickinson, Mountain View, CA]) and mouse IgG1-PECy5 (clone MOPC-21 [Caltag]). Nonspecific Fc receptor-mediated binding of conjugated antibodies was blocked by preincubating 1 mL blood with 30 μ L mouse IgG (Caltag).

Blood was drawn by venipuncture into tubes containing EDTA. Samples were stained and analyzed within 24 hours of collection. Granulocyte GPI-AP expression was evaluated after incubating for 60 minutes 100 μ L whole blood with 10 μ L CD15-PE and either 20 μ L CD66b-FITC and 5 μ L CD16-PECy5 or the appropriate isotype controls. Erythrocytes were lysed using the Q-Prep apparatus (Coulter, Hialeah, FL) and were then fixed in paraformaldehyde. All samples were analyzed using the Coulter XL flow cytometry machine equipped with a 488-nm argon laser and XLII software. Strict criteria to distinguish cells lacking cell surface GPI-APs were applied. Polymorphonuclear cells were identified on the basis of light-scatter properties that correlate with size and granularity and on the expression of the constitutively expressed granulocytic marker (CD15). We used the simultaneous absence of 2 GPI-APs, CD66b and CD16, that are constitutively and at high levels expressed on normal granulocytes to determine PNH phenotype. The control mean \pm SD of percentage of double-negative cells was established by measurements in 76 healthy volunteers. Classification of patients with a GPI-anchored protein-deficient granulocytic population was based on the percentage of double-negative granulocytes greater than mean + 2 SD of controls and the presence of a negative population clearly separate from wild-type CD16⁺/CD66b⁺ cells on a 2-dimensional histogram.^{22,23}

Statistical analysis

Two types of tests were performed to evaluate the equality of antigen frequencies (the proportion of patients with at least one copy of a specific antigen). When comparing the antigen frequencies of a diagnosis group (eg, AA) to healthy controls, one sample χ^2 tests of the equality of proportions were used. When comparing the antigen frequencies of 2 different disease groups (such as AA versus MDS), 2 sample χ^2 tests for the equality of proportions were used. Tests were not performed when the expected antigen frequency was less than 5%. Antigen frequencies of the healthy controls were based on a weighted average of antigen frequencies of healthy controls from different ethnic groups.^{29,30} The weights were chosen to match the ethnic composition of the patients used in this report. For Cw, the

control frequencies were based on National Institutes of Health blood donors whose demographics approximated those in our patient database. Antigen frequencies for the controls were calculated using gene frequencies and the assumption of a Hardy-Weinberg equilibrium. To reduce the false-positive error rate due to performing approximately 700 statistical tests, only standardized test statistics (ie, z score) larger in absolute value than 3 (equivalently a $P < .003$) are reported. A threshold of 4 for the absolute value of the z score strongly controls the type I error rate for all tests at .05.

For the analysis of HLA association with response to therapy a 2-step process was performed. In the first step, allele frequencies were compared against control proportions, and alleles that were significantly different from the control ($P < .003$) were identified. This identified 2 alleles: HLA-B14 and HLA-DR2. In the second step allele frequencies for these 2 alleles were compared between responders and nonresponders. Because the second step is statistically independent of the first step, the type I error rate was strongly controlled in this stage by use of a Bonferroni correction, thus a P of less than .05/2 was used for significance at the .05 level. To evaluate the multivariate relationship between response and potential prognostic variables, multivariate logistic regression was performed.

Results

Association of HLA type and the presence of GPI-AP-deficient clone.

Historically, PNH was diagnosed by signs of anemia resulting from intravascular hemolysis, high lactic dehydrogenase (LDH) value, low haptoglobin, and a positive Ham test. With the introduction of flow cytometry for the detection of GPI-AP-deficient blood cells, the diagnosis of PNH syndrome is made more frequently, often in patients in whom PNH is not suspected on clinical evidence or who lack symptoms and signs of hemolysis. Using this sensitive method, we have examined the association of an expanded GPI-AP clone (defined by precise diagnostic criteria) with alleles of the HLA system.

We first calculated the frequencies of HLA class I and II alleles in patients with hemolytic PNH, irrespective of associated diagnoses. By medical history, these patients had primary PNH without bone marrow failure (see "Patients, materials, and methods"), or PNH had evolved from AA (AA/PNH syndrome) and (in a minority of cases) from myelodysplasia (MDS/PNH syndrome). In comparison to controls, there was a higher incidence of HLA-DR2 within this group (Tables 2 and 3; HLA-DR2 also was more often found in patients with primary hemolytic PNH, but statistical significance

Table 2. HLA phenotypes of patients with bone marrow failure and PNH

Comparison	HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
Hemolytic PNH vs controls†	NS	NS	NS	DR2; 54% vs 28% $z = 4.1$ N = 48	NS
Expanded PNH clone present (PNH/AA, PNH/MDS, PNH) vs controls	NS	NS	NS	DR2; 58% vs 28% $z = 6.4$ N = 89	NS
PNH/AA syndrome vs controls	NS	NS	NS	DR2; 56% vs 28% $z = 4.5$ N = 48	NS
PNH/MDS vs controls	NS	B7; 48% vs 21% $z = 3.5$ N = 27	NS	DR2; 74% vs 28% $z = 5.3$ N = 29	NS

NS indicates not significant.

No statistical differences in HLA phenotype were detected between the patients with florid hemolytic PNH and all other patients in whom PNH clone was detected (64% vs 54%, $z = 0.88$).

†All patients with current florid hemolytic PNH irrespective of the original diagnosis.

Table 3. Allelic frequencies of the more common A, B, DR, DQ, and Cw alleles in patients and controls

	HLA-A				HLA-B				HLA-C				HLA-DR				HLA-DQ							
	C	AA	MDS	PNH	C	AA	MDS	PNH	C	AA	MDS	PNH	C	AA	MDS	PNH	C	AA	MDS	PNH				
A1	24	18	29	25	B5	13	11	15	14	Cw1	09	07	11	06	DR1	18	17	18	09	DQ1	68	59	66	72
A2	47	47	46	45	B7	21	23	25	31	Cw2	10	10	08	13	DR2	28	43	40	58	DQ2	40	28	31	28
A3	22	23	24	23	B8	15	16	16	16	Cw3	23	19	25	13	DR3	20	21	19	21	DQ3	48	47	50	55
A9	23	20	21	20	B12	25	21	25	14	Cw4	26	23	18	24	DR4	29	25	29	33	DQ4	06	03	04	05
A10	12	15	17	12	B13	04	05	03	04	Cw5	10	06	14	08	DR5	24	28	22	28					
A11	12	12	13	15	B14	08	15	09	08	Cw6	12	16	23	16	DR6	28	23	20	17					
A19	29	34	27	25	B15	12	10	15	10	Cw7	45	49	44	57	DR7	23	17	22	10					
A28	10	12	10	12	B16	09	06	07	09	Cw8	11	13	09	09	DR8	08	08	06	08					
					B17	10	08	10	08	Cw12	06	03	11	10	DR9	03	03	03	01					
					B18	09	08	08	11	Cw15	07	05	02	05	DR10	03	01	03	01					
					B21	06	07	06	01	Cw16	08	03	02	06										
					B22	04	05	02	05															
					B27	07	07	10	05															
					B35	19	16	15	18															
					B37	02	02	06	03															
					B40	13	16	10	16															

HLA allelic frequencies found within all patients with AA, MDS, and all those with PNH clone (in percent) in comparison to controls (C). For comparison to control groups, molecular typings were converted into serologic nomenclature (parent antigens).

was not achieved, likely due to the low numbers of patients in this group [data not shown]). When resolved at the molecular level, the great majority of the patients with HLA-DR2 were carriers of the DRB1*15 allele (within HLA-DR2, DRB1*16 was present in only 14 instances).

When the larger category of all patients with an expanded PNH clone, regardless of manifestations of hemolysis and the relative size of the GPI-AP-deficient clone, were studied as a group, there was also a strong association with HLA-DRB1*15. We then determined the frequency of HLA class I and II types separately in patients with AA/PNH and MDS/PNH syndrome and found a statistically significant, strong association with the HLA-DR2 allele (Table 2). In addition, patients with MDS/PNH syndrome were more frequently positive for HLA-B7 in comparison with the control group (Table 2).

Comparison of HLA type within subgroups of patients with bone marrow failure and PNH

To analyze whether certain HLA class II alleles are associated with a particular clinical form of bone marrow failure, we compared the frequencies of HLA alleles among these groups. HLA-DR2 correlated more with the presence of GPI-AP-deficient cells in patients with AA than with AA without a PNH clone (43%, $n = 242$, versus 28%, $z = 5.2$, for all AA patients and controls and 58%, $n = 89$, versus 37%, $n = 177$, $z = 3.4$, for all patients with a PNH clone versus patients with AA). There also was a relationship between the age of patients with AA and the presence of HLA-DR2 allele, being more frequent in adults than in children (χ^2 test,

$P = .038$; data not shown). In addition, patients with hypocellular MDS showed an increased frequency of HLA-B7 in comparison to MDS patients with hypercellular bone marrows (44% versus 16%, $z = 3.49$); patients with hypoplastic MDS were more frequently HLA-B7 positive than were patients with AA (44% versus 23%, $z = 3.00$).

Other HLA class I and II associations in patients with bone marrow syndromes

We have systematically determined the frequencies of HLA alleles in patients with these bone marrow syndromes. In addition to confirming the previously reported association with HLA-DR2, we also found a decreased frequency of HLA-DQ2 and a small but statistically increased frequency of HLA-B14 (Table 4). However, although the frequency of DQ alleles in the control population was established based on serologic testing and AA patients were typed using molecular methods, the observed difference could be due to multispecific antisera used to determine serologic DQ2 (frequency likely serologically overestimated). In MDS, HLA-Cw*06 was overrepresented. The frequency of HLA-DR2 appeared to be increased, but this trend did not reach statistical significance levels (40% versus 28%; $z = 2.89$). We also compared the frequency of HLA-DR2 in all patients with hypocellular bone marrow morphology. When compared to controls, a significant increase in the frequency of HLA-DR2 was found (38% versus 28%; $z = 3.31$). HLA-B14 was also increased in this group, at 13% versus 8% controls ($z = 3.09$).

Table 4. HLA type of patients with bone marrow failure and healthy controls

Comparison	HLA-A	HLA-B	HLA-Cw	HLA-DR	HLA-DQ
Patients with initial diagnosis of AA vs controls	NS	B14 14% vs 8% $z = 3.6$ $N = 242$	NS	DR2 43% vs 28% $z = 5.22$ $N = 242$	DQ2 28% vs 40% $z = -3.8$ $N = 221^*$
Patients with initial diagnosis of MDS vs controls	NS	NS	Cw06 ($N = 112$) 23% vs 12% $z = 3.04$	NS	NS

NS indicates not significant.

*DQ typing was not available for all patients.

Response to immunosuppressive therapy with ATG, HLA-type, and the presence of GPI-AP-deficient cells in patients with sAA

To identify HLA alleles that might predict response to treatment, we studied a cohort of patients with bone marrow failure syndromes, including AA, MDS, and PNH who were treated with immunosuppressive regimens (cyclophosphamide + CsA, ATG + CsA, ATG + CsA + MMF, or ATG alone). To select candidate HLA alleles for responsiveness to immunosuppression, we initially compared allele frequencies in this cohort of patients versus healthy controls. Two alleles, HLA-B14 and HLA-DR2, were substantially more frequent in these patients than in healthy controls (Table 5). We then separately compared the frequency of these alleles between the responders to therapy versus nonresponders (Table 6). Although HLA-B*14 was not significantly different between these groups, for the difference in the frequency of HLA-DR2, a *P* of .01 was calculated (using a Bonferroni correction for multiple testing the new *P* value was significant at .05). Again, most of the difference in the frequency of HLA-DR2 between the groups was accounted for by DRB1*15 (by molecular analysis, only 4 HLA-DR02 alleles were due to DRB1*16).

Based on the association of PNH with HLA-DR2, we hypothesized that these 2 parameters were markers of immune-mediated bone marrow failure and that their presence might predict response to treatment. To assess the simultaneous contribution of each variable on predicting response, a multivariate logistic regression analysis was performed (Table 7). Both PNH and HLA-DR2 were statistically significant predictors of response. The presence of PNH increased the odds of response by 94%, whereas the presence of DR2 increased the odds of response by 81%. Furthermore, a statistical test of interaction was not significant suggesting that the 2 variables behave as independent predictors. Therefore, the presence of both PNH and HLA-DR2 has a multiplicative effect and increased the odds of response nearly 3.5-fold ($1.94 \times 1.81 = 3.51$).

Discussion

Introduction of more sensitive flow cytometric testing for detection of GPI-AP-deficient clones facilitated our investigation of the relationship between PNH and HLA alleles. We report here a novel association of PNH with increased frequency of the HLA-DR2 allele. A population of patients with classical hemolytic PNH but also those with a flow cytometrically detected but clinically silent PNH clone showed significantly increased frequency of HLA-DR2. The highest prevalence of this allele was found among patients with the PNH/MDS overlap syndrome. This strong association across boundaries of hematologic diagnoses suggests that HLA-DR2 is a major predisposing factor for the expansion of GPI-AP-deficient cells in diverse bone marrow failure states. This

Table 5. Response to therapy and HLA phenotype

Comparison	HLA-A	HLA-B	HLA-Cw	HLA-DR	HLA-DQ
Immunosuppression vs controls	NS	B14 13% vs 8% z = 3.4 N = 305	NS	DR2 42% vs 28% z = 5.3 N = 281	NS

HLA phenotype was analyzed in patients with bone marrow failure treated with immunosuppressive therapy (as indicated in "Patients, materials, and methods") and compared to the controls. Only alleles that are substantially different from controls are shown.

Table 6. Frequency of HLA antigens in responders and nonresponders

Allele	Responders*	Nonresponders*	<i>P</i>
HLA-B14	14% (N = 159)	12% (N = 146)	NS
HLA-DR2	50% (N = 145)	34% (N = 136)	.01

Class II typing was not available for all patients.

NS indicates not significant.

*Percentage (out of N) with the allele by response to therapy.

clonal expansion may render hematopoiesis permissive for PNH evolution. When we compared the frequency of HLA-DR2 in AA patients with GPI-AP-deficient cells to those in whom these cells were absent, HLA-DR2 was more correlated with the presence of an expanded PNH clone. AA patients without evidence of PNH at any time had a significantly lower frequency of HLA-DR2 than did AA/PNH patients; it appears that this class II allele coincides more closely with the occurrence of the PNH clone than with the aplasia as defined by marrow cellularity.

In contrast to classical approaches aimed at establishment of HLA association with a particular syndrome or clinically defined disease, a simple linkage of an individual laboratory parameter, in our case flow cytometric detection of GPI-AP-deficient cells, allows for a more objective analysis of possible pathophysiologic mechanisms leading to PNH evolution. From this perspective, our results appear to link the expansion of the PNH clone itself to the HLA-DR2, rather than to the diseases in which PNH occurs.

Consistent with the results of previous smaller studies, we found that HLA-DR2 (mostly DRB1*15) was overrepresented among AA patients (about 2-fold higher than in the general population⁹⁻¹⁶). An increased frequency of HLA-DR2 alleles was seen in AA patients of different ethnic backgrounds.^{13,16,17} Possible overrepresentation of HLA-DR2 in primary MDS indicates that misdiagnosed AA may contribute to this group of patients, or alternatively that evolution of MDS from AA patients with HLA-DR2 allele is common. More intricate HLA analysis in subtypes of MDS, now in progress in our laboratory, may further clarify the relationship between AA and MDS.

HLA-DR2, reported in the paper based on the molecular typing as DRB1*15, was shown to predict response to CsA in a subset of AA patients.¹⁷⁻¹⁹ Increased frequency of HLA-DR2 within PNH/AA is consistent with the previously published positive prognostic value of the presence of GPI-AP-deficient cells with regard to response to immunosuppression.²² However, in contrast to the results obtained in CsA-dependent patients, others found no correlation between HLA-DR2 and outcome after ATG therapy.^{11,17,19} We studied a very large cohort of patients with bone marrow failure syndromes and pancytopenia treated with intense immunosuppression, usually in combination with CsA, and found that HLA-DR2 was present in a significantly higher portion of patients who improved with therapy in comparison to refractory cases. In agreement with the hypothesis that presence of an expanded PNH clone may be a marker of immune-mediated disease, this result was paralleled by a positive relationship between the presence of an expanded population of GPI-AP-deficient cells (both at presentation as well as retrospectively at

Table 7. Multivariate logistic regression analysis of factors that predict response

Factor	Odds ratio	<i>P</i>
HLA-DR2	1.81	.02
PNH	1.94	.03

Odds ratio is calculated by dividing the odds of response with factor by odds of response without factor.

later evaluations) and a favorable response to immunosuppressive drugs. Moreover, multivariate regression analysis showed that both parameters (presence of an expanded PNH clone and HLA-DR2) independently increased the odds of positive response to immunosuppression. HLA-DR2 has also been observed to correlate with responsiveness to immunosuppression in patients with MDS treated with ATG (manuscript in preparation). The difference in the odds for responding to ATG between our and previously published results may be attributed to the ethnic composition of patients studied, different etiologies of AA in different parts of the world, and also variable clinical definitions of disease and response. Note also that our large cohort of patients allowed for statistical analysis not compromised by sample size.

There was a statistically significant difference in the frequency of HLA-B14, which had been previously reported,¹³ suggesting that this is a reproducible observation, although the small numerical difference may not be clinically relevant. In contrast to one previously published study, there was no increase in the frequency of HLA-A2.^{7,10,13,14} An increased frequency of HLA-B7 in AA patients has been described in AA,¹⁵ but we found this allele to be only more prevalent in patients with the hypocellular form of MDS, potentially distinguishing them from hypercellular MDS and from patients with AA, and implying that hypocellular bone marrow may result from pathophysiologic processes that are restricted by different immunologic pathways.

In certain diseases, class II antigen presentation or class II restricted responses may play a greater pathophysiologic role than class I regulated response.³³ For example, HLA-DR1 and some forms of HLA-DR4 have been found in majority of patients with rheumatoid arthritis,^{34,35} whereas HLA-DR3 predisposed to mixed cryoglobulinemia after hepatitis C infection.^{3,4} HLA-DR2 has been described as closely associated with anti-glomerular basement membrane disease,³² multiple sclerosis,³⁶ and narcolepsy.³⁷ In general, HLA linkage to autoimmune disease is believed to arise from an HLA haplotype–determined ability to present pathogenic peptides derived from self-antigens or foreign antigens. In addition, HLA-derived peptides themselves may serve as autoantigens.

Nevertheless, the genotypic classification of HLA-DR alleles may not reflect the mechanistic basis of autoimmunity and HLA/disease association. For example, functional categorization of HLA-DR alleles according to similarity in the restrictive supertype structure (recognizing that classification of various HLA-DR alleles can be based on structural characteristics^{38,39}) would explain why several genotypically unrelated HLA-DR alleles may accommodate a specific peptide. The functional but not genotypic or serologic similarity among classes of more than 200 HLA-DR alleles may explain some of the difficulties in establishing HLA disease associations based on traditional approaches.

Although somatic mutation in the *PIG-A* gene is clearly required for the development of a PNH phenotype, immune pathogenesis plays a role in the evolution and clinical manifestations of this syndrome. The finding of an increased frequency of HLA-DR2 in association with PNH clone in patients with bone marrow failure supports this theory, but several hypotheses can be formulated as to how individual mutations in *PIG-A* gene can lead to the expansion of GPI-AP–deficient clone and, under certain circumstances, to the total replacement of normal hematopoiesis with PNH cells.²¹ For example, increased frequency of HLA-DR2 in patients with bone marrow failure and an expanded PNH clone might simply result from an extraordinarily close relationship between PNH and AA. However, the decreased frequency of HLA-DR2 within AA without GPI-AP–deficient clone present as compared to AA/PNH syndrome rather argues against this conclusion. Based on the realization that mutations in the *PIG-A* gene are not infrequent and can occur also in healthy individuals,⁴⁰ another hypothesis may also be considered: the autoimmune process, to which the HLA-DR2 allele is a predisposing factor, is the primary event facilitating the expansion of otherwise occult PNH clones. This process could be triggered by a class II–dependent antigen or alternatively, a GPI-AP could serve as an autoantigen providing a growth advantage to PNH cells. Under such circumstances, presentation of peptides derived from a putative GPI-anchored antigen in the context of HLA-DR2 may drive the autoimmune process.

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