Roles of DRB1*1501 and DRB1*1502 in the pathogenesis of aplastic anemia

TITLE PAGE

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Bylines

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This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Technology, Sports and Culture of Japan (KAKENHI 15390298) and grants from the Research Committee for the Idiopathic Hematopoietic Disorders, The Ministry of Health, Labour and Welfare, Japan.

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Category

Clinical investigations

Word counts

Total text word count: 3412 words

Abstract word count: 240 words

ABSTRACT

Objective. Although a number of reports have documented a significantly increased incidence of HLA-DR15 in aplastic anemia (AA), the exact role of HLA-DR15 in the immune mechanisms of AA remains unclear. We herein clarify the difference between DRB1*1501 and DRB1*1502, the 2 DRB1 alleles which determine the presentation of HLA-DR15, in the pathophysiology of AA. ***Materials and Methods.*** We investigated the relationships of the patients' HLA-DRB1 allele with both the presence of a small population of $CD55^{-}CD59^{-}$ (PNH-type) blood cells and the response to antithymocyte globulin (ATG) plus cyclosporine (CsA) therapy in 140 Japanese AA patients. ***Results.*** Of the 30 different DRB1 alleles, only DRB1*1501 (33.6% vs. 12.8%, P_c<0.01) and DRB1*1502 (43.6% vs. 24.4%, P_c<0.01) displayed significantly higher frequencies among the AA patients than among a control. AA patients possessing HLA-DR15 tended to be old, and especially, the frequency of DRB1*1502 in patients ≥40 years old (52.4%) was markedly higher than that in those <40 years old (16.2%, P_c<0.01). Only DRB1*1501 was significantly associated with the presence of a small population of PNH-type cells and it also showed a good response to ATG plus CsA therapy in a univariate analysis. A multivariate analysis showed only the presence of a small population of PNH-type cells to be a significant factor associated with a good response to the immunosuppressive therapy (P<0.01). ***Conclusion.*** Although both DRB1*1501 and DRB1*1502 contribute to the development of AA, the methods of contribution differ between the two alleles.

INTRODUCTION

Aplastic anemia (AA) is a syndrome characterized by pancytopenia and bone marrow hypoplasia. Although the etiology remains unclear, the immune destruction of hematopoietic stem cells has been considered the most important mechanism of bone marrow failure in AA [1]. One important finding supporting the role of such autoimmune mechanisms in AA is the high incidence of a certain HLA allele in AA patients. A number of reports have documented a significantly increased incidence of HLA-DR2 or the split antigen HLA-DR15 in AA [2-5]. We previously demonstrated a strong association between DRB1*1501 and a susceptibility to AA, in which the hematopoietic function improves with the administration of cyclosporin A (CsA) [6]. Some reports have also demonstrated that HLA-DR15 or DRB1*1501 can predict the response to immunosuppressive therapy (IST) in patients with AA and myelodysplastic syndrome (MDS) [7-9], while others have failed to identify HLA-DR15 as a predictor for the response to ATG therapy [3,10,11]. In our previous study, AA patients carrying DRB1*1502, another major allele corresponding to HLA-DR15 in Japanese, did not show a better response to CsA than those without HLA-DR15 [6]. The exact role of HLA-DR15 in the immune mechanisms of AA thus remains unclear, probably due to both the low number of patients that have been studied for DRB1 alleles and the general heterogeneity in the pathogenesis of AA.

Another interesting aspect of HLA-DR15 is the association with the expansion of paroxysmal nocturnal hemoglobinuria (PNH) clones. Several studies have revealed

the frequency of HLA-DR15 to be significantly higher in patients with AA and MDS possessing PNH-type blood cells and in florid PNH than in normal controls [10,12], however, the relationship between DRB1 alleles corresponding to DR15 and increased PNH-type cells in AA has not yet been studied in detail. The close relationship between HLA-DR15 and the expansion of PNH clones suggests that the T-cell responses against certain antigen presented by HLA-DR15 or other HLA-class II alleles in linkage disequilibrium with DR15 in hematopoietic stem cells may cause bone marrow failure, thus allowing PNH-type stem cells to survive.

We previous demonstrated the frequency of HLA-DR15 to markedly increase in patients with MDS-refractory anemia (RA) and a small population of PNH-type cells (more than 0.003% for granulocyte, more than 0.005% for RBCs), as demonstrated by sensitive flow cytometry [13]. In that study, RA patients possessing a small population of PNH-type cells displayed favorable responses to CsA. An investigation of a large number of AA patients treated with IST using the same methods to detect small populations of PNH-type cells would thus clarify the role of DRB1 alleles corresponding to HLA-DR15 and PNH-type cells in the immune mechanisms of AA and their mutual relationships. To test this hypothesis, we investigated the relationship between the DRB1 allele in such patients and both the presence of a small population of PNH-type cells and the response to ATG plus CsA therapy in 140 Japanese AA patients.

MATERIALS AND METHODS

Patients

Table 1 summarizes the patient characteristics. The 140 Japanese AA patients were diagnosed at Kanazawa University Hospital, hospitals which participate in a cooperative study led by the Intractable Disease Study Group of Japan and other referring institutions from April 1999 through November 2005. The study subject included 77 patients who were tested for any correlation between the presence of a minor population in PNH-type cells and the response to IST in our previous study [14]. The severity of AA was classified according to the criteria proposed by Camitta et al [15,16]. All participants provided written, informed consent to all procedures associated with the study, which was approved by the Ethical Committee at our institution (study number 46). This study also conforms to the recently revised tenets of the Helsinki protocol.

Detection of PNH-type cells

We performed two-color flowcytometry of the granulocytes and RBCs according to our previously described method [14,17,18]. First, 3-5 mL of heparinized blood was drawn from each patient. To detect the PNH-type granulocytes, phycoerythrin (PE)-labeled anti-CD11b monoclonal antibodies (MoAbs; Becton Dickinson, Mountain View, CA), fluorescein-isothiocyanate (FITC)-labeled anti-CD55 MoAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA), and FITC-labeled anti-CD59 MoAbs

(clone p282, mouse IgG2a; Pharmingen) were used in combination with isotype-matched control MoAbs, as previously described. To detect the PNH-type RBCs, PE-labeled anti-glycophorin A MoAbs (clone JC159, DAKO, Glostrup, Denmark) were used instead of anti-CD11b MoAbs. Fresh blood was diluted to 3% using phosphate-buffered saline (PBS), and 50 mL of diluted blood was incubated with 4 mL of PE-labeled anti-glycophorin A MoAbs, FITC-labeled anti-CD55 and anti-CD59 MoAbs on ice for 25 minutes. A total of at least 1×10^5 $CD11b^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analyzed using FACScan flow cytometry (Becton Dickinson). In order to avoid any false positive results, we excluded $CD11b^{dim}$ and glycophorin A^{dim} cells from the analyses using careful gating because these cells include damaged cells those are often mistakenly judged to be PNH-type cells due to their poor binding to anti-CD55 and anti-CD59 MoAbs. This flow cytometry method failed to detect 0.003% or more $CD55^-CD59^-CD11b^+$ granulocytes or 0.005% or more $CD55^-CD59^-$glycophorin-A^+ RBCs in any of 183 healthy individuals. We therefore defined the presence of more than 0.003% $CD55^-CD59^-CD11b^+$ granulocytes $CD55^-CD59^-$glycophorin-A^+ RBCs to be abnormal [14,18].

Determination of DRB1 alleles

DRB1 alleles of 140 AA patients and 491 healthy Japanese randomly selected from general population [19] were determined using polymerase chain reactions with

sequence-specific primers (PCR-SSP) (Micro SSP HLA DNA typing trays; One Lambda, Canoga Park, CA). Genomic DNA was prepared from blood samples using a DNA extraction kit (Generation capture column kit; Gentra, Minneapolis, MN).

ATG plus CsA therapy and response criteria

Seventy-seven of 140 patients (55.0%) were treated with antithymocyte globulin (ATG, Lymphoglobuline, Aventis Behring, King of Prussia, PA, 15 mg/kg/day, 5 days) and cyclosporin (CsA, Novartis, Basel, Switzerland, 6 mg/kg/day) within 1 year of diagnosis. The dose of CsA was adjusted to maintain trough levels at between 150 and 250 ng/mL and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (G-CSF, filgrastim, 300 $\mu g/m^2$ or lenograstim, 5 $\mu g/kg$) was administered to some patients. The response to ATG plus CsA therapy was evaluated according to the response criteria described by Camitta [20]. A complete response (CR) was defined as hemoglobin normal for age, neutrophil count more than $1.5 \times 10^9/L$, and platelet count more than $150 \times 10^9/L$. A partial response (PR) was defined as transfusion independent and no longer meeting the criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling or the normalization of at least one cell line or an increase in the baseline hemoglobin of more than 30 g/L (if initially less than 60 g/L), a neutrophil count of more than $0.5 \times 10^9/L$ (if initially less than 0.5

x 10^9/L), and a platelet count of more than 10 x 10^9/L (if initially <20 x 10^9/L) in patients with moderate AA.

Statistical analysis

The allele frequency defined as the proportion of patients with at least one copy of a specific gene was determined by direct counting. The χ^2 test compared the allele frequencies of HLA-DRB1 between the patient groups and a Japanese control population, composed of 491 healthy unrelated individuals selected at random from the general population [19]. The corrected value of P (P_c) was calculated by multiplying P with the number of alleles tested (n=30). The χ^2 test, Fisher exact test and logistic procedures [21] analyzed associations between the prevalence of increased PNH-type cells and genetic factors, and between individual pretreatment variables and the response to ATG plus CsA therapy. The Kaplan-Meier methods graphically compared the cumulative incidence of the response to ATG and CsA therapy and the time to event, while the log-rank test analyzed differences between the patients who possess HLA-DRB1*1501, DRB1*1502 and DRB1 alleles other than these two alleles. All statistical analyses were performed using the JMP version 5.0.1J software program (SAS Institute, Cary, NC).

RESULTS

Frequencies of DRB1 alleles in AA patients

Table 2 summarizes the frequencies for the 30 different DRB1 alleles identified in the 140 AA patients and 491 controls. Only the frequencies of DRB1*1501 (33.6% vs. 12.8%, P_c<0.01, Odds ratio=3.43) and DRB1*1502 (43.6% vs. 24.4%, P_c<0.01, Odds ratio =2.39) were significantly higher among the AA patients than among controls. Figure 1 illustrates the numbers of patients with DRB1*1501 and/or DRB1*1502 and the patients without either of the two alleles in the different age groups. Two peaks in the age distribution of the patients were noted, namely, at 20 to 29-years-old and at 60 to 79-years-old. After dividing the patients into young (<40-years-old, n=37) and old (≥40-years-old, n=103) groups, 82.5% of patients in the older group carried at least one of DRB1*1501 or DRB1*1502. The frequency of DRB1*1502 in the older group (54 of 103 patients, 52.4%) was significantly higher (P_c=0.03) than that in the younger group (6 of 37 patients, 16.2%). No significant difference in the frequency of DRB1*1501 was identified between the two groups (36 of 103 patients, 35.0% vs. 11 of 37 patients, 29.7%, P=0.56).

Prevalence of patients possessing PNH-type cells

A wide range of PNH-type granulocytes (0.005% to 23.0%; median, 0.153%) and PNH-type RBCs (0.007% to 6.57%; median, 0.094%) were detected in 92 of 140 (65.7%) AA patients. When the patients were divided into four groups according to the presence of DRB1*1501 and DRB1*1502, the proportions of PNH^+ patients were

66.7% (4 of 6 patients) in the DRB1*1501^{+}1502^{+} patients, 85.3% (35 of 41 patients) in DRB1*1501^{+}1502^{-}, 59.3% (32 of 54 patients) in DRB1*1501^{-}1502^{+} and 53.8% (21 of 39 patients) in DRB1*1501^{-}1502^{-}.

Allele frequencies in the PNH^{+} and PNH^{-} AA patients

We next divided the 140 AA patients for whom both DRB1 alleles were determined into PNH^{+} patients (n=92) and patients without a small population of PNH-type cells (PNH^{-} patients, n=48), and then compared the frequency of each DRB1 allele among the three different groups including the PNH^{+} patients, PNH^{-} patients and controls (Fig. 2). The frequency of DRB1*1501 compared to the controls was significantly higher in only the PNH^{+} patients (39 of 92 patients, 42.4%, P_c<0.01), not in PNH^{-} patients (8 of 48 patients, 16.7%). On the other hand, the frequency of DRB1*1502 in comparison to the controls was higher in both the PNH^{+} patients (37 of 92 patients, 40.2%, P_c =0.05) and PNH^{-} patients (24 of 48 patients, 50.0%). The frequencies of other DRB1 alleles, including DRB1*0405, were similar among PNH^{+} patients, PNH^{-} patients, and controls.

Correlation of HLA-DR15 alleles with the prevalence of increased PNH-type cells in AA patients

We analyzed the associations between the prevalence of PNH-type cells and genetic factors, such as age, sex, severity, chromosomal abnormality and HLA-DRB1 allele

to determine which factors might contribute to a slight increase in PNH-type cells in our AA patients. The presence of DRB1*1501 (P<0.01, Odds ratio=3.68) was the only significant factor associated with an increase in the proportion of PNH-type cells based on a univariate analysis, and a multivariate analysis confirmed this result (P<0.01). The presence of DRB1*1502 was not considered to be a contributing factor.

Favorable factors affecting response to ATG plus CsA therapy

Fifty-five of 77 patients (71.4%) improved with ATG plus CsA therapy. The factors favorably affecting the response to IST in the AA patients were examined under a univariate and multivariate analysis (Table 3). Only the presence of PNH-type cells was significantly associated with the response to IST based on a multivariate analysis. After taking into account the kinetics of the response to treatment, we made Kaplan-Meier curves to determine the probability of response to IST in 3 different groups of patients as defined by DRB1 alleles (Fig. 3). There were significant differences in the probability of the response to IST between the DRB1*1501^{+}1502^{-} patients and either the DRB1*1501^{-}1502^{+} patients (P<0.01) or the DR15^{-} patients (P=0.01) (Fig. 3A). However, these differences in the probability of response IST were no longer observed when the probability of response was compared in either the PNH^{+} patients or the PNH^{-} patients (Fig. 3B, C).

DISCUSSION

This study demonstrated for the first time that, in addition to DRB1*1501, which is a major DRB1 allele determining the presentation of HLA-DR15 in Caucasian [2,3] and Chinese populations [4], DRB1*1502 is frequently present in Japanese AA patients. This finding, based on a large number of patients, suggests that the DR15 molecule plays a definite role in the development of a subset of AA. Another novel finding in the present study was that the significantly increased frequency of HLA-DR15 was only observed in old AA patients. The frequency of HLA-DR15 reached up to 80% in AA patients ≥40-years old. The apparent age-dependent differences in HLA-DR15 frequency suggest that the pathophysiology of AA in older patients may therefore differ from that in younger patients. Several studies of Japanese pediatric patients have revealed a relatively high incidence of MDS secondary to AA compared to adult patients [22-24]. Given the lower frequency of HLA-DR15, pediatric AA may thus display a higher proportion of bone marrow failure caused by non-immune mechanisms than adult AA.

In contrast to the findings of previous reports, DRB1*1501 appeared to confer a better chance of response to regimens including ATG than other DRB1 alleles, including DRB1*1502. We previously demonstrated that DRB1*1501 predicts the response to CsA, but not to ATG [11]. In the previous study, only 6 of 59 ATG-treated patients received CsA. The combined use of CsA and the larger number of ATG-treated patients in the present study probably accounts for the different findings

regarding the role of DRB1*1501 in predicting the response to ATG therapy. DRB1*1501 may affect the response of AA to ATG therapy only when CsA is administered in combination with ATG.

Several previous studies failed to confirm the role of HLA-DR15 in predicting the response to ATG [3,10]. Most previous studies analyzed DRB1 alleles using low-resolution methods that are unable to sufficiently distinguish DRB1*1502 from DRB1*1501. DRB1*1502 accounts for 3-7% of the DRB1 alleles corresponding to DR15 even in Caucasians [25], and this frequency may even be higher in AA patients, particularly among AA patients ≥40-years-old. As a result, some patients with DR15 who did not respond to ATG in previous studies may have been DRB1*1502^{+}, rather than DRB1*1501^{+}. The results of this study indicate the importance of accurately determining the DRB1 alleles using high-resolution methods to clarify the role of HLA-DR15 in predicting a response to IST.

A higher frequency of HLA-DR15 among PNH^{+} patients in comparison to PNH^{-} patients has been reported by Maciejewsky et al. in 2001 [26]. The present study confirmed this finding using a different flow cytometry assay that distinguished PNH^{+} patients from PNH^{-} patients using lower levels of glycosylphosphatidyl inositol-anchored protein-deficient cells than the assay used in the previous study. Our methods also identified a significant difference between DRB1*1501 and DRB1*1502 in the minimal expansion of PNH clones. The frequencies of both alleles increased in the PNH^{+} patients in comparison to the normal controls, thus

supporting the preliminary results of our study of 23 patients with refractory anemia [13]. However, only DRB1*1501 represented a genetic factor significantly associated with an increase in the proportion of PNH-type cells in AA patients in the present study because the frequency of DRB1*1502 was high in both PNH^+ and PNH^- AA patients, thus indicating that the minimal expansion of PNH clones is not affected by DRB1*1502. Together with the difference in the response rate to IST between $DRB1*1501^+$ and $DRB1*1502^+$ AA patients, all these findings suggest that DRB1*1501 and DRB1*1502 therefore play a different role in the pathogenesis of AA.

In AA patients carrying DRB1*1501, the presentation of autoantigen by this molecule may readily induce a cell-mediated attack against hematopoietic stem cells that may be associated with the minimal expansion of a PNH clones. Previous studies have demonstrated that the presence of a $CD4^+$ T-cell attack against hematopoietic stem cells allows the survival of PNH-type stem cells [27,28]. On the other hand, polymorphic gene alleles of myelosuppressive cytokines, in linkage disequilibrium with DRB1*1502 may predispose individuals with HLA-DRB1*1502 towards the development of AA. In keeping with this hypothesis, a recent study on diabetes mellitus patients revealed that a haplotype of TNFa12-DRB1*1502 was therefore more frequent in patients likely to develop insulin-dependency than in those who do not develop insulin-dependency [29]. Several reports have demonstrated TNFa12 to be associated with a higher secretion of TNF-alpha [30].

HLA-DR15 molecules derived from DRB1*1502 differ from those derived from DRB1*1501 in only one amino acid at position 86 (valine for DRB1*1502 and glycine for DRB1*1501) of the beta-chain [31]. This structural similarity indicates that antigenic epitopes presented by these molecules are common [32,33]. For most autoimmune diseases where DRB1*1501 is associated with susceptibility in patients from Western countries, DRB1*1502 is expected to play the same role as DRB1*1501 in Japanese patients. However, in Japanese patients with multiple sclerosis, the frequency of DRB1*1502 is not increased in comparison to that in the controls [34,35]. As a result, DRB1*1502 appears to contribute to the development of some autoimmune diseases via different mechanisms to DRB1*1501. In AA patients carrying DRB1*1501, certain antigens of which presentation requires position 86 of the beta-chain to be glycine may likely induce an immune system attack to hematopoietic progenitor cells. It is also possible that DRB5*0101 and DRB5*0102, which are in complete linkage disequilibrium with DRB1*1501 and DRB1*1502, respectively, in the Japanese population [19] may be responsible for the difference because DRB5*0101 differs from DRB5*0102 by 3 amino acids in the antigen-peptide binding domain.

Our data may be relevant to the management of AA. Although the incidence of HLA-DR15 is significantly higher in AA patients than in the normal controls, only DRB1*1501 was found to be a predictive marker for a good response to ATG plus CsA therapy. AA patients with DRB1*1502 who do not show an increased

proportion of PNH-type cells may not benefit from IST. HLA-DR typing has been considered to be useful for predicting a good response to IST in AA patients[7,8], but this costly test may not be necessary in the circumstance where the highly sensitive flow cytometry is available because the presence of a small population of PNH-type cells is the only significant factor that affects the response to ATG plus CsA therapy based on the findings of our multivariate analysis. Prospective studies are called for to confirm these findings.

Acknowledgements

We wish to express our sincere thanks to Ms. M. Yoshii for technical assistance. In addition, we would also like to thank the following physicians for providing us with patient samples and clinical information: M. Ueda and M. Yamaguchi of Ishikawa Prefectural Central Hospital, H. Yamauchi of Kurobe City Hospital, H. Kobayashi of Nagano Red Cross Hospital, A. Urabe of NTT Kanto Hospital, A. Matsuda of Saitama Medical University Hospital, T. Matsunaga of Sapporo Medical University Hospital, K. Ohyashiki of Tokyo Medical University, M. Teramura of Tokyo Women's Medical University, Y. Terasaki of Toyama City Hospital, and T. Yoshida of Toyama Prefectural Central Hospital.

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FIGURE LEGENDS

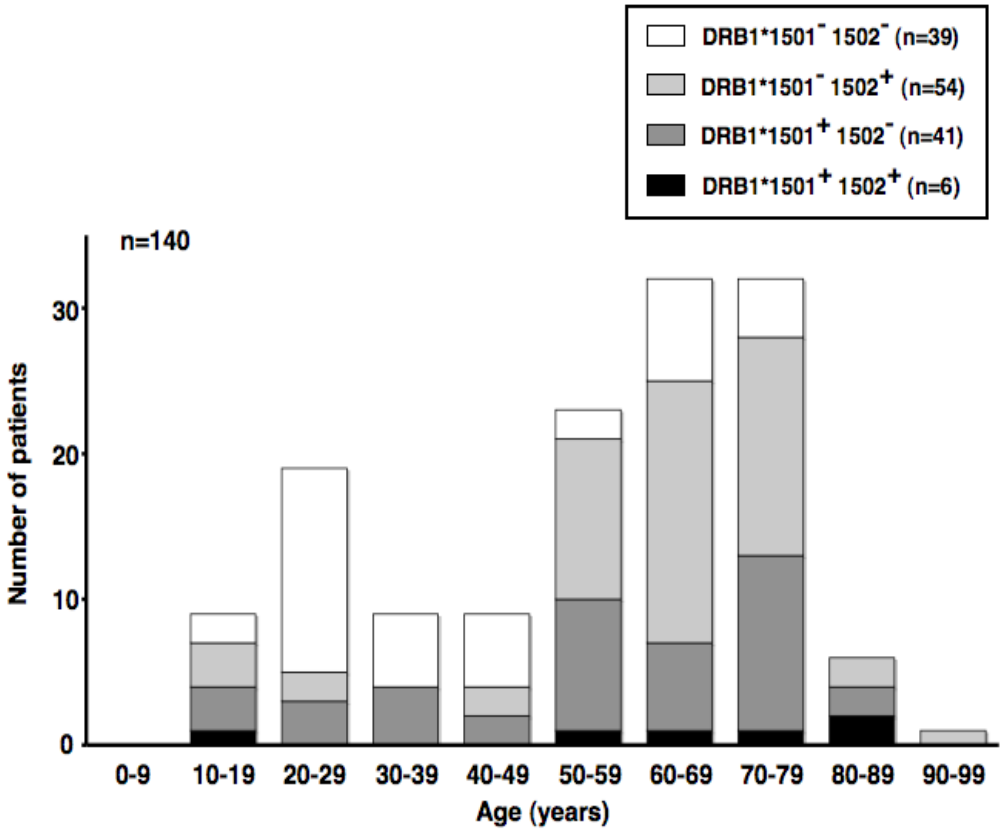

Figure 1: Age distribution of AA patients with or without HLA-DR15

The number of AA patients with or without HLA-DR15 in different age groups is shown. DRB1*1501^{+}1502^{+}, patients with both DRB1*1501 and DRB1*1502; DRB1*1501^{+}1502^{-}, patients with DRB1*1501 but not DRB1*1502; DRB1*1501^{-}1502^{+}, patients with DRB1*1502 but not DRB1*1501; DRB1*1501^{-}1502^{-}, patients with neither DRB1*1501 nor DRB1*1502.

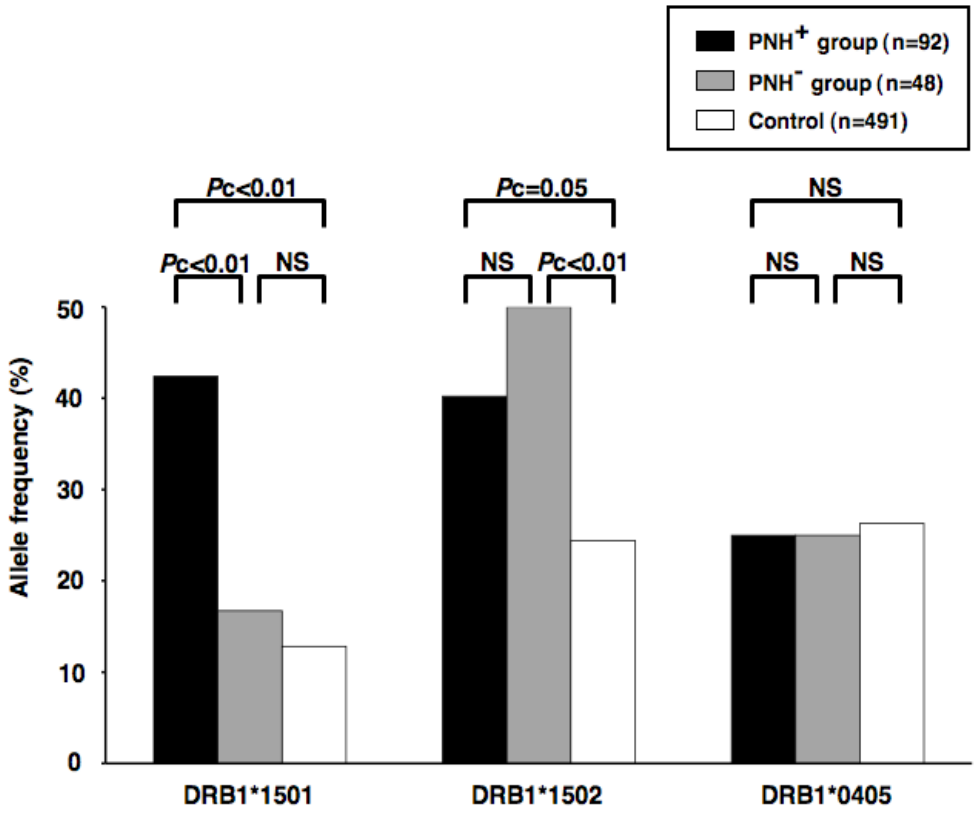

Figure 2. HLA-DRB1 allele frequencies in PNH$^+$ and PNH$^-$ AA patients

Frequencies of the three alleles, DRB1*1501, DRB1*1502, and DRB1*0405 are compared in the PNH$^+$ AA patients, PNH$^-$ AA patients, and controls.

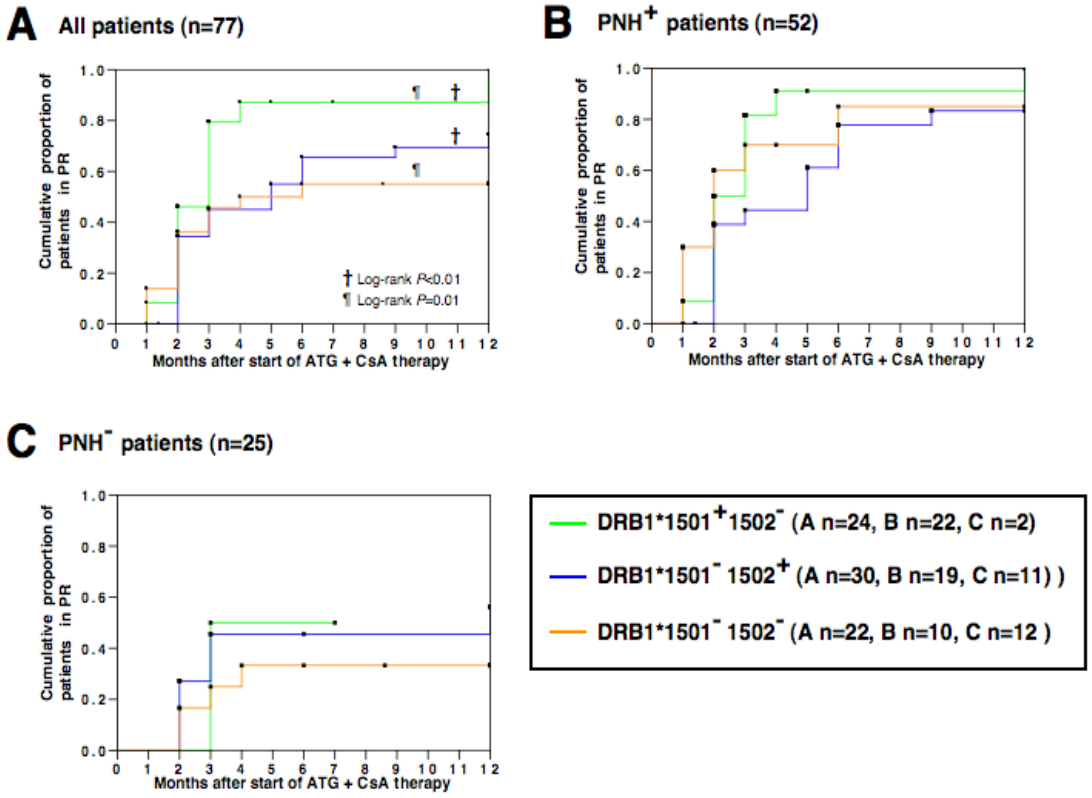

Figure 3. Kinetics of response to ATG plus CsA therapy.

Kaplan-Meier curves for the response in the different groups of patients based on the DRB1 alleles are shown. DRB1*1501^{+}1502^{+} patients were not showed in this figure because only one patient (he was PNH^{+}) was available for the analysis. A, All patients; B, PNH^{+} patients; C, PNH^{-} patients.

Table 1. Patient characteristics

Characteristics	Number	Range
Total (n)	140	NA
Age at diagnosis (y)	60	12-92
Gender: Male/female	65/75	NA
Severity: Severe/moderate	65/75	NA
Neutrophil count (x 10^9/L)	720	0-2226
Platelet count (x 10^9/L)	20	2-118
Reticulocyte count (x 10^9/L)	28	2-106
No. of patients with clonal abnormality (n)	11	NA

NA indicates not applicable.

Table 2. Frequencies of HLA-DRB1 alleles in Japanese AA patients and controls

HLA-DRB1 allele	AA patients (n = 140)		Controls (n = 491)		*P*c value**
	No of patients	(%)*	No of patients	(%)*	
0101	10	7.1	64	13.0	NS
0301	0	0.0	4	0.8	NS
0401	2	1.4	17	3.5	NS
0403	4	2.9	18	3.7	NS
0404	0	0.0	2	0.4	NS
0405	35	25.0	129	26.3	NS
0406	5	3.6	32	6.5	NS
0407	2	1.4	2	0.4	NS
0409	0	0.0	1	0.2	NS
0410	1	0.7	17	3.5	NS
0701	0	0.0	2	0.4	NS
0801	0	0.0	0	0.0	NS
0802	6	4.3	36	7.3	NS
0803	8	5.7	84	17.1	NS
0901	36	25.7	148	30.1	NS
1001	2	1.4	2	0.4	NS
1101	7	5.0	22	4.5	NS
1201	7	5.0	34	6.9	NS
1202	2	1.4	12	2.4	NS
1301	0	0.0	4	0.8	NS
1302	11	7.9	61	12.4	NS
1401	2	1.4	21	4.3	NS
1402	0	0.0	2	0.4	NS
1403	4	2.9	13	2.6	NS
1405	4	2.9	18	3.7	NS

1406	2	1.4	10	2.0	NS
1407	0	0.0	1	0.2	NS
1501	47	33.6	63	12.8	<0.01
1502	61	43.6	120	24.4	<0.01
1602	2	1.4	4	0.8	NS

*Allele frequencies were determined by dividing the number of patients carrying one or two specific alleles by the total number of individuals.

**Corrected P value (*P*c) was calculated by multiplying the P value with the number of alleles (n=30) tested.

Table 3. Pre-treatment variables associated with a response to ATG plus CsA therapy

Favorable factors	P value	
	univariate*	multivariate**
Gender (male vs. female)	0.32	0.47
Age (at least 40 y vs. younger)	0.79	0.37
Severity (severe vs. moderate)	0.61	0.86
HLA-DRB1*1501 (positive vs. negative)	0.03	0.19
HLA-DRB1*1502 (positive vs. negative)	0.61	0.46
PNH-type cells (positive vs. negative)	<0.01	<0.01

**P* value derived from Fisher's exact probability test.

***P* value derived from the Wald c^2 test for a logistic regression model.