Table 1. Laboratory features at the time of diagnosis and clinical features in patients with RA classified according to the FAB criteria

	Japan, n = 131	Germany, n = 597	P
Sex, male/female	70/61	309/288	.73
Age, y	57 (12-88)	71 (7-93)	< .001
Neutrophil count, × 10^9/L	1.58 (0.05-10.24)	1.98 (0.06-23.00)	< .001
Hemoglobin concentration, g/L	84 (25-143)	94 (30-169)	.002
Platelet count, × 10^9/L	41 (4-390)	127 (2-1540)	< .001
2- or 3-lineage cytopenias, %*	68	39	< .001
Abnormal karyotype, %	29	53	< .001
Median survival, mo	175	40	< .001

Values for presentation characteristics are given as median and range (in parentheses) where applicable.

*Cytopenia according to IPSS: hemoglobin concentration less than 100 g/L, absolute neutrophil count less than 1.5 × 10^9/L, platelet count less than 100 × 10^9/L.

than that of German patients with FAB-RA ($P < .001$). The sex ratios were not significantly different between the 2 countries. Japanese patients with FAB-RA had significantly lower absolute neutrophil counts (ANCs), lower hemoglobin (Hb) concentrations, lower platelet (PLT) counts, and higher frequency of 2 or 3 lineage cytopenias according to the IPSS definition than did German patients with FAB-RA (Table 1). Cytogenetic analysis was performed in 102 Japanese and 199 German patients. In the Japanese FAB-RA group, the frequency of cytogenetic abnormalities was 30 patients (29%). In contrast, cytogenetic abnormalities were found in 105 (53%) of the German patients with FAB-RA. Japanese patients with FAB-RA had a significantly lower frequency of cytogenetic abnormalities than did German patients with FAB-RA. The subgroups of cytogenetic abnormalities according to IPSS are summarized in Table 2. The distribution of the cytogenetic subgroups according to IPSS showed no significant difference between Japanese and German patients with FAB-RA. Japanese patients with FAB-RA had a significantly lower frequency of FAB-RA associated with an isolated del(5q) cytogenetic abnormality (5q− syndrome) than did German patients with FAB-RA. Japanese patients with FAB-RA were highly categorized into the intermediate-1 (INT-1) risk subgroup, whereas German patients were equally categorized into the low-risk and INT-1 risk subgroups. The frequency of patients with intermediate-2 (INT-2) risk was low in both countries (Table 3).

Prognosis. Follow-up periods ranged from 1 to 292 months (median, 69 months) in Japanese patients with FAB-RA. Follow-up periods in German patients with FAB-RA ranged from 0 to 313 months (median, 13 months). During the follow-up period, 50 Japanese patients and 252 German patients died, and 10 Japanese patients and 56 German patients transformed to acute leukemia. Japanese patients showed a significantly lower cumulative risk of acute leukemia evolution than did German patients (Figure 1). Concerning causes of death, German patients were classified as 153 cases of MDS death (50 acute leukemia, 25 bleeding, 64 infection, 14 heart failure), 24 cases of non-MDS death, and 75 cases of unclear death. Japanese patients were classified as 40 cases of MDS death (11 acute leukemia, 9 bleeding, 19 infection, 1 heart failure), 7 cases of non-MDS death, and 3 cases of unclear death. In both OS and modified survival, all Japanese patients with FAB-RA had a more favorable prognosis than did all German patients with FAB-RA (OS median survival: Japan, 175 months; Germany, 40 months; $P < .001$; modified survival median survival: Japan, 202 months; Germany, 73 months; $P < .001$) (Figure 2A). In OS, for those aged 60 years or younger, Japanese patients with FAB-RA had a more favorable OS than did German patients with FAB-RA (median survival: Japan, 217 months; Germany, 66 months; $P < .001$) and for those aged older than 60 years, Japanese patients with FAB-RA had a more favorable OS than did German patients with FAB-RA (median survival: Japan, 59 months; Germany, 35 months; $P = .025$). In modified survival, for those aged 60 years or younger, Japanese patients with FAB-RA had a more favorable modified survival than did German patients with FAB-RA (median survival: Japan, > 292 months; Germany, 108 months; $P < .001$). However, for those aged older than 60 years, Japanese patients with FAB-RA did not show a more favorable modified survival than did German patients with FAB-RA (median survival: Japan, 102 months; Germany, 69 months; $P = .46$) (Figure 2B-C).

Prognostic factors. In Japanese patients with FAB-RA, the clinical variables of age older than 60 years and Hb concentration less than 70 g/L were significantly correlated with OS. Sex, Hb concentration less than 100 g/L, PLT count fewer than 100 × 10^9/L, ANC fewer than 1.5 × 10^9/L, cytopenias (2 or 3 lineages), and IPSS cytogenetic subgroups were not significantly correlated with OS (Table 3). In German patients with FAB-RA, age older than 60 years, Hb concentration less than 100 g/L, PLT count fewer than 100 × 10^9/L, cytopenias (2 or 3 lineages), and IPSS cytogenetic subgroups were significantly correlated with OS. Sex and ANC fewer than 1.5 × 10^9/L were not significantly correlated with OS (Table 3). The IPSS cytogenetic subgroups and IPSS subgroup were significantly correlated with cumulative risk of acute leukemia evolution in Japanese patients with FAB-RA (Table 3). The other clinical variables in Table 3 were not significantly correlated with cumulative risk of acute leukemia evolution. ANC fewer than 1.5 × 10^9/L, PLT count fewer than 100 × 10^9/L, cytopenias (2 or 3 lineages), IPSS cytogenetic subgroups, and IPSS subgroup were significantly correlated with cumulative risk of acute leukemia evolution in German patients with FAB-RA. Age, sex, and Hb concentrations were not significantly correlated with cumulative risk of acute leukemia evolution (Table 3).

In the age- and sex-adjusted multivariate analyses for OS, there was no clinical parameter that associated with OS in Japanese patients in all models, whereas cytopenias (especially, thrombocytopenia and anemia) and poor IPSS cytogenetic subgroup, and INT-1 and INT-2 IPSS risk subgroups retained as significantly adverse clinical parameters for OS in German patients. For acute leukemia evolution, poor IPSS cytogenetic subgroup and INT-2 IPSS risk subgroup were retained as significant parameters in the cumulative risk of acute leukemia evolution in Japanese patients after age and sex adjustment, whereas in German patients ANC fewer than 1.5 × 10^9/L was no longer associated with acute

Table 2. Cytogenetic findings at the time of diagnosis in patients with RA classified according to the FAB criteria

	Japan, n = 102	Germany, n = 199
Good, no. (%)	79 (77.5)	143 (71.8)
Normal	72	94
−Y	1	4
del(5q)	3	39
del(20q)	3	6
Intermediate, no. (%)	15 (14.7)	31 (15.6)
Poor, no. (%)	8 (7.9)	25 (12.6)
Complex (3 or more abnormalities)	5	16
Chromosome 7 anomalies	3	9

Intermediate indicates other abnormalities not listed in good and poor classifications.

Table 3. Univariate analysis of overall survival and cumulative risk of acute leukemia in patients with RA classified according to the FAB criteria

Variable, by country of origin	No. of patients	Percentile of OS (mo)		P	Percentile of cumulative risk of AML (mo)			P
		75%	50%		10%	25%	50%	
Japanese patients								
Age, y								
60 or younger	72	114	217	< .001	NR	NR	NR	.12
Older than 60	59	18	59		51	NR	NR	
Sex								
Male	70	42	176	.85	74	NR	NR	.53
Female	61	53	129		104	NR	NR	
Neutrophil count								
Fewer than 1.5 × 10^9/L	63	52	157	.84	51	NR	NR	.16
At least 1.5 × 10^9/L	68	53	176		NR	NR	NR	
Hemoglobin concentration								
Less than 100 g/L	81	52	114	.24	92	NR	NR	.95
At least 100 g/L	50	53	202		38	NR	NR	
Hemoglobin concentration								
Less than 70 g/L	45	23	100	.01	104	NR	NR	.81
At least 70 g/L	86	62	202		92	NR	NR	
Platelet count								
Fewer than 100 × 10^9/L	109	52	175	.37	92	NR	NR	.35
At least 100 × 10^9/L	22	54	109		14	NR	NR	
Cytopenia (IPSS)								
0/1	42	53	202	.84	NR	NR	NR	.83
2/3	89	52	157		92	NR	NR	
Chromosome (IPSS)								
Good	79	76	175	.17	104	NR	NR	< .001
Intermediate	15	19	NR		NR	NR	NR	
Poor	8	27	102		4	37	NR	
IPSS*								
Low	21	76	202	.29	NR	NR	NR	< .001
INT-1	73	52	175		104	NR	NR	
INT-2	8	27	102		4	22	NR	
German patients								
Age, y								
60 or younger	133	26	66	< .001	13	91	NR	.85
Older than 60	461	14	35		21	136	173	
Sex								
Male	309	16	41	.92	21	78	173	.41
Female	288	16	43		19	NR	NR	
Neutrophil count								
Fewer than 1.5 × 10^9/L	162	14	43	.54	17	52	173	.014
At least 1.5 × 10^9/L	301	16	37		25	NR	NR	
Hemoglobin concentration								
Less than 100 g/L	337	9	30	< .001	14	136	NR	.18
At least 100 g/L	217	23	57		42	173	NR	
Hemoglobin concentration								
Less than 90 g/L	235	8	29	< .001	17	136	NR	.15
At least 90 g/L	319	20	51		40	173	NR	
Platelet count								
Fewer than 100 × 10^9/L	207	9	23	< .001	11	50	136	< .001
At least 100 × 10^9/L	339	23	53		35	NR	NR	
Cytopenias (IPSS)								
0/1	288	23	55	< .001	63	NR	NR	< .001
2/3	188	7	22		10	28	136	
Chromosome (IPSS)								
Good	143	27	66	< .001	25	NR	NR	< .001
Intermediate	31	26	44		10	91	91	
Poor	25	7	16		4	14	52	
IPSS*								
Low	82	43	82	< .001	NR	NR	NR	< .001
INT-1	78	12	31		10	27	91	
INT-2	11	4	7		2	5	52	

Variables are defined in Tables 1 and 2.

OS indicates overall survival; AML, acute myeloid leukemia; NR, not reached.

*Low indicates 0; INT-1, 0.5-1.0; and INT-2, 1.5-2.0, according to IPSS score.

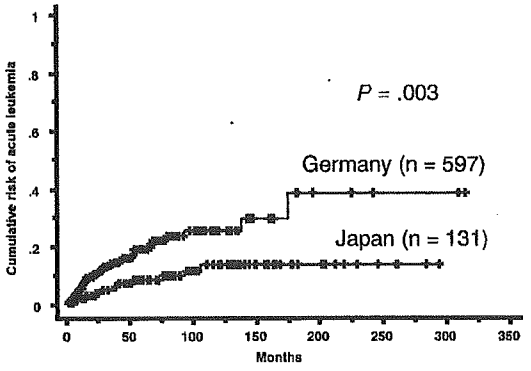

Figure 1. Cumulative risk of acute leukemia evolution of patients with FAB-RA. Japanese patients had a lower cumulative risk of acute leukemia evolution than did German patients ($P = .003$).

leukemia evolution, but other parameters in the univariate analyses were retained as poor prognostic factors (Table 4).

WHO classification. The original diagnoses according to the WHO classification by each group in the present series show that the frequency of WHO-RA in Japanese patients with FAB-RA (73%) was significantly higher than in the German patients with FAB-RA (24%) ($P < .001$). In Japanese patients, patients with WHO-RA were significantly younger and had significantly lower PLT counts than did patients with RCMD. The OS of Japanese patients with WHO-RA was significantly more favorable than that of Japanese patients with RCMD (Table 5). The OS of all Japanese patients with WHO-RA was significantly more favorable than that of all German patients with WHO-RA (Figure 3A). For those aged 60 years or younger, the OS of Japanese patients with WHO-RA was significantly more favorable than that of German patients with WHO-RA. However, for those older than 60 years, Japanese patients with WHO-RA did not show a more favorable OS than did German patients with WHO-RA (Figure 3B-C). Frequencies of poor karyotype according to IPSS in Japanese patients with WHO-RA and RCMD were 4% and 20%, respectively. Japanese patients with WHO-RA had a lower cumulative risk of acute leukemia evolution than did Japanese patients with RCMD (10% cumulative risk: WHO-RA, not reached; RCMD, 38 months; 25% cumulative risk: RCMD, 104 months; $P = .018$).

Discussion

Different clinical features between Asian and Western patients with MDS have been reported by several studies.[10,17] However, these data are based on local series of patients. Speculation about certain differences is problematic because there might be differences in the interpretation of dysplasia in blood and bone marrow by different observers. The present study aimed to characterize the racial features of Western and Asian MDS cases. We thought that an assessment of interpretation of morphologic findings and definition of diagnostic criteria was warranted to check that the diagnoses by the Japanese group were in line with those of the German group, before comparing the clinical features between Japanese and German patients with FAB-RA. In the present study, the agreement of morphologic diagnosis between Japanese and German hematologists was 98.4%. It was confirmed that the diagnoses according to FAB classification or AA were not different between the Japanese and German groups. After morphologic consensus was obtained at the first joint review meeting, we performed this separate review. The concordance rate according to the FAB classification of morphologic diagnosis between Japanese and German hematologists was thus excellent. However, the subjects of this separate review were only FAB-RA, FAB-RARS, and AA cases that had already been diagnosed by the Japanese or German groups. We think that it is most difficult to distinguish FAB-RA and disorders

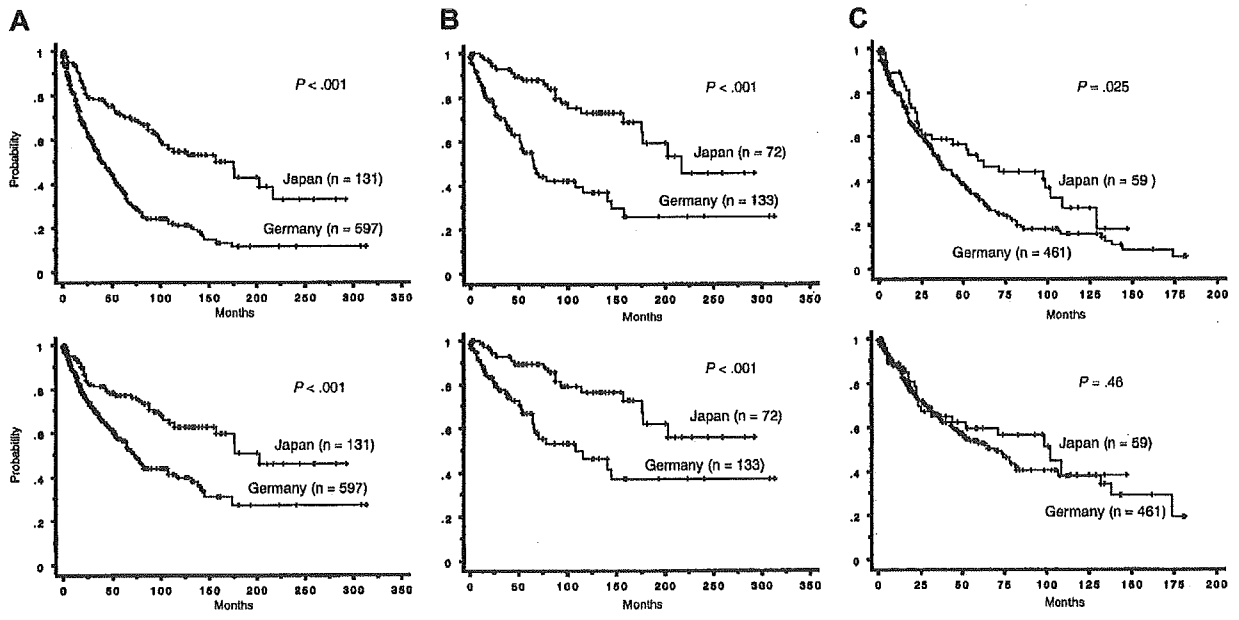

Figure 2. Cumulative survival of patients with FAB-RA. (Top) Overall survival (OS). (Bottom) Modified survival. (A) In all patients with FAB-RA, Japanese patients had a more favorable prognosis than did German patients in OS ($P < .001$). Japanese patients had a more favorable prognosis than did German patients in modified survival ($P < .001$). (B) In patients aged 60 years or younger, Japanese patients had a more favorable prognosis than did German patients in OS ($P < .001$). Japanese patients had a more favorable prognosis than did German patients in modified survival ($P < .001$). (C) In patients aged older than 60 years, Japanese patients had a more favorable prognosis than did German patients in OS ($P = .025$). Japanese patients did not show a more favorable prognosis than did German patients in modified survival ($P = .46$).

Table 4. Multivariate analysis of parameters that affected overall survivors and acute leukemia evolution in patients with RA classified according to the FAB criteria

Characteristic, by model	Overall survival		Leukemic transformation	
	Japanese HR (95% CI)	German HR (95% CI)	Japanese HR (95% CI)	German HR (95% CI)
Model A				
Age older than 60 y	5.1 (2.6-9.9)*	2.2 (1.5-3.0)*	1.6 (0.4-6.4)	1.7 (0.9-3.3)
Sex, male	1.2 (0.7-2.2)	1.0 (0.8-1.4)	1.6 (0.4-7.1)	1.2 (0.7-2.1)
ANC fewer than 1.5×10^9/L	1.2 (0.7-2.2)	1.0 (0.7-1.3)	2.0 (0.5-8.2)	1.7 (0.9-3.1)
Platelet count fewer than 100×10^9/L	1.3 (0.6-2.7)	1.9 (1.4-2.5)*	0.4 (0.1-1.9)	2.2 (1.2-4.1)*
Hemoglobin concentration less than 100 g/L	1.5 (0.8-2.8)	1.8 (1.4-2.4)*	1.0 (0.2-3.9)	1.9 (1.1-3.5)*
Chromosome (IPSS), intermediate	1.5 (0.6-3.6)	1.1 (0.6-1.9)	1.5 (0.2-14)	2.3 (0.9-5.6)
Chromosome (IPSS), poor	1.4 (0.5-4.2)	2.8 (1.6-4.9)*	11.9 (2.4-59)*	6.6 (2.8-16)*
Model B				
Age older than 60 y	4.6 (2.5-8.7)*	2.1 (1.5-2.9)*	1.7 (0.5-6.3)	1.6 (0.8-3.0)
Sex, male	1.1 (0.6-2.0)	1.2 (0.9-1.6)	1.7 (0.4-6.7)	1.2 (0.7-2.1)
IPSS, INT-1	1.0 (0.5-1.8)	1.4 (1.0-2.0)*	1.0 (0.2-4.7)	3.1 (1.6-5.7)*
IPSS, INT-2	1.6 (0.5-4.7)	4.0 (2.0-8.0)*	8.6 (1.7-43)*	9.5 (3.2-27)*

Model A included age category, sex, dichotomized peripheral blood counts, and chromosome category of IPSS. Model B included age category, sex, and IPSS score.

HR indicates hazard ratio; 95% CI, 95% confidence interval; ANC, absolute neutrophil count; IPSS, International Prognosis Score System; INT-1, Intermediate-1; INT-2, intermediate-2.

*Statistically significant hazard ratio.

with secondary dysplasia (collagen diseases, viral infectious diseases, and liver cirrhosis, etc). If we included these diseases in the present separate review, the concordance rate of morphologic diagnosis would likely have been lower.

Our results indicate that the clinical features of Japanese FAB-RA cases differ from those of German cases. Comparing Japanese and German FAB-RA cases we found that the median age of Japanese patients with FAB-RA was lower than that of German patients with FAB-RA. The population pyramids (negative growth type) and life expectancies (Japan, 80.7 years; Germany, 77.4 years) at 2000 in Japan and Germany are almost the same.[19] Therefore, we think that this difference of median age is real. Furthermore, Japanese patients with FAB-RA had more pronounced cytopenia, especially more severe thrombocytopenia, and a higher frequency of pancytopenia or bicytopenia, as compared with German patients with RA. Also the cytogenetic characteristics differed between Japanese and German RA cases. Although there was no difference in the distribution of cytogenetic subgroups according to IPSS, the frequency of chromosomal abnormalities was lower in Japanese patients with RA; notably that of isolated del (5q) was lower in Japan. Toyama et al[18] and Matsushima et al[20] reported that Japanese patients with MDS had a lower frequency of isolated del (5q) than Western reports (2.0% and 1.5%, respectively). Morel et al[21] and Greenberg et al[5] reported that the frequencies of isolated del (5q) in all MDS cases were 4.7% and 5.9%, respectively. The majority of patients with 5q− syndrome are diagnosed as FAB-RA at diagnosis. If the percentage that patients with FAB-RA compared with all MDS is assumed to be 35%, that of 5q− syndrome in the present German patients with FAB-RA becomes 6.9% of all MDS. Although this frequency of 5q− syndrome present in German patients with FAB-RA was slightly higher than the reports of Morel et al[21] and Greenberg et al,[5] we believe that the result of the present study supported Japanese previous reports.

Table 5. Laboratory features at the time of diagnosis and clinical features in Japanese patients with WHO-RA and RCMD classified according to the WHO criteria

	WHO-RA, n = 96	RCMD, n = 32	*P*
Sex, male/female	53/43	15/17	.41
Age, y	55 (12-86)	66 (16-88)	.038
Neutrophil count, $\times 10^9$/L	1.62 (0.26-4.69)	1.28 (0.05-10.24)	.76
Hemoglobin concentration, g/L	87 (30-143)	71 (25-140)	.094
Platelet count, $\times 10^9$/L	38 (4-246)	127 (13-390)	.026
2- or 3-lineage cytopenias, %*	67	75	.38
Abnormal karyotype, %	24	36	.26
Median survival, mo	176	52	.023

Values for presentation characteristics are given as median and range (in parentheses) where applicable.

*Cytopenia according to IPSS: hemoglobin concentration less than 100 g/L, absolute neutrophil count less than 1.5×10^9/L, platelet count less than 100×10^9/L.

In OS, regardless of age, Japanese patients with FAB-RA had a more favorable prognosis than did their German counterparts. In modified survival, for those aged 60 years or younger, Japanese patients with FAB-RA had a more favorable modified survival than did German patients with FAB-RA. Therefore, we believe that the favorable prognosis of younger patients with FAB-RA (≤ 60 years) is certain. In modified survival, for those older than 60 years, Japanese patients with FAB-RA did not show a more favorable modified survival than did German patients with FAB-RA. Therefore, the prognostic difference between Japan and Germany may result from the characteristics of young Japanese patients with FAB-RA (≤ 60 years).

Characteristics of Japanese patients with WHO-RA were younger and had lower PLT counts. These characteristics were similar to those of MDS responders for immunosuppressive therapy (IST) in a report by Molldrem et al.[22] The response rate for IST from a Japanese report[23] was higher than from Western reports.[22,24] However, only 8 Japanese cases received IST and only 3 responded in our present study. We think that a large-scale study is necessary to establish the relationship between Japanese WHO-RA and response for IST.

In the present study, IPSS was useful for assessing OS in German FAB-RA cases but not in Japanese FAB-RA cases. This was mainly due to the lack of a significant correlation between the number and degree of cytopenias and OS in Japanese patients with FAB-RA. In the IPSS publication, the researchers reported that cytopenias (2 or 3 lineages) were related with poor survival. In this study, however, Japanese patients showed more favorable prognoses despite possessing more pronounced cytopenia. Management of thrombocytopenia seems to be similar between Japan and

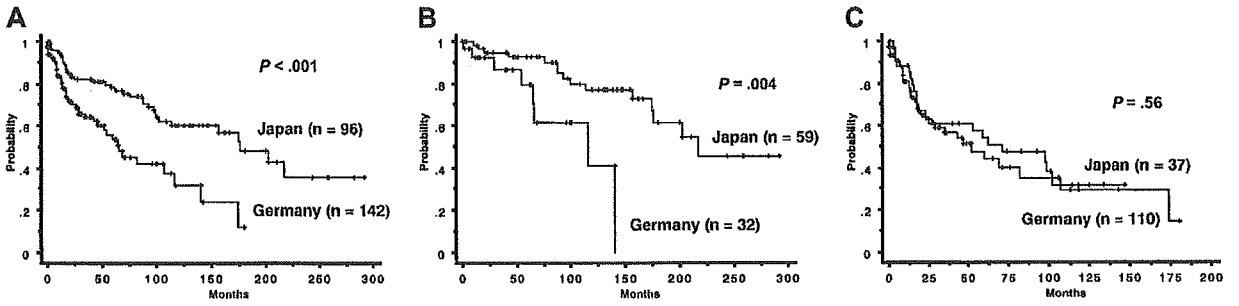

Figure 3. Cumulative overall survival of patients with WHO-RA. (A) Among all patients with WHO-RA, Japanese patients had a more favorable prognosis than did German patients ($P < .001$). (B) In patients aged 60 years or younger, Japanese patients had a more favorable prognosis than did German patients ($P = .004$). (C) In patients aged older than 60 years, Japanese patients did not show a more favorable prognosis than did German patients ($P = .56$).

Germany. Concerning the prognostic effect of Hb concentration, the threshold was different between Japanese and German patients with FAB-RA. Most of the Japanese patients with Hb concentrations greater than 70 g/L had no symptoms related to anemia and did not require red cell transfusion. In fact, most Japanese patients with Hb concentration lower than 70 g/L had received red cell transfusion. In contrast, most German patients with Hb concentration lower than 90 g/L had received red cell transfusion. We presumed that the cause of the different prognostic Hb concentration thresholds by Japanese and German patients may be related to these red cell transfusion procedures. We also presume that the difference in Hb concentration used as a threshold for red cell transfusion may be related to the different general characteristics among races rather than the different characteristics of FAB-RA between Asian and Western patients with FAB-RA. The Italian guideline recommends that all patients with Hb concentration lower than 80 g/L should receive red cell transfusion.[25] Japanese patients with FAB-RA with Hb concentration greater than 70 g/L do not usually require regular red cell transfusion. We compared Japanese patients with RA with Hb concentrations greater than 100 g/L and those with Hb concentrations of 70 to 100 g/L. In fact, the latter group (70-100 g/L) did not differ in clinical course from patients with Hb concentrations greater than 100 g/L ($P = .86$). Moreover, Japanese patients with Hb concentrations of 70 to 100 g/L had a significantly more favorable prognosis than those with Hb concentrations lower than 70 g/L ($P = .039$) (Figure 4). This result indicates that the Hb threshold below which transfusion should be recommended may be different between Asian and Western patients with FAB-RA.

We think that our results concerning the prognostic OS effect of chromosomal findings may be insufficient and may include some problematic issues. In particular, the observation periods of Japanese patients with poor karyotype according to IPSS may be problematic. Four of 8 Japanese patients with poor karyotype are surviving. However, the observation periods of the 2 surviving patients were insufficient (1 and 6 months, respectively). Concerning acute leukemia evolution, the effect of chromosomal findings was not different between Japanese and German patients. We think that the prognostic effect on OS of chromosomal findings may not be different between Japanese and German patients, if sufficient observation periods for Japanese patients with poor karyotype are available.

We made great efforts to achieve morphologic consensus in the present study. The original diagnoses according to FAB and WHO classifications were not different between the Japanese and German groups. In the present series, the original diagnoses according to the WHO classification by each group show the frequency of WHO-RA in Japanese patients to be higher than that in German patients. In Japanese patients, the prognosis of patients with WHO-RA was more favorable than that of patients with RCMD, and patients with WHO-RA had a lower cumulative risk of acute leukemia evolution than did patients with RCMD. In a previous report of a German group,[7] the same results had been reported. This finding indicates that one reason for the better prognosis of Japanese patients may be the different distribution of subgroups by WHO classification between Asian and Western patients with FAB-RA, namely a higher frequency of patients with WHO-RA in Japan. In Japanese patients, patients with WHO-RA were younger and had lower PLT counts than did patients with RCMD, significantly. Furthermore, the prognosis of Japanese patients with WHO-RA was significantly more favorable than that of Japanese patients with RCMD. For those aged 60 years or younger, the prognosis of Japanese patients with WHO-RA was significantly more favorable than that of German patients with WHO-RA. However, for those older than 60 years, Japanese patients with WHO-RA did not show a more favorable prognosis than did German patients with WHO-RA. These findings in young Japanese patients with WHO-RA ($\leq$ 60 years) might indicate the differences in clinical features between Japanese and German patients with FAB-RA.

This is the first report to compare clinical features between Asian and Western patients with FAB-RA after confirming a morphologic consensus. Our results indicate that the clinical features of Japanese FAB-RA cases differ from those of German cases. These differences are not due to the different interpretation of morphologic features by different observers. Several guidelines[25,26] have been published in Western countries. To adapt these Western guidelines to Asian patients, some modifications may be required, taking into account ethnic characteristics.

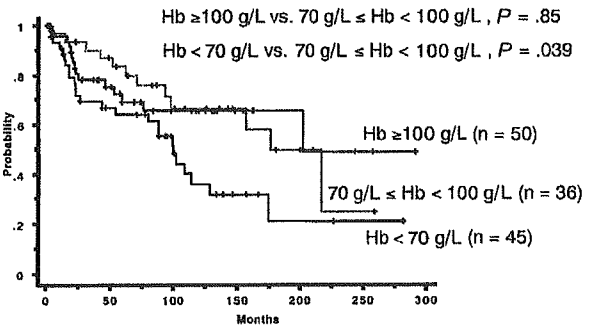

Figure 4. Cumulative overall survival of Japanese patients with FAB-RA. The group with hemoglobin concentration of 70 to 100 g/L showed no significant prognostic difference from the group with hemoglobin greater than 100 g/L in patients with FAB-RA ($P = .85$). The group with hemoglobin concentrations of 70 to 100 g/L had a more favorable prognosis than did the group with hemoglobin concentrations lower than 70 g/L in patients with FAB-RA ($P = .039$).

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Progress in Hematology

International Journal of HEMATOLOGY

Immune Pathophysiology of Aplastic Anemia

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Received July 28, 2005; accepted August 1, 2005

Abstract

Acquired aplastic anemia (AA) is considered an immune-mediated disease because approximately 70% of AA patients improve with immunosuppressive therapy. However, little is known about the inciting antigens or the mechanisms responsible for the destruction of hematopoietic stem cells by immune system attack. Recent advances in immunologic techniques have promoted our understanding of the pathogenesis of AA and have provided evidence that AA is an organ-specific T-cell–mediated disease localized in the bone marrow. Moreover, antibody screening of patients' serum with a complementary DNA library derived from hematopoietic cells has identified several proteins as candidate autoantigens in AA.
Int J Hematol. 2005;82:196-200. doi: 10.1532/IJH97.05116

Key words: Aplastic anemia; T-cell repertoire; Autoantigen; Paroxysmal nocturnal hemoglobinuria

1. Introduction

Immune-mediated suppression of hematopoiesis has been considered the most important mechanism underlying acquired aplastic anemia (AA) [1]. This concept is based on the clinical finding that approximately 70% of patients improve with immunosuppressive therapy (IST) comprising antithymocyte globulin (ATG) and cyclosporine (CsA) [2-4]. Despite such firm evidence for the immune pathophysiology in AA, little is known about the inciting antigens or the precise immune mechanisms that lead to bone marrow failure. The lack of good animal models and assays for primitive hematopoietic stem cells of humans have hindered the clarification of immune mechanisms in AA. However, the recent development of new immunologic methods have promoted our understanding of the immune mechanisms responsible for the decrease in the number of hematopoietic stem cells in AA. In this review article, we summarize the new laboratory findings that provide evidence for the presence of such immune mechanisms and discuss the clinical significance of these findings.

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2. Induction of the Immune System Attack against Hematopoietic Stem Cells

For hematopoietic stem cells to be attacked by the immune system, a breakdown of tolerance to certain antigens on stem cells and the subsequent proliferation of T-cells specific to the antigens need to occur at the initial step of pathogenesis.

2.1. Breakdown of Tolerance to Autoantigens

What induces the breakdown of immune tolerance to antigens on hematopoietic stem cells, as in the case of other autoimmune diseases, is totally unknown. Unknown viruses may induce immune responses toward some cryptic antigens on hematopoietic stem cells or altered self antigens mimicking viral antigens, although epidemiologic studies have failed to identify any viruses related to the development of AA, including hepatitis-associated AA [5].

The primary immune response may be directed not to antigens restricted to hematopoietic stem cells but rather to antigens broadly expressed by immature hematopoietic cells in the bone marrow. Such immune responses potentially lead to the production of inflammatory cytokines capable of inhibiting the growth of hematopoietic stem cells. It is possible that the bystander effect of these cytokines is responsible for the abrupt loss of hematopoietic cells in the bone marrow, as is often seen in drug-induced AA or transfusion-induced graft-versus-host disease.

2.2. Genetic Factors Affecting Susceptibility to AA

The frequency of HLA-DR15 is significantly higher in AA patients than in control populations [6,7]. The frequency is particularly high in Japanese adult patients with AA who are aged more than 40 years [8]. Because the presentation of this DR allele is associated with a good response to IST, HLA-DR15 is thought to be related to the immune mechanisms of AA [9]. The presentation of HLA-DR15 in Japanese patients is determined by 2 DRB1 alleles, DRB1*1501 and DRB1*1502. Although the 2 alleles differ by only 1 amino acid residue, they appear to contribute to the development of AA in different ways. Most patients carrying DRB1*1501 show an increase in the fraction of glycosylphosphatidylinositol-anchored protein–deficient ($GPI\text{-}AP^-$) cells and respond to IST [10]. On the other hand, the prevalence of patients showing an increase in $GPI\text{-}AP^-$ cells (paroxysmal nocturnal hemoglobinuria–positive [PNH^+] patients) and the rate of response to IST in patients carrying DRB1*1502 are similar to those in patients without HLA-DR15 [8]. An increase in the proportion of $GPI\text{-}AP^-$ cells is thought to reflect the presence of antigen-specific T-cells that inhibit hematopoietic stem cells in patients with bone marrow failure. An autoantigen of AA may have a T-cell epitope with a high affinity to DRB1*1501 and may therefore be likely to induce expansion of myelosuppressive T-cells in individuals carrying DRB1*1501. In contrast, an epitope presented by DRB1*1502 may stimulate T-cells to produce myelosuppressive cytokines such as interferon γ (IFN-γ) and tumor necrosis factor (TNF) and thereby cause profound bone marrow failure that is difficult to reverse with IST.

2.3. Proliferation of Antigen-Specific T-Cells in the Bone Marrow

In a subset of AA patients, certain antigens are thought to stimulate autoreactive T-cells to proliferate and attack hematopoietic stem cells. A murine study has demonstrated that a small number of $CD4^+$ cells can directly kill hematopoietic stem cells through cell-to-cell contact [11]. PNH^+ AA patients with HLA-DRB1*1501 are a good model to study the role of antigen-specific T-cells in the development of bone marrow failure. When bone marrow T-cells of untransfused AA patients who meet these conditions are studied with a complementarity-determining region 3 (CDR3) size distribution analysis, clonal T-cell proliferation is often detected in a limited number of T-cell receptor Vβ families [12]. Figure 1 shows an example of CDR3 size distribution patterns in such a patient with immune-mediated AA. Skewed patterns of CDR3 size distribution were corrected when the patient obtained remission after CsA therapy, suggesting that the clonal proliferation of the bone marrow T-cells was related to the pathogenic mechanisms of bone marrow failure [12]. Similar findings indicating antigen-driven T-cell proliferation in the bone marrow of AA patients have been reported by other researchers [13-15].

Figure 2 compares CDR3 size distribution patterns for the peripheral blood and the bone marrow of a PNH^+ AA patient who carries HLA-DRB1*1501. Marked skewing of the CDR3 size distribution was seen only in bone marrow T-cells (C.S. et al, unpublished observation). This finding strongly indicates that PNH^+ AA with HLA-DRB1*1501 is an organ-specific autoimmune disease restricted to the bone marrow. Some AA patients with DRB1*1501 share the CDR3 structure among the T-cells that show clonal proliferation in the bone marrow. If these T-cell clones could be isolated from the bone marrow, it might be possible to identify the target antigens of these T-cells [13]. We previously isolated such a $CD4^+$ T-cell clone possessing the CDR3 amino acid sequence DLTSGP from the bone marrow of an AA patient [12]. This $CD4^+$ T-cell clone expressed Vβ15, Vα2, and Vα4. However, we have not been able to identify a target antigen of the T-cell clone because of the lack of good assays to determine the corresponding antigen of a $CD4^+$ T-cell whose specificity is unknown.

2.4. Autoantibodies in AA Patients

T-cell suppressants such as ATG and CsA often produce a remission of AA, but therapies such as plasma apheresis and infusion of anti-CD20 antibodies that aim for the elimination of antibodies are rarely effective in restoring the hematopoietic function of AA patients. Therefore, T-cells, not antibodies, have been accepted to be responsible for the pathogenesis of AA. However, autoantibodies are often

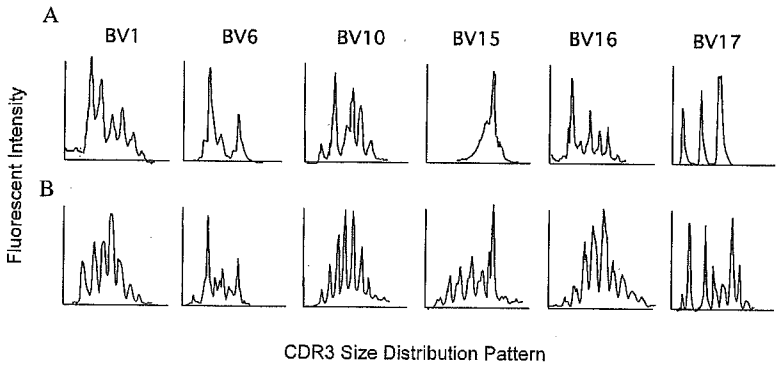

Figure 1. Changes in complementary-determining region 3 (CDR3) size distribution patterns of bone marrow T-cells associated with cyclosporine therapy [12]. Skewing of CDR3 size distribution patterns in BV10, BV15, and BV16 families were ameliorated by successful cyclosporine therapy. A, Before therapy. B, Four years after therapy.

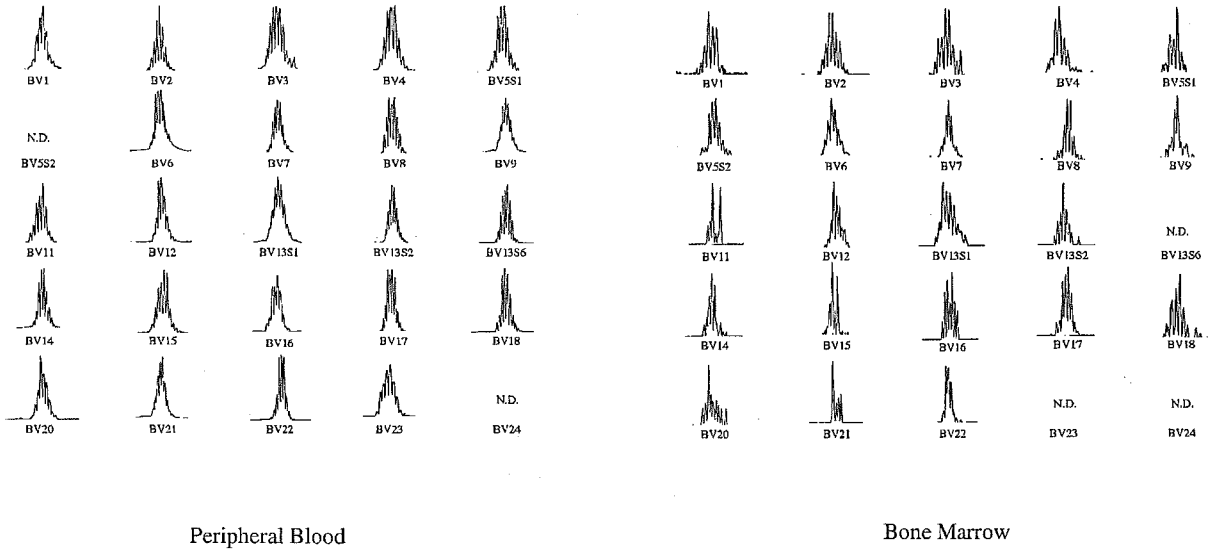

Figure 2. T-cell repertoire in the peripheral blood and bone marrow of a patient with immune-mediated aplastic anemia. Skewing of the complementarity-determining region 3 (CDR3) size distribution was evident only in bone marrow T-cells, indicating that antigen-driven T-cell proliferation predominantly occurs in the bone marrow.

detected in organ-specific autoimmune diseases such as insulin-dependent diabetes mellitus and multiple sclerosis, in which T-cells play an essential role in their pathogenesis [16,17]. If autoantibodies can be detected in the serum of AA patients, the corresponding antigens may also serve as antigens of T-cells that are responsible for AA pathogenesis.

Hirano et al of Harvard University screened a complementary DNA (cDNA) library derived from human fetal liver cells for antigens recognized by serum immunoglobulin G (IgG) of an AA patient and identified kinectin [18]. Kinectin is abundantly expressed by a limited number of human tissues, including the liver, the brain, the testis, and $CD34^+$ cells of the bone marrow. Antibodies to kinectin were detectable in 7 (39%) of 18 AA patients and were not detectable in healthy individuals and in heavily transfused patients with hereditary hemolytic anemia. Cytotoxic T-lymphocytes (CTLs) specific for a kinectin peptide showing a high affinity to HLA-A2 were generated from an individual carrying HLA-A2, and these T-cells inhibited the growth of granulocyte-macrophage progenitor cells in vitro. These findings suggest that anti-kinectin antibodies reflect not only the presence of immune pathophysiology in certain AA patients but also the role of kinectin as inciting antigens in the pathogenesis of AA.

We used a cDNA library derived from leukemia cell line UT-7 to screen antigens recognized by serum IgG of a PNH^+ AA patient who carried HLA-DRB1*1501. Among the several antigens identified, only antibodies to diazepam-binding inhibitor–related sequence 1 (DRS-1) were detected in different PNH^+ AA patients [19]. The DRS-1 gene and protein were highly expressed by leukemia cell lines and by $CD34^+$ cells from healthy individuals. Figure 3 shows the titers of anti–DRS-1 antibodies for different diseases, as determined by enzyme-linked immunosorbent assay. Significantly high titers of anti–DRS-1 antibodies were detected in 38% of PNH^+ AA patients, 6% of AA patients not showing an increase in the proportion of GPI-AP^- cells (PNH^- patients), and 39% of PNH^+ patients with refractory anemia of myelodysplastic syndrome (MDS), but such antibodies were undetectable in any of the patients with PNH^- refractory anemia. Approximately half of the patients who were positive for anti–DRS-1 antibodies had high antibody titers to a DRS-1 peptide comprising amino acid residues 178 to 198, and this peptide overlapped with a putative T-cell epitope comprising residues 191 to 204, a peptide showing high affin-

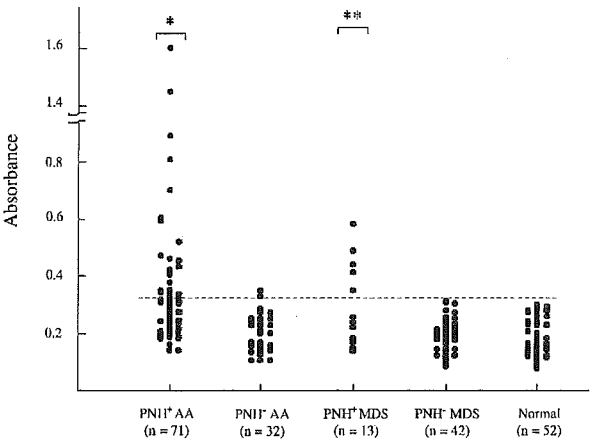

Figure 3. Titers of antibodies to diazepam-binding inhibitor–related sequence 1 (anti–DRS-1) in the different subsets of bone marrow failure [19]. High titers of anti–DRS-1 antibodies were primarily detected in aplastic anemia (AA) and refractory anemia patients showing an increase in the proportion of glycosylphosphatidylinositol-anchored protein–deficient (GPI-AP^-) cells. PNH^+ AA indicates AA showing an increase in the proportion of GPI-AP^- cells; PNH^- AA, AA not showing an increase in GPI-AP^- cells; MDS, myelodysplastic syndrome. $^*P < .001$; $^{**}P < .05$.

ity to DRB1*1501. When peripheral blood T-cells of 2 AA patients who showed high titers of anti-DRS-1 antibodies were examined with the enzyme-linked immunospot assay, both patients showed an increase in the frequency of T-cell precursors specific for the peptide comprising amino acid residues 191 to 204. DRS-1-specific $CD4^+$ T-cells were able to respond specifically to antigen-presenting cells transfected by the DRS-1 gene. These findings suggest that the breakdown of tolerance to DRS-1 may give rise to T-cells specific to hematopoietic progenitor cells in individuals carrying HLA-DR15 and that DRS-1 may be one of the autoantigens that incite the development of AA.

3. Immune Mechanisms Responsible for the Loss of Hematopoietic Stem Cells in AA

In contrast to the few studies that have focused on the mechanisms responsible for the induction of immune system attack against hematopoietic stem cells, a large number of studies have focused on the effector mechanisms that lead to a decrease in hematopoietic stem cells. However, most of the studies used committed progenitor cells as target cells because of the lack of good assays for enumerating primitive hematopoietic stem cells in humans, and so the results may not reflect the effector mechanisms of bone marrow failure in vivo [20,21].

3.1. Suppression of Hematopoiesis by Inflammatory Cytokines

Inflammatory responses to certain cells in the bone marrow lead to the secretion of cytokines from T-cells and monocytes in the bone marrow. These cytokines may play a major role in the development of bone marrow failure. A low amount of constitutively produced IFN-γ or TNF in the bone marrow may be able to induce the exhaustion of hematopoietic stem cells in vivo [22-24]. However, whether these cytokines can induce persistent bone marrow failure by themselves is unknown. The administration of antibodies that neutralize myelosuppressive cytokines may provide insights into their role in the development of bone marrow failure. The administration of infliximab, an antibody that neutralizes TNF, is reported to ameliorate the chronic anemia associated with rheumatoid arthritis or MDS [25,26]. However, the TNF receptor (p75)-Fc fusion protein failed to improve anemia in a larger number of MDS patients [27,28]. Thus, there appears to be no clinical evidence at present to indicate that myelosuppressive cytokines play a major role in the development of bone marrow failure.

3.2. Myelosuppression by Antigen-Specific T-Cells

A small number of $CD4^+$ T-cells may directly kill hematopoietic stem cells, as has been demonstrated with a murine model [11]. We have isolated a $CD4^+$ T-cell clone from the bone marrow of an AA patient whose bone marrow function depended on the administration of low-dose CsA [29]. This T-cell clone killed autologous hematopoietic cells in a Ca^{2+}-dependent way, and the cytotoxicity was restricted by HLA-DRB1*0405 [30]. It is thus possible that an attack on hematopoietic stem cells by a low number of CTLs causes the depletion of hematopoietic stem cells in some patients with immune-mediated AA [31].

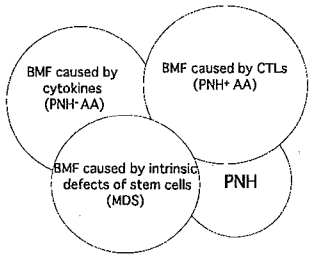

Figure 4. Mutual relationships among the different subsets of bone marrow failure. BMF indicates bone marrow failure; PNH^- AA, aplastic anemia (AA) not showing an increase in glycosylphosphatidylinositol-anchored protein-deficient ($GPI\text{-}AP^-$) cells; PNH^+ AA, AA showing an increase in the proportion of $GPI\text{-}AP^-$ cells; CTLs, cytotoxic T-lymphocytes; MDS, myelodysplastic syndrome; PNH, paroxysmal nocturnal hemoglobinuria.

3.3. Markers for the Presence of CTLs in AA Patients

Because immune mechanisms underlie bone marrow failure in only 70% of AA patients, predicting the response to IST with markers that reveal immune mechanisms is essential for the management of AA. One of the best predictive markers is an increase in the percentage of $GPI\text{-}AP^-$ blood cells [32,33].

Although a small number of $GPI\text{-}AP^-$ stem cells exists in healthy individuals [34], these cells remain at a very low frequency (<0.002%) because of their low proliferative potential. In circumstances in which CTLs against hematopoietic stem cells exist, $GPI\text{-}AP^-$ stem cells may survive longer than $GPI\text{-}AP^+$ cells because of lower sensitivity to CTLs [35]. If this hypothesis is correct, patients with bone marrow failure who show an increased proportion of $GPI\text{-}AP^-$ cells are more likely to respond to IST than patients who do not show an increase in the fraction of $GPI\text{-}AP^-$ cells. Indeed, PNH^+ AA patients in our study showed a better response to ATG plus CsA than PNH^- patients did. These findings suggest that an increase in the fraction of $GPI\text{-}AP^-$ cells reflects the immune pathophysiology of AA mediated by antigen-specific T-cells. Figure 4 illustrates the mutual relationships among the different subsets of bone marrow failure.

4. Conclusions

AA has been regarded as a vague syndrome that can be diagnosed only by ruling out the other hematologic diseases characterized by pancytopenia and bone marrow hypoplasia. The use of markers for immune pathophysiology, such as a small number of $GPI\text{-}AP^-$ cells and autoantibodies, may help physicians to positively diagnose immune-mediated AA. Further studies on PNH^+ AA patients will be useful in clarifying immune mechanisms of bone marrow failure.

Acknowledgments

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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International Journal of
HEMATOLOGY

Case Report

Isolated Hyperkalemia Associated with Cyclosporine Administration in Allogeneic Stem Cell Transplantation for Renal Cell Carcinoma

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Received August 2, 2004; received in revised form October 27, 2004; accepted November 17, 2004

Abstract

Two patients with advanced renal cell carcinoma underwent allogeneic hematopoietic stem cell transplantation and received cyclosporine (CSP) as part of their immunosuppressive therapy. Despite adequate renal function, both patients developed hyperkalemia. CSP was the only pharmaceutical agent to which this electrolyte abnormality could be attributed. Evaluation of renal tubule function suggested that CSP-associated isolated hyperkalemia resulted from tubular resistance to aldosterone. We propose that the presence of a single functional kidney may be a risk factor for isolated hyperkalemia due to CSP.
Int J Hematol. 2005;81:159-161. doi: 10.1532/IJH97.04113

Key words: Cyclosporine; Renal cell carcinoma; Stem cell transplantation; Hyperkalemia; TTKG

1. Introduction

Cyclosporine (CSP), the first calcineurin inhibitor available for clinical use, was introduced in the 1980s and radically changed the field of organ transplantation [1]. However, the use of CSP has been associated with certain side effects. Of major concern is nephrotoxicity [1,2]. CSP-associated renal insufficiency is due to vasoconstriction of the afferent glomerular arterioles, with resultant reductions in renal blood flow and glomerular filtration rate [2]. CSP can also cause hyperkalemia while preserving renal function, seemingly owing to inhibition of potassium secretion by the distal nephron [2-6]. Hyperkalemia with adequate renal function is called isolated hyperkalemia. Despite its common occurrence in renal transplant recipients treated with CSP [4,7,8], reports of cases in the setting of allogeneic hematopoietic stem cell transplantation (SCT) are limited. Only 7 cases of isolated hyperkalemia are attributable to CSP: in 2 patients with myelodysplastic syndrome, 4 with acute myeloid leukemia, and 1 with chronic myelogenous leukemia [5,6,9]. The etiology of this discrepancy has not been well delineated. Recently, the use of allogeneic SCT has expanded into the treatment of renal cell carcinoma (RCC) [10]. We report 2 cases of CSP-induced hyperkalemia in the setting of adequate renal function after allogeneic SCT for RCC. This report is the first to describe isolated hyperkalemia in RCC patients after allogeneic SCT. We suggest that the development of isolated CSP-induced hyperkalemia is primarily attributable to a shortage of renin and aldosterone in patients with a single kidney.

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2. Case Reports

2.1. Case 1

A 56-year-old man with clear cell RCC of his right kidney underwent a right nephrectomy. Six years later, the patient had metastatic diseases in the right upper jaw and pancreas that were partially removed. The remaining metastases grew, and new metastases developed in the left lung, retroperitoneal space, and subcutaneous space. These metastases were refractory to interferon alfa, interferon gamma, and interleukin 2 treatments. In view of the low probability of response to further conventional treatment for metastatic RCC, the patient was referred to our institute at the age of 69 years. The patient gave written informed consent to participate in an investigational protocol approved by the ethics committee of

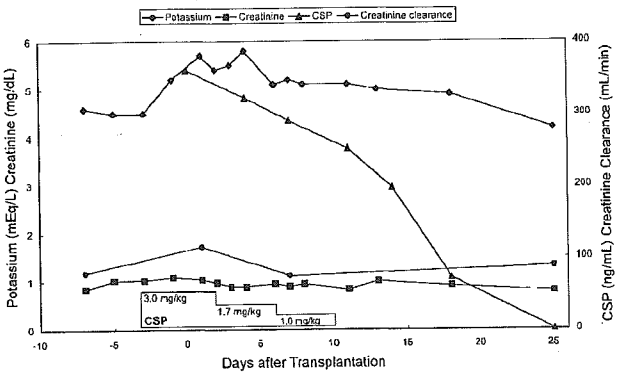

Figure 1. Blood cyclosporine (CSP), serum creatinine, creatinine clearance, and potassium levels in case 1. The serum potassium level returned to within the normal range with the lowering of CSP levels.

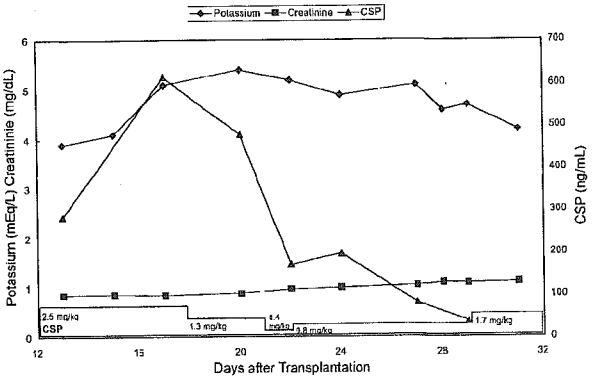

Figure 2. Blood cyclosporine (CSP), serum creatinine, and potassium levels in case 2. Serum potassium levels returned to within the normal range following the reduction of CSP levels.

Kanazawa University Graduate School of Medicine. The preparative regimen consisted of a single dose of 50 mg/kg cyclophosphamide on day −6, 40 mg/m^2 fludarabine daily for 5 days (days −6 to −2), and a single dose of 200 cGy total body irradiation (day −1). Unrelated cord blood (UCB), phenotypically mismatched at 1 HLA-B antigen and 1 DRB1 antigen, was obtained from the Hokkaido Cord Blood Bank. The patient received a UCB graft at a dose of 1.95×10^7 nucleated cells/kg recipient body weight. Rejection of the graft and graft-versus-host disease (GVHD) were prevented by intravenous administration of CSP (1.5 mg/kg twice a day) beginning 3 days before transplantation. Blood CSP levels were monitored by enzyme-linked multiple immunoassay, and the dosage was adjusted to maintain the trough blood level between 200 and 300 μg/L. In addition, the patient received 15 mg/kg mycophenolate mofetil (MMF) orally twice daily from days −3 to +30. For antimicrobial prophylaxis, 600 mg ciprofloxacin, 400 mg fluconazole, and 1000 mg acyclovir were given daily. Trimethoprim-sulfamethoxazole (320 mg trimethoprim and 1600 mg sulfamethoxazole) was given daily between days −7 and −2 to prevent *Pneumocystis carinii* infection. Daily administration of 300 μg granulocyte colony-stimulating factor was started on day +1. The serum potassium level showed a gradual increase from 4.5 mEq/L starting on day −3 to 5.8 mEq/L on day +4 (Figure 1), and this change was associated with CSP levels between 360 and 322 ng/mL. The patient did not receive any parenteral fluid or antibiotics containing potassium during this period. At the time of the peak potassium level, the serum creatinine level and creatinine clearance were 0.89 mg/dL and 75 mL/min, respectively. The transtubular potassium concentration gradient (TTKG) was calculated to be 2.4, and the plasma aldosterone level and plasma renin activity were 32.8 pg/mL (normal range, 30-255 pg/mL) and 1.8 ng/mL per hour (normal range, 0.4-2.7 ng/mL per hour), respectively. The serum chloride, bicarbonate, and blood pH were 104 mEq/L, 20 mmol/L, and 7.356, respectively. As the dose of CSP was reduced, a decrease in the serum potassium level was observed. It returned to within the normal range on day +11, when the CSP level was 197 ng/mL (Figure 1). During this time no hypertension developed.

2.2. Case 2

A 44-year-old man with clear cell RCC of the right kidney underwent a right nephrectomy. Nineteen years later, metastatic RCC developed in his right lung and bones and progressed despite treatment with interferon alfa and a cytotoxic drug. Because the patient's disease was unlikely to respond to further conventional treatment, he was referred to our institute at 64 years of age. The patient and donor gave written informed consent to participate in an investigational protocol approved by the ethics committee of Kanazawa University Graduate School of Medicine. Following conditioning therapy with 60 mg/kg cyclophosphamide administered intravenously on days −7 and −6 and then 25 mg/m^2 fludarabine administered intravenously daily for 5 days, the patient received from his HLA-identical brother an allogeneic peripheral blood SCT containing 5.6×10^6 CD34$^+$ cells/kg. Twice-daily doses of intravenous CSP (1.5 mg/kg) were started on day −4. The blood CSP level was monitored and adjusted as described for case 1. As antimicrobial prophylaxis, 800 mg ciprofloxacin, 200 mg fluconazole, and 1000 mg acyclovir were given daily. Trimethoprim-sulfamethoxazole (320 mg trimethoprim and 1600 mg sulfamethoxazole) was given daily between days −10 to −2 as prophylaxis for *P carinii* infection. The patient's serum potassium level rose from 4.1 mEq/L on day +17 to 5.4 mEq/L on day +20 in parallel with an increase in blood CSP from 284 to 614 ng/mL (Figure 2). No potassium-containing parenteral fluid or antibiotics were used during this period. At the peak serum potassium level on day +17, the serum creatinine and serum chloride levels were 0.84 mEq/L and 97 mEq/L, respectively. The CSP dose was reduced, and the return of the patient's serum potassium level to the normal range on day +29 was associated with a CSP level of 81 ng/mL (Figure 2). No hypertension was seen during this time.

3. Discussion

In the present 2 cases, hyperkalemia developed in parallel with high blood CSP levels and disappeared as CSP levels were reduced. During this time, renal function remained nor-

mal, and no medication or parenteral nutrition that would cause hyperkalemia was used, indicating that CSP had induced isolated hyperkalemia. The TTKG is a simple index that permits semiquantitative evaluation of potassium secretion with respect to aldosterone bioactivity [3-6]. Hyperkalemia associated with renal insufficiency has a TTKG value of >10. An abnormally low TTKG value <8 and normal or elevated plasma aldosterone levels in a hyperkalemic patient, such as in case 1, are indicative of low aldosterone bioactivity due to tubular dysfunction [3,6]. CSP is known to decrease renal tubule sensitivity to aldosterone at the distal nephron in renal transplant recipients [4] and allogeneic SCT recipients [5,6,9]. Thus, it is reasonable to suppose that the isolated hyperkalemia in case 1 resulted from CSP-induced tubular resistance to aldosterone. Although the onset of hyperkalemia in case 2 was slightly different from that in case 1, the 2 cases were similar with respect to other clinical parameters, such as the association of isolated hyperkalemia with blood CSP levels, intravenous infusions of CSP, and no medications or parenteral nutrition that could cause hyperkalemia, all of which imply the same mechanism of hyperkalemia in case 2 as in case 1, despite the lack of TTKG information for case 2.

There are many in vivo and in vitro data indicating the activation of the renin-angiotensin-aldosterone system by CSP, including increased plasma renin activity and juxtaglomerular apparatus hyperplasia in renal transplant recipients on CSP, a decrease of renin-containing cells in renal allografts following conversion from CSP to azathioprine treatment, and CSP promotion of renin synthesis and release in cultured juxtaglomerular cells [2,11]. Accordingly, CSP-induced hyperreninemia may counterbalance CSP's inhibitory effect on distal renal tubular function in most SCT patients. The fact that plasma renin activity and plasma aldosterone concentrations were both normal in case 1 implies that such a shortage of plasma renin activity and a low aldosterone concentration overcame renal tubular resistance to aldosterone induced by CSP. This hypothesis is supported by findings of hyporeninemic hypoaldosteronism in patients on CSP who exhibited isolated hyperkalemia after renal transplantation [8,12] and after SCT for myelodysplastic syndrome [9].

Since 2000, 5 postnephrectomy RCC patients have undergone allogeneic SCT in our institute, 2 (40%) of whom developed isolated hyperkalemia in association with CSP administration. There is a possibility that drug interaction between CSP and MMF caused hyperkalemia in case 1. However, among the 74 patients with hematologic diseases who received allogeneic SCT during the same period, including 3 patients who received both MMF and CSP as GVHD prophylaxis as in case 1, no such cases of hyperkalemia were observed. There were no reports indicating a drug interaction between CSP and MMF. Because isolated hyperkalemia is common among renal transplant recipients on CSP [4], the development of CSP-induced isolated hyperkalemia may be associated with the reliance of the patient on a single kidney. A single kidney could cause insufficient renin synthesis and release in response to CSP, leading to the inability to offset CSP-induced tubular resistance to aldosterone, thereby resulting in decreasing potassium secretion. It is unclear whether a single functional kidney could be a risk factor for isolated hyperkalemia due to CSP in allogeneic stem cell recipients with RCC. More cases are required for the evaluation of hyperkalemia. Immunotherapy with allogeneic SCT is promising for treating advanced RCC not curable by any other treatment and is most often administered to patients who have only a single kidney. Our experience shows the importance of the awareness of CSP as a risk factor for isolated hyperkalemia in such patients.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare. The authors wish to thank Megumi Yoshii and Sadako Yoshioka for their excellent technical assistance as well as their patience in the preparation of the manuscript.

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Minor population of CD55$^-$CD59$^-$ blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia

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We investigated the clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria (PNH)–type blood cells in patients with acquired aplastic anemia (AA). We quantified CD55$^-$CD59$^-$ granulocytes and red blood cells (RBCs) in peripheral blood from 122 patients with recently diagnosed AA and correlated numbers of PNH-type cells and responses to immunosuppressive therapy (IST). Flow cytometry detected 0.005% to 23.1% of GPI-AP$^-$ cells in 68% of patients with AA. Sixty-eight of 83 (91%) patients with an increased proportion of PNH-type cells (PNH$^+$) responded to antithymocyte globulin (ATG) + cyclosporin (CsA) therapy, whereas 18 of 39 (48%) without such an increase (PNH$^-$) responded. Failure-free survival rates were significantly higher (64%) among patients with PNH$^+$ than patients with PNH$^-$ (12%) at 5 years, although overall survival rates were comparable between the groups. Numbers of PNH-type and normal-type cells increased in parallel among most patients with PNH$^+$ who responded to IST, suggesting that these cells are equally sensitive to immune attack. These results indicate that a minor population of PNH-type cells represents a reliable marker of a positive IST response and a favorable prognosis among patients with AA. Furthermore, immune attack against hematopoietic stem cells that allows PNH clonal expansion might occur only at the onset of AA. (Blood. 2006;107:1308-1314)

Introduction

Immunosuppressive therapy (IST) with antithymocyte globulin (ATG) plus cyclosporin (CsA) is the standard approach to treating acquired aplastic anemia (AA).[1-5] Approximately 70% of patients respond to this therapy and achieve remission. However, for the remaining 30%, IST might even be harmful because of an increased risk of opportunistic infections, particularly in the absence of any remission. The immune pathophysiology of patients should thus be understood at diagnosis, and IST should be applied only to those with immune-mediated AA. Several factors have been proposed as good markers that appear to reflect the immune pathophysiology of AA. These factors include an increased ratio of activated T cells,[6] increased interferon-γ expression in bone marrow,[7] and peripheral-blood T cells,[8] as well as increased expression of heat-shock protein 70.[9] Although these markers are useful in predicting responses to IST, few patients with AA have been tested, and the assays applied to detect these abnormalities are vulnerable to the effects of artifacts and the transportation of test samples. Consequently, none of the markers have been practically applied to predict responses to IST. Because of this, patients with AA are placed on IST without understanding the underlying pathophysiology.

One marker closely associated with immune pathophysiology in bone marrow failure is a small number of cells that are glycosylphosphatidylinositol-anchored membrane protein-deficient (GPI-AP$^-$), namely paroxysmal nocturnal hemoglobinuria (PNH)–type cells.[10-14] Dunn et al[11] have demonstrated that an increase in CD15$^-$CD66b$^-$CD16$^+$ granulocytes is associated with a good response to ATG among patients with myelodysplastic syndrome (MDS). Using 2-color flow cytometry that can distinguish proportions of CD55$^-$CD59$^-$CD11b$^+$ granulocytes and CD55$^-$CD59$^-$ glycophorin A$^+$ red blood cells (RBCs) below 0.1%, we also demonstrated that a population of 0.01% to 6% PNH-type cells among granulocytes and red blood cells predicts a response to CsA in patients with MDS.[15] Although one study group did not find a correlation between PNH-type cells and response to ATG in patients with AA,[14] an increase in the proportion of PNH-type cells was correlated with a good response to IST among our patients with AA[16] as well as those in another report.[12] However, the significance of a minor population of PNH-type cells in the management of patients with AA has remained obscure because the number of patients with recently diagnosed AA has been small and follow-up periods have not been long enough. Our sensitive flow cytometric protocol has not become popular despite its potential clinical usefulness, perhaps because of the lower cut-off values (0.003% for granulocytes and 0.005% for RBCs) than previous assays.[11,12,17,18]

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Submitted June 22, 2005; accepted September 14, 2005. Prepublished online as *Blood* First Edition Paper, September 22, 2005; DOI 10.1182/blood-2005-06-2485.

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, Ministry of Health, Labor, and Welfare, Japan.

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The outcome of IST in patients with AA is negatively affected by the length of time from diagnosis to treatment.[19] To clarify the role of a marker that would predict a good response to IST, the marker should be tested on patients who have been recently diagnosed with AA and before they receive therapy, and then the marker should be correlated with the subsequent response to IST. Since 1999, we have been studying the presence of PNH-type cells in peripheral blood using flow cytometry in 241 patients who had not yet undergone therapy and who were diagnosed with AA. The present study focuses on 122 patients who were treated with ATG and CsA within 1 year of the diagnosis of AA and compares the response rates to IST and subsequent survival between patients with (PNH^+) and without (PNH^-) an increased proportion of PNH-type cells. We also examined changes in the number of PNH-type cells after successful IST to characterize the immune system attack against hematopoietic stem cells that confers a survival advantage on PNH-type stem cells in immune-mediated AA.

Patients, materials, and methods

Patients

We evaluated PNH-type cells in peripheral-blood samples from 122 Japanese patients (55 men and 67 women; median age, 56 years) with idiopathic AA (75 severe and 47 moderate AA) before they received IST. The patients were diagnosed with AA at Kanazawa University Hospital, hospitals participating in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions. The severity of AA was classified according to the criteria proposed by Camitta et al.[20] All patients were treated with ATG Lymphoglobuline (Aventis Behring, King of Prussia, PA) 15 mg/kg/d, 5 days; plus CsA (Novartis, Basel, Switzerland) 6 mg/kg/d; within 1 year of diagnosis between April 1999 and December 2004. The dose of CsA was adjusted to maintain trough levels 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. Response to IST was evaluated according to the response criteria described by Camitta.[21] Complete response (CR) was defined as hemoglobin normal for age, neutrophil count more than 1.5×10^9/L, and platelet count more than 150×10^9/L. Partial response (PR) was defined as transfusion independent and no longer meeting criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling or normalization of at least one cell line or increase in baseline hemoglobin of more than 30 g/L (if initially less than 60 g/L), neutrophil count of more than 0.5×10^9/L (if initially less than 0.5×10^9/L), and platelet count of more than 10×10^9/L (if initially less than 20×10^9/L) in patients with moderate AA. The patients provided written, informed consent to participate in all procedures associated with the study, which was reviewed and approved by the ethical committee of Kanazawa University Hospital (study no. 46). The study also conforms to the recently revised tenets of the Helsinki protocol.

High-resolution 2-color flow cytometry

We improved the 2-color flow cytometry developed by Araten et al[22] as follows. Briefly, 3 to 5 mL heparinized blood was drawn from each patient. To detect PNH-type granulocytes, RBCs were lysed in NH_4Cl 8.26 g/L, $KHCO_3$ 1.0 g/L, and EDTA · E4Na 0.037 g/L (lysis buffer). After a saline wash, 50 μL leukocyte suspension was incubated with 4 μL phycoerythrin (PE)–labeled anti-CD11b monoclonal antibodies (mAbs; Becton Dickinson, Franklin Lakes, NJ), fluorescein-isothiocyanate (FITC)–labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen) on ice for 30 minutes.[13] To detect PNH-type RBCs, PE-labeled anti–glycophorin A mAbs (clone JC159; DAKO, Glostrup, Denmark) were included instead of anti-CD11b mAbs.[15] Fresh blood was diluted to 3% in phosphate-buffered saline (PBS), and then 50 μL was incubated with 4 μL PE-labeled anti–glycophorin A mAbs, FITC-labeled anti-CD55, and anti-CD59 mAbs on ice for 30 minutes. A total of at least 1×10^5 $CD11b^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometry. To exclude damaged cells that often produce false-positive results, all samples were treated for flow cytometry within 24 hours after collection, and SSC^{dim} and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs on the histograms were excluded from the analyses by careful gating as shown in Figure 1A. On the basis of analytic results from 68 healthy individuals, the presence of greater than 0.003% $CD11b^+$ granulocytes and 0.005% glycophorin A^+ RBCs was considered abnormal. Both thresholds greatly exceeded the mean + 4 SDs for $GPI\text{-}AP^-$ granulocytes (0.0025%) and RBCs (0.0032%) determined in healthy individuals.[13,15] When PNH-type cells were increased in only 1 of the 2 cell lineages, another sample was collected, and the patient was deemed PNH^+ only when the second sample produced similar results.

We compared the sensitivity of detecting a few PNH-type cells in this manner with that of a low-resolution method[23] by analyzing the blood of some patients by 2-color flow cytometry using both PE-labeled anti-CD55

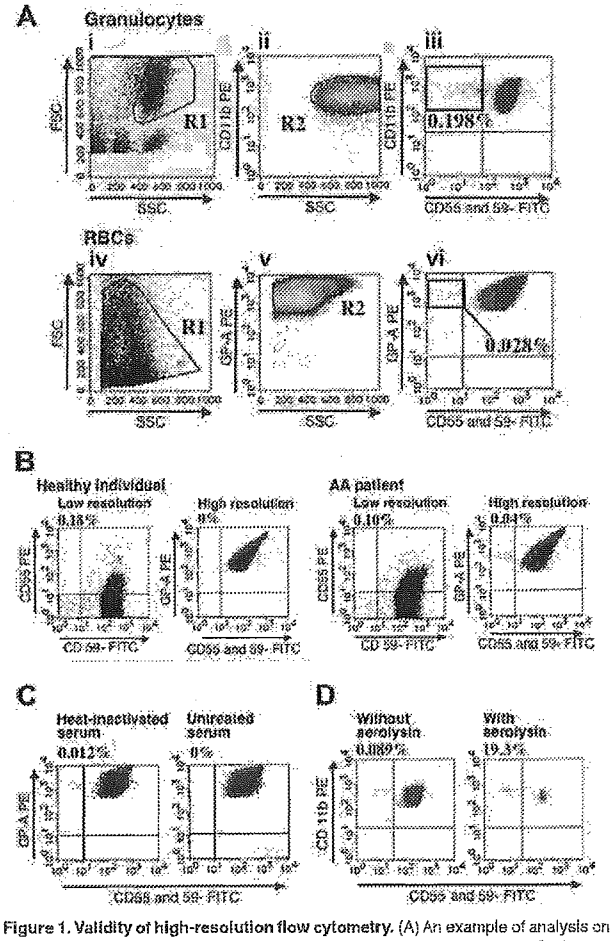

Figure 1. Validity of high-resolution flow cytometry. (A) An example of analysis on a patient with PNH^- AA is shown. Gates were set up to exclude SSC^{dim} (i) and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs (ii,v). Cells within rectangles showing horizontal distribution represent PNH-type cells. (B) RBCs from a healthy individual and a patient with AA were examined using a low-resolution assay and the high-resolution assay. Numbers on histograms denote the percentages of $CD55^-CD59^-$ cells in total RBCs for the low-resolution assay, and in glycophorin A^+ RBCs for the high-resolution assay. (C) RBCs from a patient with PNH^+ AA were incubated in acidified saline containing heat-inactivated or untreated serum.$CD55^-CD59^-$ RBCs were then quantified. (D) PNH^- AA WBCs were incubated with or without 0.5×10^{-8} M aerolysin and analyzed by flow cytometry.

and FITC-labeled anti-CD59 mAbs. This assay defines the presence of 1% or more PNH-type cells as a significant increase.

Modified Ham test

Peripheral blood of patients with AA with a low proportion (< 0.1%) of $CD55^-CD59^-$ RBCs was washed with saline and suspended in saline at a hematocrit of 50%. The RBC suspension (15 μL) was incubated with 80 μL heat-inactivated fetal calf serum (FCS) for 10 minutes at 4°C for sensitization by anti–human heteroantibodies and then washed with saline. Human AB serum as a source of complement (0.5 mL) and 55 μL 0.2 N HCl were then added to the cell suspension. The negative control included heat-inactivated human AB serum instead of untreated human AB serum. These RBC suspensions were incubated for 60 minutes at 37°C and washed with PBS, and then the RBCs were analyzed by flow cytometry as described in "High resolution 2-color flow cytometry."

Aerolysin treatment of granulocytes

Peripheral blood from patients with AA with a low proportion of PNH-type granulocytes was lysed as described in "High resolution 2-color flow cytometry," and suspended in PBS at a density of 2×10^5 cells/mL. The leukocyte suspension was split into 2 portions; one was incubated for 15 minutes with and the other without 0.5×10^{-8} M aerolysin at 37°C.[24] Before and after the incubation with aerolysin, the suspension was examined by flow cytometry to detect $CD55^-CD59^-CD11b^+$ granulocytes as described in "High resolution 2-color flow cytometry."

Statistics

The Mann-Whitney test compared clinical characteristics between patients with PNH^+ and patients with PNH^-. Fisher exact test and logistic regression modelling[25] analyzed associations between individual pretreatment variables with response to IST. Kaplan-Meier methods graphically compared the cumulative incidence of the response with IST and time to event, and differences between patients with PNH^+ and patients with PNH^- were assessed by the log-rank test. A paired *t* test analyzed changes in the proportions of PNH-type cells associated with IST. All statistical analyses were performed using JMP version 5.0.1J software (SAS Institute, Cary, NC).

Results

Validity of high-resolution flow cytometry

Figure 1B shows that a low-resolution assay using PE-labeled anti-CD55 and FITC-labeled anti-CD59 mAbs detected greater than 0.1% PNH-type RBCs in the peripheral blood of a healthy individual, whereas our assay of the same sample detected 0% PNH-type cells. Thus, the low-resolution assay could not discriminate a patient with AA with 0.1% PNH-type cells from a healthy individual, whereas our method revealed 0.04% PNH-type RBCs in the same patient, indicating a diagnosis of PNH^+ AA. When the sensitivity of RBCs to complement-mediated lysis was examined using the modified Ham test, almost all RBCs in the glycophorin $A^+CD55^-CD59^-$ fraction disappeared after an incubation in acidified saline containing human AB serum, verifying the reliability of our method for detecting PNH-type RBCs (Figure 1C). Conversely, when granulocytes from a patient with PNH^+ AA were treated with aerolysin, approximately 99% of granulocytes in the $CD11b^+CD55^+CD59^+$ fraction disappeared, whereas almost all cells in the $CD11b^+CD55^-CD59^-$ fraction remained unchanged (Figure 1D), indicating that the few granulocytes in the $CD11b^+CD55^-CD59^-$ fraction had the properties of PNH-type cells.

Proportions of PNH-type cells in patients with AA

The proportion of PNH-type cells was increased in 83 (68%) patients. Among these patients with PNH^+, the number of PNH-type cells was increased in both the granulocytes and RBCs of 69 (83%) of them, in only the granulocytes of 12 (15%), and in only the RBCs of 2 (2%). Figure 2A shows the proportions of PNH-type granulocytes and histograms from 2 patients with PNH^+. Notably, the proportions of PNH-type granulocytes were below 0.1% in greater than 40% of patients with PNH^+. Table 1 compares the clinical characteristics between patients with PNH^+ and PNH^-. Although the PNH^+ group tended to be older and have higher WBC and MCV values than the PNH^- group, the clinical and hematologic parameters did not significantly differ between them.

Response to ATG and CsA therapy

Sixty-eight of 83 (91%) patients with PNH^+ improved with IST and achieved PR or CR at 12 months. However, only 18 of 39 (48%) patients with PNH^- responded to IST. Kaplan-Meier analysis showed that the chance of achieving PR was significantly better among patients with PNH^+ than among patients with PNH^- (Figure 3A). The rate of obtaining CR at 5 years was also significantly higher in patients with PNH^+ (36%) than in patients with PNH^- (3%) (Figure 3B). Multivariate analysis showed that among sex (male or female), age (older or younger than 40 years), severity (severe or moderate), presence or absence of chromosomal abnormalities, and presence or absence of increased PNH-type cells, only the presence of increased PNH-type granulocytes was a significant factor associated with good response to IST ($P < .001$). When patients with PNH^+ were classified into 5 subgroups according to the proportions of PNH-type granulocytes (0.003%-0.01% in 7, 0.01%-0.1% in 21, 0.1%-1.0% in 22, 1.0%-10.0% in 13, 10.0%-23.1% in 3), the response rates to IST at 6 months did not significantly differ (88%, 74%, 90%, 81%, and 100%, respectively) among these subgroups. The responses of all of these subpopulations were significantly better than that of patients with PNH^-.

Prognosis after IST

The median follow-up period was 26.4 months (range, 0.1 to 71.4 months). In contrast to the response rates, the rates of overall survival at 5 years were comparable between patients with PNH^+ (77%) and with PNH^- (71%) (Figure 4A). However, the probability of surviving failure free at 5 years was significantly higher in patients with PNH^+ (64%) than in patients with PNH^- (12%) when

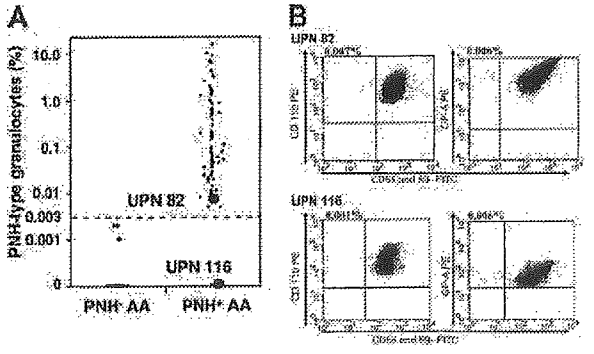

Figure 2. Proportions of PNH-type granulocytes. (A) Proportions of $CD55^-CD59^-$ granulocytes in each patient. (B) Histograms from one patient with PNH^- (UPN 82) with minimal PNH-type cells and from another patient with increased PNH-type cells only in RBCs (UPN 116).

Table 1. Clinical characteristics of PNH⁺ and PNH⁻ patients

	PNH⁺	PNH⁻	*P*
No. of patients	83	39	NA
Median age, y (range)	57 (13-83)	54 (12-83)	.16
Sex, M/F	36/47	19/20	.58
Severity, severe/moderate	53/30	22/17	.43
Chromosome abnormality, no. of patients	7	3	.88
−7	0	1	
+8	2	1	
Y	3	0	
Others	2	1	
Median WBC count, × 10^9 (range)	2.1 (0.5-4.3)	1.9 (0.7-3.2)	.15
Median neutrophil count, × 10^9/L (range)	0.53 (0.02-2.2)	0.49 (0.01-2.7)	.65
Median hemoglobin level, g/L (range)	67 (32-140)	67 (40-108)	.92
Mean corpuscular volume, fL (range)	101.5 (84.2-123.5)	98.5 (77.2-116.0)	.13
Median platelet count, × 10^9/L (range)	14.0 (2.0-50.0)	16.0 (1.0-87.0)	.65
Median reticulocyte count, × 10^9/L (range)	19.0 (3.0-90.0)	24.0 (2.0-106.0)	.50
Median time from diagnosis to IST, d (range)	30 (1-334)	33 (2-268)	.46
No. of patients who received G-CSF during IST	25	12	.94

NA indicates not applicable.

failure-free survival was calculated based on time to treatment failure. This was defined as whichever came first among time from the first day of treatment until salvage treatment for nonresponse, relapse, development of a clonal hematologic disease (PNH, MDS, leukemia), solid tumor, or disease- or treatment-related death (Figure 4B). Although the probability of evolution into florid PNH or MDS at 5 years after IST did not significantly differ between patients with PNH⁺ (6% and 3%) and patients with PNH⁻ (0% and 4%) (Figure 4C), the probability of relapse tended to be higher in patients with PNH⁻ (36%) than in patients with PNH⁺ (21%) (Figure 4D). Two (2%) patients with PNH⁺ and 7 (18%) with PNH⁻ underwent allogeneic bone marrow transplantation (BMT) from related (n = 6) or unrelated (n = 3) donors because of failure to respond to IST (n = 6) and relapse of AA (n = 3). Rates of survival after BMT did not significantly differ between the 2 groups (data not shown).

Changes in PNH-type granulocytes after IST

The presence of PNH-type cells after IST was serially tested in the peripheral blood of 53 of 122 patients. To characterize immune attack against hematopoietic stem cells that favors PNH-type cell clonal expansion, we examined the numbers of PNH-type cells in responsive patients. Figure 5A shows that the proportions of PNH-type granulocytes remained almost constant in 32 of 33 patients with PNH⁺ who responded to IST and decreased from 0.045% to 0% in only 1 patient (UPN 25). This indicates that the absolute number of PNH-type as well as of normal-type granulocytes increased in most responsive patients after IST. We compared the ratio of the degree of the increase in the absolute count between PNH-type (a) and normal-type (b) granulocytes before IST. The PNH-type granulocyte-to-normal-type granulocyte ratio in 32 patients ranged from 0.07 to 38.1 with a median of 1.06 (Figure 5B). The proportions of PNH-type cells did not change in 4 patients with PNH⁺ who were refractory to IST (Figure 5A-B). Sixteen patients with PNH⁻ were also tested after 6 to 24 months of IST. Only one patient who had achieved PR became PNH⁺ at 24 months and then relapsed with AA at 29 months after IST.

The proportions of PNH-type granulocytes were repeatedly determined in 23 patients for more than 24 months after IST. Figure 5C shows that the proportions remained constant over a long period in most patients including one (UPN 106) who had 0.1% PNH-type granulocytes (Figure 5D). The proportion of PNH-type granulocytes significantly increased from 3.31% to 76.0% in only one patient during the 4-year observation period.

Discussion

An increase in the proportion of PNH-type cells in peripheral blood has been implicated in the immune pathophysiology of bone marrow failure.[16] Several studies including our previous investigation found a correlation between an increase in the proportion of PNH-type cells and a favorable response to IST among patients with MDS[11,12,15] and with AA.[16,26] However, the clinical application of these findings has been hampered. Small patient cohorts and the relatively low prevalence of an increased number of PNH-type cells in these studies have led to concerns about unreliability of the correlation. The present study based on a larger number of patients with recently diagnosed AA conclusively demonstrated that a minor population of PNH-type cells predicts a good response to IST as well as good prognosis for patients with AA after IST.

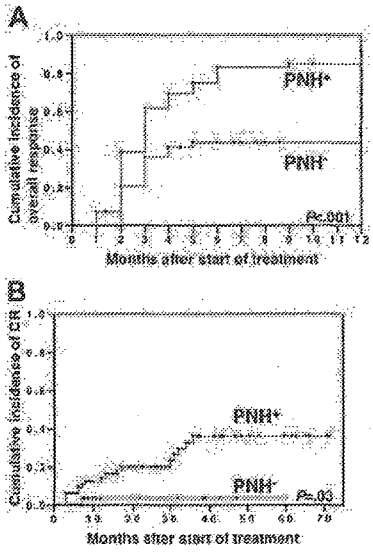

Figure 3. Response to immunosuppressive therapy. Incidence of overall (A) and complete (B) responses in patients with PNH⁺ and PNH⁻.

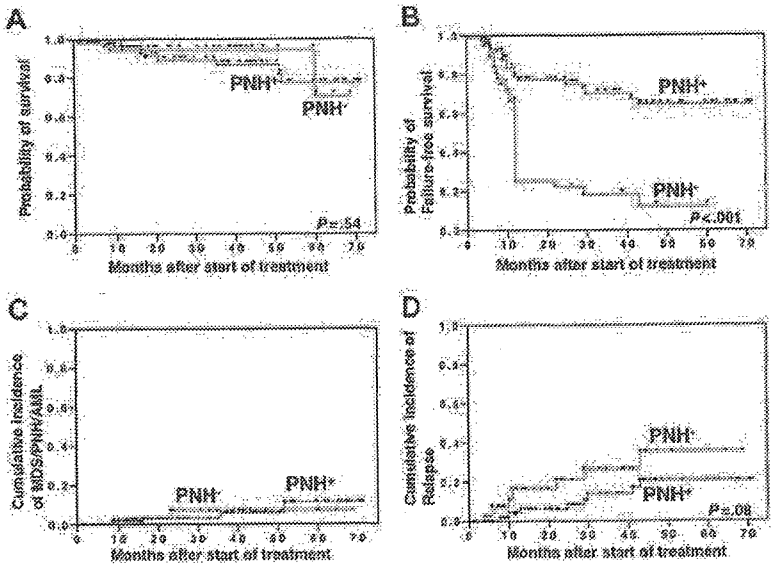

Figure 4. Prognosis after IST compared between patients with PNH$^+$ and with PNH$^-$. (A) Overall survival; (B) failure-free survival; (C) incidence of clonal hematologic disorders, including PNH, myelodysplastic syndrome, and acute myelogenous leukemia; and (D) incidence of relapse.

The reliability of our high-resolution flow cytometry, which was verified by the modified Ham test and by aerolysin treatment, revealed an increase in the number of PNH-type cells in 68% of the patients with AA. This was considerably higher than the reported prevalence.

The clinical features and overall survival rates did not significantly differ between patients with PNH$^+$ and patients with PNH$^-$ in the present study. However, failure-free survival was obviously better among patients with PNH$^+$ than patients with PNH$^-$. This indicated that, although patients with PNH$^-$ can survive as long as patients with PNH$^+$ after IST, they often require salvage or supportive treatment such as allogeneic stem cell transplantation and blood transfusions, because of a partial response to IST or a high rate of relapse. Contrary to the expectation based on the presence of abnormal hematopoietic clones such as PNH-type cells, the probability of evolving into clinical PNH or MDS in patients with PNH$^+$ was comparable to that in patients with PNH$^-$. The proportions of PNH-type granulocytes remained stable over a period of 1 to 66 months in most patients with PNH$^+$, a finding consistent with previous reports.[26,27] These findings indicate that

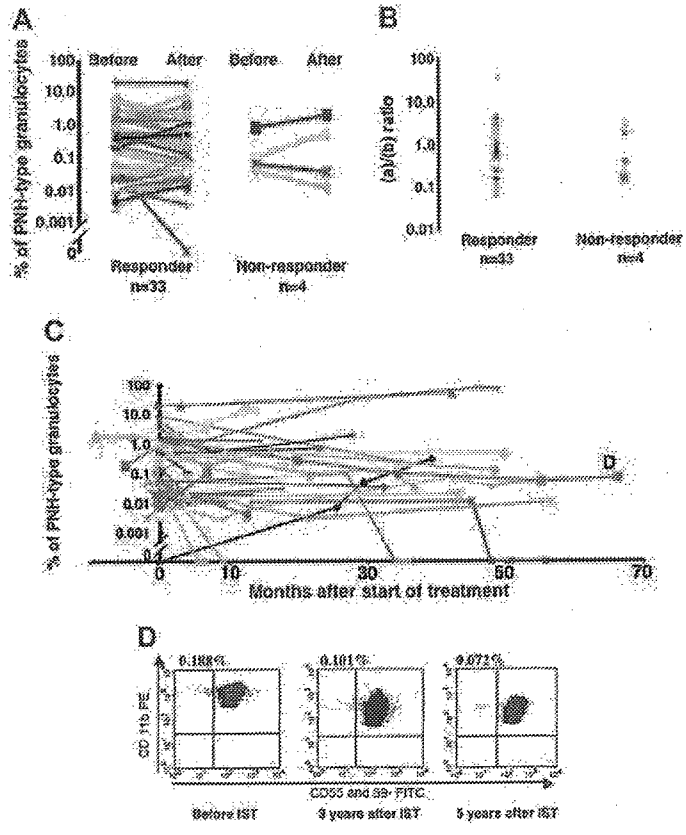

Figure 5. Changes in proportions of PNH-type granulocytes associated with responses to IST. (A) Change in responders and nonresponders. (B) Proportions of granulocyte counts after and before IST determined for PNH-type (a) and normal-type (b) granulocytes and ratios of PNH-type granulocytes (a) to normal-type granulocytes (b) were plotted. (C) Longitudinal analysis of PNH-type granulocytes. Proportions of PNH-type granulocytes of 37 patients with PNH$^+$ and 1 patient with PNH$^-$ who became PNH$^+$ (black line) were displayed. (D) Changes in proportions of PNH-type granulocytes over 5 years in patient UPN 106 with AA (shown as D in Figure 5C).

the presence of an increased proportion of PNH-type cells predicts not only a positive response but also a good quality of response to IST among patients with AA.

The significantly high response rate to IST among patients with PNH⁺ AA suggests that PNH⁺ AA is an authentic type of immune-mediated marrow failure. In line with this hypothesis, patients with PNH⁺ AA often have a specific HLA-DR allele (HLA-DR15) and antigen-driven T-cell proliferation in the bone marrow.[12,28] Furthermore, antibodies against diazepam-binding inhibitor-related sequence-1 (DRS-1), a peroxisomal protein abundantly expressed by hematopoietic progenitor cells, are frequently detected in sera from patients with PNH⁺ AA.[29] However, the relatively low response rate to IST among patients with PNH⁻ AA indicates that a heterogeneous pathophysiology might underlie this subset of AA. In line with this notion as described in our previous study,[16] clonal hematopoiesis arose more frequently in patients with PNH⁻ AA than in patients with PNH⁺ AA. Even among patients who responded to IST, patients with PNH⁻ AA rarely achieved complete recovery of hematopoiesis and were susceptible to AA relapse. Immune mechanisms that are not associated with an increase in the proportion of PNH-type cells might damage hematopoietic stem cells more profoundly than those in PNH⁺ AA.

PNH-type stem cells might acquire a survival advantage over normal-type stem cells when T or natural killer (NK) cells attack hematopoietic stem cells.[30-32] The high response rate to IST in patients with PNH⁺ AA indicates that such an immune mechanism is functional in this subset of AA. If the immune mechanisms were responsible for bone marrow failure, IST would more efficiently induce expansion of normal-type than of PNH-type stem cells. However, in most patients with PNH⁺, successful IST resulted in a similar increase in the number of both PNH-type and normal-type granulocytes, which contradicts the immune escape theory. A similar finding has been reported by Maciejewski et al[26] for patients with AA with 1% or more CD15⁺CD66b⁻CD16⁻ granulocytes. One possible explanation for this discrepancy is as follows. An immune attack against hematopoietic stem cells at the onset of AA that allows PNH-type stem cells to survive does not contribute to the subsequent progression of bone marrow failure, which is caused by different immune mechanisms targeting epitopes other than those that induce disease. Such epitope spreading occurs in the development of other immune diseases such as multiple sclerosis.[33] Alternatively, the suppression of hematopoiesis after the clonal expansion of PNH-type cells might be caused by myelosuppressive cytokines rather than antigen-specific T cells.

The presence of a few PNH-type cells has profound significance for the management of patients with recently diagnosed AA. Although those who have PNH⁻ AA can improve with IST, the maximal response rate is 50% and the rate of failure-free survival at 5 years is below 20%. Therefore, allogeneic BMT is recommended more often than IST for young patients with PNH⁻ who have HLA-compatible sibling donors. Conversely, IST is more frequently recommended than BMT for patients with PNH⁺, particularly when the likelihood of BMT-related mortality is high. Among patients with AA who are unresponsive to the initial ATG and CsA therapy, those who benefit from a second IST might be PNH⁺. Conventional flow cytometry capable of detecting 1% or more PNH-type cells would also be clinically useful in predicting response to IST because the response to IST does not change according to the proportion of PNH-type cells. The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in AA identified here warrants a further worldwide prospective study on non-Japanese patients with AA.

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