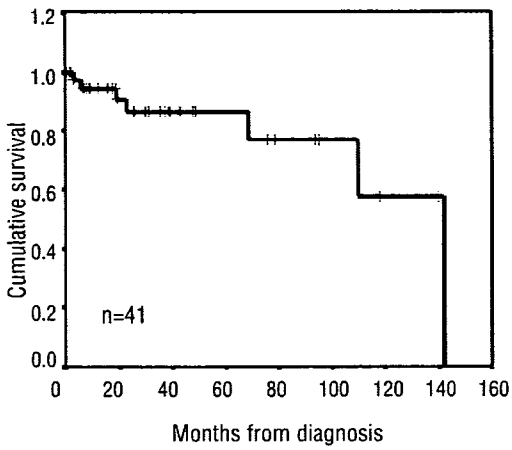

Figure 3. Overall survival of all patients with thymoma-associated PRCA (n=41).

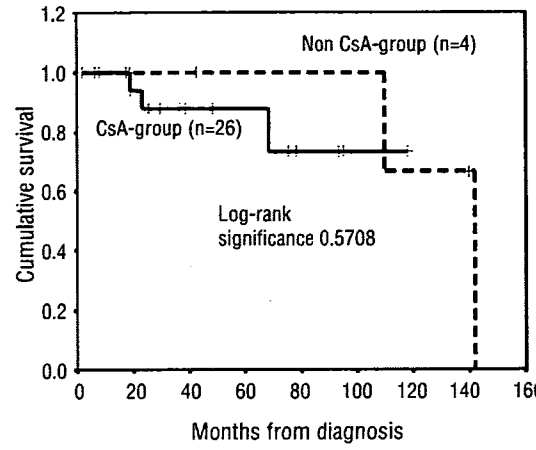

Figure 4. Comparison of the overall survival between the cyclosporine-group and the non-cyclosporine-group.

The patients were classified into a cyclosporine group and a non-cyclosporine group according to the agent used for maintenance therapy, regardless of the drugs that had successfully induced remission. Comparing the duration of response, there was a significant difference between the two groups (Figure 2; p=0.0065). However, 22 out of the 26 patients in the cyclosporine group were still on maintenance cyclosporine therapy (median dose of cyclosporine, 2.5 mg/kg; range, 0.5 to 5.0 mg/kg; median duration of maintenance, 18 months). Three patients who had received corticosteroids and additional cyclosporine were continuously given corticosteroids after cessation of the cyclosporine, and remained in remission (duration of cyclosporine maintenance; 7, 10 and 16 months). Only two patients (one treated with cyclosporine and one with cyclophosphamide) were alive in CR after stopping all immunosuppressive therapy (19 and 67 months).

The estimated median overall survival of all patients with thymoma-associated PRCA was 142 months (Figure 3). Overall survival did not differ substantially between the cyclosporine group and non-cyclosporine-group (Figure 4, p=0.5708). Seven patients died: the causes of death were infection in four patients and malignant thymoma in one patient. Two patients treated with cyclosporine and one with corticosteroids died of infection during hematologic remission. In two patients, the cause of death could not be identified.

Discussion

Cyclosporine produced excellent responses in patients with thymoma-associated PRCA, although most patients continued to receive cyclosporine for maintenance therapy. This suggests that most physicians consider it difficult to discontinue maintenance cyclosporine therapy. Sawada *et al.* reported that discontinuation of maintenance cyclosporine therapy was strongly correlated with relapse in patients with idiopathic PRCA.[11] As for idiopathic PRCA, remissions from thymoma-associated PRCA may also be cyclosporine-dependent. If this is true, other therapeutic modalities may be required to cure thymoma-associated PRCA.

Continuous immunosuppression is associated with an increased risk of infection and malignancy.[13,14] Among the patients with a good clinical response to immunosuppressive therapy, four patients died and in three of these, death was associated with infection during remission of anemia. The age of patients who died of infection ranged from 48 to 61 years, suggesting that infection may not necessarily be a complication of elderly patients only and that adequate prevention and treatment of infection are requisites for the successful management of patients with thymoma-associated PRCA. Although we have not yet had a report of malignancy secondary to immunosuppressive therapy in the present cohort of patients, continuous careful follow-up is required for patients receiving long-term cyclosporine therapy.

Limited information suggests that patients with thymoma-associated PRCA have a poor prognosis.[15-17] The median age of the patients in the present cohort was 66 years, and the estimated overall survival time was 12 years. Life expectancies of average 65-year-old Japanese males and females are 18 and 23 years, respectively (*http://www.mhlw.go.jp/english/index.html*), thus suggesting that the life expectancy of thymoma-associated PRCA patients is shorter than that of the

average Japanese population. It should be noted that the outcome of thymoma-associated PRCA can be affected by the histology of the thymoma.

Surgery is performed in thymoma-associated PRCA with the expectation of an improvement of anemia, and thymomectomy has been reported to result in occasional improvement of PRCA.[1] Surgical resection of the thymoma has been recommended as the initial treatment of thymoma-associated PRCA, with an expected hematologic response rate of 25-30%.[18] In our cohort, five patients received surgical care alone after the diagnosis of anemia; two patients did not show any improvement of anemia, and the clinical response to removal of the thymoma could not be evaluated in the other three patients. Thompson *et al.* recently reported that surgical resection of thymoma was insufficient for normalization of erythropoiesis in all 13 patients so treated, but immunosuppressive therapy was effective as an adjuvant treatment.[10] As described earlier, many patients developed PRCA at long intervals after removal of the thymoma, raising the question as to whether thymoma indeed plays a role in the pathogenesis of anemia. Masuda *et al.* reported the case of a patient with thymoma-associated PRCA and clonal T-cell expansions in both the thymoma and circulating blood,[19] whereas we have recently described a patient with a clonal T-cell expansions in the blood but not in the thymoma.[20] Thus, the role of thymoma in providing an environment for clonal expansions of pathogenic T cells may be different among individuals.

In conclusion, we have deermined, for the first time, the long-term response and outcome of patients with thymoma-associated PRCA receiving immunosuppressive therapy. Although cyclosporine produces excellent responses in thymoma-associated PRCA, it can lead to infectious complications and careful follow-up is recommended. It remains unknown whether cyclosporine can induce a maintenance-free hematologic response. Although adequate prevention of infection will be essential, the efficacy of newly developed agents such as campath-1 may be evaluated in patients refractory to cyclosporine treatment.[21]

Authorship and Disclosures

MH performed the research, collected and analyzed the data, and wrote the paper; K-iS designed the study, analyzed the data and revised the manuscript; NF collected and analyzed the data. SN, AU, KD, SF, YY, FK, MO and KO collected and analyzed the data, and revised the manuscript. All authors are responsible for the scientific content of this manuscript and approved the manuscript to be published. The authors reported no potential conflict of interest.

Appendix

The following institutions participated in the Collaborative Study Group: Aichi Medical School, Akita University, Asahikawa Medical School, Chiba University, Dokkyo Medical School, Ehime University, Fujita Health University, Fukui University, Fukui National Hospital, Fukuoka University, Fukushima Medical University, Gifu University, Gunma University, Hamamatsu Medical School, Hirosaki University, Hiroshima University, Hokkaido University, Hyogo Medical University, Iwate Medical School, Jichi Medical School, Jikei University, Juntendo University, Kagawa Childrens' Hospital, Kagawa University, Kagoshima University, Kanazawa University, Kanazawa Medical School, Kansai Medical University, Kawasaki Medical School, Keio University, Kinki University, Kitazato University, Kobe University, Kochi University, Kumamoto University, Kurume University, Kyoto Prefectural University, Kyoto University, Kumamoto Medical Center, Kyushu University, Mie University, Nagasaki University, Nagoya City University, Nagoya Medical Center, Nagoya University, Nara Medical University, National Cancer Center, National Institute of Infectious Diseases, Niigata University, Nishi Sapporo National Hospital, Nippon Medical School, Nippon University, NTT Kanto Medical Center, Oita University, Okayama Medical Center, Okayama University, Osaka City University, Osaka Medical School, Osaka National Hospital, Osaka University, Ryukyu University, Saga University, Saitama Medical School, Sapporo Medical School, Sendai Medical Center, Shimane University, Shinsyu University, Showa University, St. Marianna University, Teikyo University, Toho University, Tohoku University, Tokai University, Tokushima University, Tottori University, Tokyo Medical Center, Tokyo Medical School, Tokyo Medical and Dental University, Tokyo University, Tokyo Women's Medical School, Tsukuba University, University of Occupational and Environmental Health, Wakayama Medical University, Waseda University, Yamagata University, Yamaguchi University, Yamanashi University, Yokohama City University.

References

1. Dessypris EN. Pure red cell aplasia. Johns Hopkins Univ Press, 1988; Baltimore.
2. Dessypris EN, Lipton JM. Red cell aplasia. In: Greer JP, Foerster J, Lukens JN, et al (eds): Wintrobe's Clinical Hematology, 11th ed, Lippincott Williams & Wilkins, 2004;1421-7, Philadelphia.
3. Raghavachar A. Pure red cell aplasia: Review of treatment and proposal for a treatment strategy. Blut 1990; 61:47-51.
4. Marmont AM. Therapy of pure red cell aplasia. Semin Hematol 1991;28: 285-97.
5. Charles RJ, Sabo KM, Kidd PG, Abkowitz JL. The pathophysiology of pure red cell aplasia: implications for therapy. Blood 1996;87:4831-8.
6. Lacy MQ, Kurtin PJ, Tefferi A. Pure red cell aplasia: association with large granular lymphocyte leukemia and the prognostic value of cytogenetic abnormalities. Blood 1996;87: 3000-6.
7. Maeda T, Shiokawa S, Yoshikawa Y, Hiroyama T, Nakajima Y, Muta H, et al. Successful treatment of pure red cell aplasia with cyclosporin A and erythropoietin after thymectomy in a 88-year old woman. Haematologica 2004;89:ECR17.
8. Marmont A, Peschele C, Sanguineti M, Condorelli M. Pure red cell aplasia (PRCA): response of three patients of cyclophosphamide and/or antilymphocyte globulin (ALG) and demonstration of two types of serum IgG inhibitors to erythropoiesis. Blood 1975;45:247-61.
9. Garcia Vela JA, Monteserin MC, Oña F, Barea LM, Lastra A, Pérez V. Cyclosporine A used as a single drug in the treatment of pure red cell aplasia associated with thymoma. Am J Hematol 1993;42:238-9.
10. Thompson CA, Steensma DP. Pure red cell aplasia associated with thymoma: clinical insights from a 50-year single-institution experience. Br J Haematol 2006;135:405-7.
11. Sawada K, Hirokawa M, Fujishima N, Teramura M, Bessho M, Dan K, et al. Long-term relapse-free survival and overall survival of patients with acquired primary idiopathic PRCA receiving cyclosporine A. A nationwide cohort study in Japan for the PRCA Collaborative Study Group. Haematologica 2007; 92:1021-8.
12. Rosai J, Sobin LH. Histological typing of tumors of the thymus. WHO international histological classification of the tumors. 2nd ed. Berlin: Springer-Verlag, 1999.
13. Opelz G, Dohler B. Lymphomas after solid organ transplantation: a collaborative transplant study report. Am J Transplant 2004;4:222-30.
14. Bustami RT, Ojo AO, Wolfe RA, Merion RM, Bennett WM, McDiarmid SV, et al. Immunosuppression and the risk of post-transplant malignancy among cadaveric first kidney transplant recipients. Am J Transplant 2004;4: 87-93.
15. Souadjian JV, Enriquez P, Silverstein MN, Pépin JM. The spectrum of diseases associated with thymoma: coincidence or syndrome? Ann Intern Med 1974;134:374-9.
16. DeSevilla E, Forrest JV, Zivnuska FR, Sagel SS. Metastatic thymoma with myasthenia gravis and pure red cell aplasia. Cancer 1975;36:1154-7.
17. Masaoka A, Hashimoto T, Shibata K, Yamakawa Y, Nakamae K, Iizuka M. Thymomas associated with pure red cell aplasia: histologic and follow-up studies. Cancer 1989;64: 1872-8.
18. Zeok JV, Todd EP, Dillon M, DeSimone P, Utley JR. The role of thymectomy in red cell aplasia. Ann Thorac Surg 1979;28:257-60.
19. Masuda M, Arai Y, Okamura T, Mizoguchi H. Pure red cell aplasia with thymoma: evidence of T-cell clonal disorder. Am J Hematol 1997;54:324-8.
20. Fujishima N, Hirokawa M, Fujishima M, Wada C, Toyoshima I, Watanabe S, Sawada K. Oligoclonal T cell expansion in blood but not in the thymus from a patient with thymoma-associated pure red cell aplasia. Haematologica 2006; 91: ECR 47.
21. Willis F, Marsh JC, Bevan DH, Killick SB, Lucas G, Griffiths R. The effect of treatment with Campath-1H in patients with autoimmune cytopenias. Br J Haematol 2001;114:891-8.

Long-term outcome of patients with acquired primary idiopathic pure red cell aplasia receiving cyclosporine A. A nationwide cohort study in Japan for the PRCA Collaborative Study Group

Ken-ichi Sawada, Makoto Hirokawa, Naohito Fujishima, Masanao Teramura, Masami Bessho, Kazuo Dan, Hisashi Tsurumi, Shinji Nakao, Akio Urabe, Mitsuhiro Omine, Keiya Ozawa for the PRCA Collaborative Study Group

From the Division of Haematology, Dept. of Medicine III, Akita University School of Medicine, Akita, Akita 010-8543, Japan (K-iS, MH, NF); Dept. of Haematology, Tokyo Women's Medical University, Tokyo 162-8666, Japan (MT); Haematology Division, Dept. of Internal Medicine, Saitama Medical University, Saitama 350-0495, Japan (MB); Dept. of Haematology, Nippon Medical School, Tokyo 113-8602, Japan (KD); First Dept. of Internal Medicine, Gifu University School of Medicine, Gifu 501-1194, Japan (HT); Dept. of Cellular Transplantation Biology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8641, Japan (SN); Division of Haematology, NTT Kanto Medical Center, Tokyo 141-0022, Japan (AU); Division of Haematology, Internal Medicine, Showa University Fujigaoka Hospital, Yokohama 227-8501, Japan (MO); Division of Haematology, Department of Medicine, Jichi Medical School, Tochigi 329-0498, Japan (KO).

Funding: supported in part by a research grant from the Idiopathic Disorders of Haematopoietic Organs Research Committee of the Ministry of Health and Welfare of Japan.

Manuscript received December 28, 2006.
Manuscript accepted April 23, 2007.

Correspondence:
Ken-ichi Sawada, M.D., Division of Haematology and Oncology, Department of Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan.
E-mail: ksawada@doc.med.akita-u.ac.jp

ABSTRACT

Background and Objectives

Cyclosporine A (CsA) has become one of the leading agents for the treatment of pure red cell aplasia (PRCA). However, further studies are necessary to determine the relapse-free survival (RFS) and overall survival (OS) of patients treated with this drug, the minimum duration of therapy for induction of remission, and whether or not there is need for maintenance treatment.

Design and Methods

We conducted a nationwide survey in Japan. From a total of 185 patients (with 73 primary idiopathic PRCA and 112 with secondary PRCA), we evaluated 62 patients with primary idiopathic PRCA for this report.

Results

The remission induction therapy for these patients included CsA (n=31), corticosteroids (CS) (n=20) or other drugs (n=11). CsA and CS produced remissions in 23 (74%) and 12 (60%) patients, respectively. The salvage treatment produced remissions in 58 patients (94%). Forty-one and 15 patients were maintained on CsA±CS (CsA-containing group) or CS alone (CS group), respectively. The median RFS in the CsA-containing group was 103 months, longer than that seen in the CS group (33 months) ($p<0.01$). Of 14 patients whose CsA was discontinued, 12 patients (86%) relapsed after a median of 3 months (range 1.5 to 40 months), while only 3 of 27 patients (11%) relapsed during CsA-containing maintenance therapy. Thus, the discontinuance of maintenance therapy was strongly correlated with relapse ($p<0.001$). Four patients in the CsA-containing group died; however, the OS of this group was not significantly different from that of the CS-groups ($p=0.104$).

Interpretation and Conclusions

CsA-containing regimens sustain prolonged RFS more effectively than CS in primary idiopathic PRCA and seem to be important to prevent relapse.

Key words: pure red cell aplasia, cyclosporine A, relapse-free survival, maintenance therapy.

Haematologica 2007; 92:1021-1028

Pure red cell aplasia (PRCA) is characterized by severe normochromic, normocytic anemia associated with reticulocytopenia and absence of erythroblasts from an otherwise normal bone marrow.[1-4] The acquired form of chronic PRCA may present as a primary hematologic disorder in the absence of any other disease, or secondary to neoplasms, infections, collagen vascular diseases, chronic hemolytic anemias, or after exposure to a variety of drugs and chemicals. Primary or secondary PRCA not responding to treatment of the underlying diseases is treated as an immunologically-mediated disorder.[1-4] Remissions have been achieved by treatment with corticosteroids (CS), cyclophosphamide, cyclosporine A (CsA), anti-thymocyte globulin (ATG), splenectomy, and plasmapheresis.[1-7] More recently, the anti-CD20 monoclonal antibody rituximab[8,9] and the anti-CD52 monoclonal antibody alemtuzumab (campath-1H)[10] have been reported to induce the remission of therapy-resistant PRCA. In general, remission induction can be easily achieved in the majority of patients. However, in the era before CsA became available, Clark *et al.* clearly showed that 80% of patients relapsed during the 24 months after having achieved remission.[11] Up to the present, the efficacy of CS, cyclophosphamide and CsA for patients with primary or secondary PRCA has been reported to be between 30-56%, 7-20% and 75-87%, respectively.[1-7,11] CsA has become established as one of the leading agents for the treatment of PRCA since the first, successfully treated cases in 1984.[12] However, it is unclear how many patients treated with CsA achieve a sustained remission and how many relapse. Up to the present, very few studies on the long-term follow-up of patients treated with CsA have been reported. Moreover, comparing one therapeutic approach to another for the treatment of PRCA is almost impossible since this disease is so rare that controlled studies are practically impossible to perform. We, therefore, conducted a nationwide survey of PRCA cases in Japan to elucidate the current status of immunosuppressive therapy for PRCA.

Design and Methods

Patients

The first questionnaires were sent to hematology departments in Japan to estimate the number of patients, aged 15 years and over, with newly diagnosed acquired chronic PRCA, excluding those with human parvovirus B19 infection. Secondary questionnaires were sent to collect data on underlying diseases, laboratory findings (including peripheral blood cell count with reticulocyte count and leukocyte differentials), findings of bone marrow examinations, immunological and cytogenetic parameters and the efficacy and the side effects of immunosuppressive therapy. Secondary questionnaires did not collect information on the trough concentration of CsA. The recommended dose of CsA in Japan is 6 mg/kg, to provide a trough concentration ranging from 150 to 250 ng/mL. The first period of the survey was between January 1990 and December 2004 across 47 institutions and the second period was between January 1990 and March 2006 across 109 institutions, including a follow-up survey of the patients identified in the first period. All combined, a total of 273 patients were enrolled from 45 institutions in response to the first questionnaires. A total of 185 patients were enrolled in response to the second questionnaires.

Classification of PRCA

There are several proposed classifications of PRCA. One is based on pathophysiology[5] and another, on underlying diseases.[2] The prognosis of patients with PRCA, which is one of the most important end-points of this study, depends on the nature of their underlying diseases.[11] We, therefore, classified our patients with PRCA based on their underlying diseases, according to the classification proposed by Dessypris and Lipton[2] with some modifications. In this classification, primary PRCA comprises preleukemic, autoimmune and idiopathic forms. The patients with definite cytogenetic abnormalities were classified as having secondary PRCA as either myelodysplastic syndrome (MDS) or preleukemia. Primary autoimmune PRCA is defined as the cases in which an immune pathogenic mechanism can be established by *in vitro* assay. Secondary questionnaires did not collect information on *in vitro* assays, therefore, cases of idiopathic PRCA in this study may include primary autoimmune PRCA.

Data analysis

The secondary questionnaires collected data on the reticulocyte count and a bone marrow examination at onset of aplasia but not at recovery. Remission was defined as no need for any further transfusions, whereas relapse was defined as the need to receive transfusions. The period to achieve maximum response varied from patient to patient; therefore, the date of remission was defined as that of the last transfusion after the initiation of remission induction therapy. Complete remission (CR), partial remission (PR) and no response (NR) were defined as the achievement of normal hemoglobin levels without transfusion, the presence of anemia without transfusion dependence, and the continued need for transfusions, respectively. It is difficult to determine the efficacy of each agent precisely when the patients are either concomitantly or sequentially treated with several agents. Moreover, the first agent(s) given may contribute to the efficacy of the agent(s) given subsequently. Therefore, in this study, the efficacy of the agent(s) reported in secondary questionnaires was re-evaluated according to the following criteria. In a simultaneous combination, the efficacies of all of the agents were determined as the same. In sequential administration and in a later on combination, the efficacy of each agent was determined depending on the response obtained during the period of administration, except for ATG and methylprednisolone. ATG and methylprednisolone usual-

ly do not produce immediate remission; therefore, the efficacies of these agents were evaluated together with the agent(s) used concomitantly and/or sequentially. The minimum period required for an evaluation of the response of an agent was defined as 2 weeks; therefore, an agent combined later on, within 2 weeks, was, for the purposes of the analysis, considered a simultaneous combination with the preceding agent(s).

Regarding maintenance treatment, the patients were classified according to the agent used for maintenance therapy as receiving CsA±CS (CsA-containing group) or CS alone (CS group) regardless of the agent(s) used for successful remission induction. The agents for remission induction and salvage therapy were defined as those used initially and those used either sequentially or in a later on combination, respectively. The agent for maintenance therapy was defined as that used or tailed off after successful remission induction. The RFS was estimated as transfusion-free survival. The overall survival and RFS were estimated by the Kaplan-Meier method and statistical differences were calculated by the log-rank test and χ^2 test.

Results

Classification of PRCA

According to the criteria of Dessypris and Lipton,[2] of the total of 185 collected patients with PRCA, 73 (39%) were classified as having primary idiopathic PRCA and 112 (61%) as having secondary PRCA (Table 1). From the 73 patients with primary idiopathic PRCA, 11 patients were excluded from further analysis because of insufficient data (nine patients) or too short an observation period after initiation of immunosuppressive therapy (two patients; 1 and 8 days of observation). Finally, 62 patients with primary PRCA were eligible for further analysis. The patients' age at the onset of anemia ranged from 18 to 89 years (55±18, mean ± standard deviation, SD) with a 23:39 (1:1.7) male to female ratio (Figure 1). The year at onset of PRCA was 1998±5 (mean±SD), ranging from 1990 to 2005.

Rate of response to the remission induction therapy

The remission induction therapy for these patients included CsA (n=31), CS (n=20), cyclophosphamide (n=3), anabolic steroids (n=1), or a simultaneous combination of CsA and anabolic steroids or CS (n=7) (Figure 1 and Table 2). CsA, as a remission induction therapy, produced CR or PR in 23/31 patients (74%). The initial dose of CsA for the responding patients was 4.8±1.2 mg/kg (mean±SD, n=23) with a range of 2.9 to 7.6 mg/kg body weight, which was higher than that for non-responding patients (3.9±1.3 mg/kg with a range of 2.1 to 5.6 mg/kg, n=8), although the difference was not statistically significant. When the patients who were treated with CsA alone were evaluated (n=23), the time for transfusion-independence from the start of therapy was 82±200 days (range, 0 to 910 days). Fifteen patients (65%) achieved transfusion-independence within 2 weeks, 17 patients (74%) within 1 month, 18 patients (78%) within 3 months and 20 patients (87%) within 6 months. CS, as a remission induction therapy, produced a CR or PR in 12/20 patients (60%). The initial dose of prednisolone in patients who responded to CS was 0.8±0.2 mg/kg (mean±SD, n=12) with a range of 0.5 to 1.0 mg/kg. There was no significant difference in the dose between the responders and non-responders. When the patients who were treated by CS alone were evaluated (n=9), the time for transfusion-independence from the start of therapy was 65±101 days (range, 0 to 311 days). Three patients (33%) achieved transfusion-independence within

Table 1. Classification of 185 patients with aquired pure red cell aplasia.

Causes of pure red cell aplasia	*Patients* *Number*	*Percent*
Primary		
Idiopathic	73	39.5%
Secondary, associated with		
Thymoma	42	22.7%
Hematologic malignancies		
Chronic lymphocytic leukemia		
B-cell type	1	0.5%
Large granular lymphocyte leukemia	14	7.6%
Macroglobulinemia	3	1.6%
Malignant lymphoma	8	4.3%
Myelodysplastic syndrome	11	5.9%
Acute myeloblastic leukemia	1	0.5%
Preleukemic	1	0.5%
Solid tumors	5	2.7%
Autoimmune, collagen vascular diseases		
Rheumatoid arthritis	7	3.8%
Systemic lupus erythematosus	1	0.5%
Systemic sclerosis	1	0.5%
Sjögren's syndrome	2	1.1%
Polymyalgia rheumatica	1	0.5%
Autoimmune hemolytic anemia	1	0.5%
Evans' syndrome	1	0.5%
Type 1 diabetes mellitus	1	0.5%
Myasthenia gravis	1	0.5%
Chronic thyroiditis	1	0.5%
Autoimmune hepatitis	2	1.1%
Drugs	2	1.1%
Chronic renal failure	5	5.7%

Table 2. Response to remission induction therapy.

Initial agent(s)	*No. of patients*	*Response, No. (%)* *CR*	*PR*	*CR+PR*	*NR*
CsA	31	10 (32%)	13 (42%)	23 (74%)	8 (26%)
CS	20	4 (20%)	8 (40%)	12 (60%)	8 (40%)
CY	3	0	0	0	3 (100%)
AS	1	0	0	0	1 (100%)
CsA+CS	4	0	4 (100%)	4 (100%)	0
CsA+AS	1	0	1 (100%)	1 (100%)	0
CS+AS	2	1 (50%)	1 (50%)	2 (100%)	0
Total	62	15 (24%)	27 (44%)	42 (68%)	20 (32%)

CsA: cyclosporine A; CS: corticosteroid including methyl-prednisolone and prednisolone; CY: cyclophosphamide; AS: anabolic steroid; CR: complete remission; PR: partial remission; NR: no response.

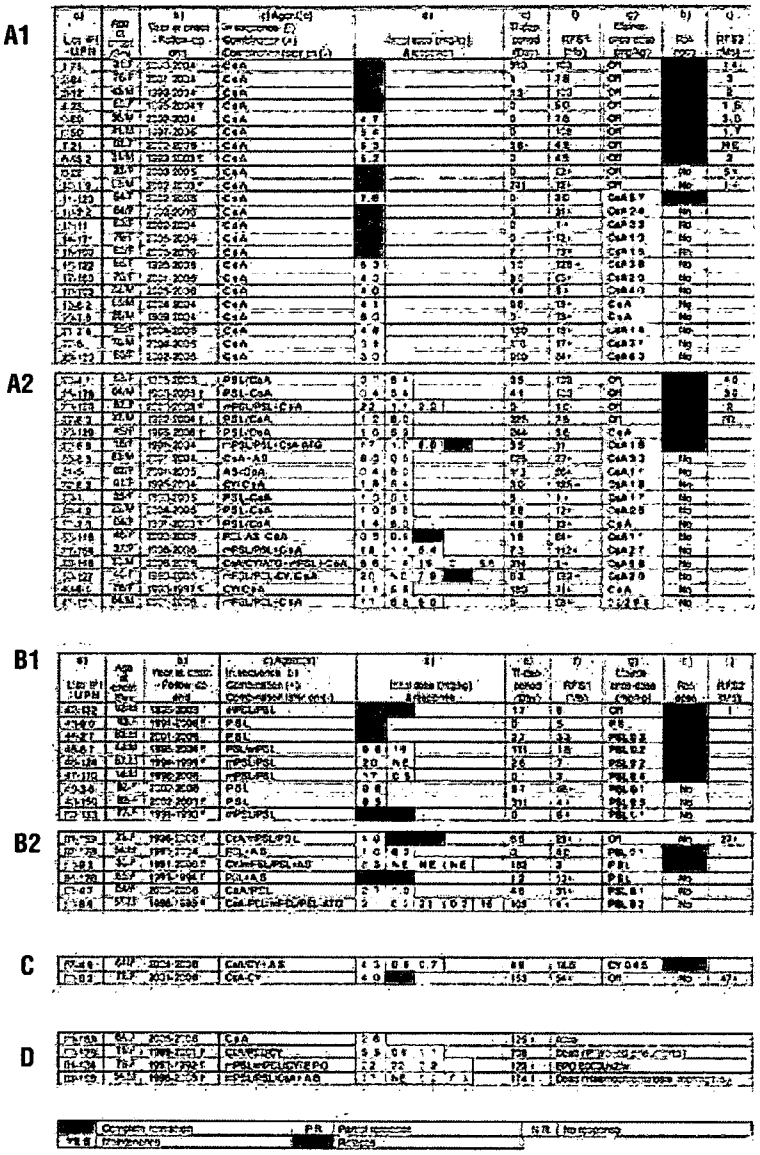

Figure 1. Immunosuppressive therapy in patients with primary idiopathic PRCA. (A) Cyclosporine A (CsA)-containing group: (A1) CsA alone, (A2) CsA in combination with other agents. (B) corticosteroid (CS) group: (B1) CS alone, (B2) CS in combination with other agents. (C) cyclophosphamide (CY) group. (D) Transfusion-dependent patients (non-responders). Abbreviations in each column; a) List #1; list number in Figure 1 and UPN (unspecified patient's number), b)¶Year at end of follow-up; †Death, c) agents are listed in order, (/); in sequential administration, (+); in simultaneous combination, (-); in combination later on, CsA; cyclosporine A, PSL; prednisolone, mPSL; methylprednisolone pulse therapy; ATG; antithymocyte globulin, CY; cyclophosphamide, AS; anabolic steroid, d) The initial dose and response to the agent; the order of agents corresponds to that shown in column c) and doses indicated are in mg/kg body weight/day, the color of each box shows response as indicated in the figure, e) Transfusion-dependent period (days) after the initiation of remission induction therapy, NE; not evaluable, f) RFS1; relapse-free survival (months) estimated as transfusion-free survival is shown as the period before the discontinuation of maintenance therapy, g) Off; tapered off, ‡doses of prednisolone/CsA in order, h) Relapse was defined as reappearance of transfusion requirement, i) RFS2; RFS after the discontinuation of maintenance therapy. EPO; erythropoietin.

2 weeks, six patients (67%) within 1 month and eight patients (89%) within 6 months. A simultaneous combination of CS and CsA produced remission in 4/4 patients. Cyclophosphamide was tried in three patients at a dose of 0.3 to 1.8 mg/kg, but no obvious responses were observed.

Salvage therapy

Twenty patients failed to respond to remission induction therapy. The effective salvage therapies for these patients are summarized in Figure 2 in which the period of administration of the initial agent(s) is also shown. The remission induction agent was rapidly discontinued in several patients. Among eight patients who failed to respond to initial CsA, six patients responded to CS (Figure 1-B2, 51-159, 55-47 and 56-69), cyclophosphamide (Figure 1C, 57-49 and 58-62), or a simultaneous combination of ATG+methylprednisolone+CsA (Figure 1-A2, 38-146). Of the remaining two CsA non-responders, one patient (Figure 1D, 59-163) was treated with CsA at a dose of 2.8 mg/kg/day but was still transfusion-dependent after 125 days, and the other did not respond to salvage therapies with a sequential administration of prednisolone and cyclophosphamide and eventually died due to *Pneumocystis jiroveci* pneumonia (Figure 1D, 60-138). Eight patients did not respond to CS; five patients responded to CsA (Figure 1-A2, 24-41, 25-179, 27-80, 28-129 and 35-20) and one other responded to cyclophosphamide (Figure 1-A2, 39-127). Of the remaining two CS non-responders, one patient was lost to the follow-up during the administration of erythropoietin (Figure 1D, 61-134), and the other did not respond to salvage therapies with a combination of CsA and anabolic steroids, received 240 units of red blood cell transfusion, and eventually died due to bacterial meningitis (Figure 1D, 62-158). There were three patients who did not respond to cyclophosphamide; two patients responded to CsA (Figure 1-A2, 32-63 and 40-81) and the other

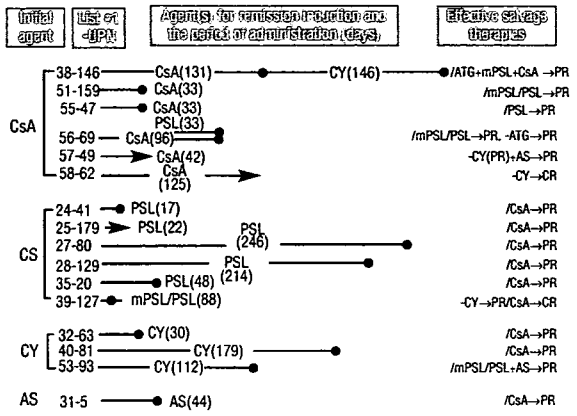

Figure 2. Effective salvage therapies for patients who failed to respond to the remission induction therapy. The initial agent(s) that failed to produce remission was discontinued (-O) or continued (→). Agents for salvage therapy were started in combination later on with the initial agent (-), simultaneously (+) or sequentially (/). The abbreviations are the same as those in the legend to Figure 1.

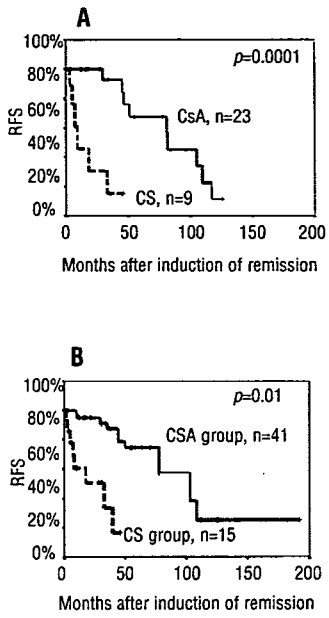

Figure 3. Relapse-free survival (RFS) of patients with primary idiopathic PRCA. RFS after induction of first remission was estimated as transfusion-free survival. A. The RFS of the patients treated with cyclosporine A (CsA) alone (solid line) (Patients listed in Figure 1-A1, n=23) is compared to that of the patients treated with corticosteroids (CS) alone (broken line) (Patients listed in Figure 1-B1, n=9). B. The RFS in the CsA-containing group (solid line) (patients listed in Figure 1-A1+A2, n=41) is compared to that of the CS group (broken line) (patients listed in Figure 1-B1+B2, n=15). There was a statistically significant difference between the duration of remission in the two groups based on the generalized Wilcoxon's test ($p<0.0001$ for A and $p<0.01$ for B).

responded to a combination of CS and anabolic steroids (Figure 1-B2, 53-93). A patient who was refractory to anabolic steroids responded to CsA (Figure 1-A2, 31-5). Finally, 58/62 patients (94%) with primary idiopathic PRCA responded to immunosuppressive therapy.

Relapse-free survival

Figure 3A illustrates the duration of RFS of the patients treated with CsA alone or CS alone (Figure 3A) after the first remission was induced. Among the 23 patients in CsA alone group, the estimated median RFS was 82 months, with a median observation period of 34 months (range, 1 to 126 months). On the other hand, among the nine patients in the group treated with CS alone, the estimated median RFS was 9 months, with a median observation period of 7 months (range, 3 to 46 months). The duration of initial remission was, therefore, longer after CsA than after CS, and the differences was statistically significant ($p<0.0001$).

Exposure to other agents might affect the efficacy of CsA to sustain remission. Figure 3B illustrates the duration of RFS among patients in the CsA-containing group (Figure 1, A1 plus A2) and the CS-group (Figure 1, B1 plus B2) after the first remission had been induced. Among the 41 patients in the CsA-containing group, the estimated median RFS was 103 months, with a median observation period of 45 months (range, 1 to 196 months). On the other hand, among the 15 patients in the CS group, the estimated median RFS was 33 months, with a median observation period of 9 months (range, 1 to 55 months). The group of patients who achieved remission with a CsA-containing regimen had a longer duration of initial remission in comparison to the CS group, with the difference being statistically significant ($p<0.01$). There was no difference in the age at onset between the CsA-containing group and CS group, which was 54±18 years old (mean±SD), with a range from 18 to 82 and 56±20 years old with a range from 18 to 89, respectively. It was difficult to derive any conclusions on RFS in the cyclophosphamide group because there were only two patients in this group.

Factors related to first relapse

Twenty-four out of 58 patients (41%) have had at least one relapse (Figure 1). Fifteen out of these 24 relapsed patients were in the CsA-containing group (Figure 1A). When the rate of first relapse was evaluated in relation to maintenance CsA therapy, it was found that of the 14 patients whose CsA was discontinued, 12 (86%) relapsed after a median period of 3 months (range, 1.5 to 40 months), while only 3 of 27 patients (11%) relapsed during maintenance therapy (Figure 1A). This indicates that maintenance CsA therapy prevents relapse ($p<0.001$, χ^2 test). The other agents used for remission induction might have affected the efficacy of CsA as maintenance therapy. However, the efficacy of CsA at preventing relapse was also noted in the patients who were treated with CsA alone (Figure 1-A1) ($p<0.01$) as well as in the patients who were treated with CsA and the other agents (Figure 1-A2) ($p<0.05$). In contrast, 8/15 patients in the CS group (53%) relapsed within 2 to 40 months after remission and 7/8 patients (88%) relapsed during maintenance prednisolone therapy, thus suggesting the difficulty of maintaining remission with prednisolone.

Relapse-free period after discontinuation of CsA

The relapse-free period after discontinuation of CsA therapy (shown as RFS2 in Figure 1) was 10±14 months (n=10), with a range of 1.5 to 40 months, indicating that

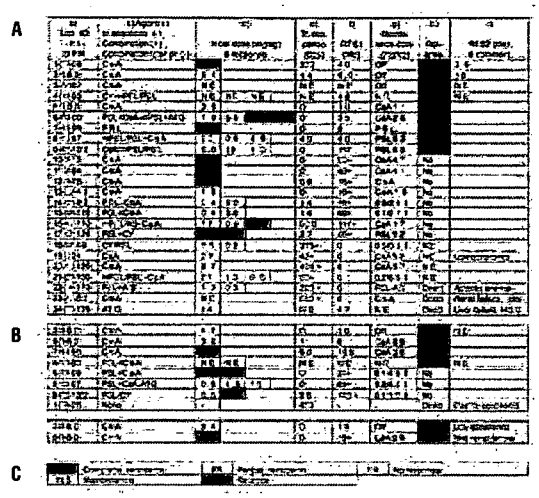

Figure 4 (*left*). Patients in first relapse (A), second relapse (B) and third relapse (C). Abbreviations in each column are the same as those shown in the legend to Figure 1 except for a) list #2; list number in this figure followed by the list number shown in Figure 1 (#1) and UPN, g) [s]doses of prednisolone/CsA in order, [r]doses of cyclophosphamide/prednisolone in order, §doses of prednisolone/cyclophosphamide in order, i) MN; membranous nephropathy, HBV; hepatitis B virus infection.

relapse can occur even 3 years after the discontinuation of CsA. Two patients have maintained remission after discontinuation of CsA therapy (Figures 1A, 9-26 and 10-19); however, the relapse-free periods after discontinuation of CsA therapy are only 1 and 5 months.

Duration of CsA therapy

The mean duration of CsA therapy in patients who relapsed after discontinuation of CsA was 76±32 months, with a range of 10 to 108 months (n=12). In contrast, the mean duration of CsA therapy in patients who are in remission under CsA therapy was 45±48 months (n=24), with a range of 1 to 192 months. The mean dosage of CsA in patients who are in continuing remission for more than 24 months was 2.2±0.8 mg/kg (n=10), 40% of the beginning dose, with a range from 1.1 to 3.8 mg/kg (Figure 1A), excluding one patient (23-130) whose dose of CsA had gradually been increased.

Response of patients in first relapse to different therapies

All patients who had a first relapse were re-treated in an attempt to re-induce remission, and this treatment was successful in 18/24 patients (75%) (Figure 4A, 1-26-28 to 17-46-124 and 24-28-129; corresponding to list No (#2) in Figure 4-list No(#1) in Figure 1-UPN in order). In the 15 relapsed patients in the CsA-containing group, CsA alone was again tried as the initial re-induction therapy for 11 patients, and this treatment was successful in eight of these 11 patients (73%). Three patients did not respond to CsA; one patient with low adherence (frequent self-discontinuation of CsA) (19-7-21), one patient whose dose of CsA was low due to renal dysfunction associated with membranous nephropathy (23-27-80), and one patient who seemed to be resistant to CsA (20-11-120). The remaining four patients were retreated by sequential administration of immuran and ATG (24-28-129), CS concomitantly with anabolic steroids (22-25-179) or CsA (6-29-68), or CS in combination later on with CsA (21-26-160). The two patients treated with ATG (24-28-129) or with a combination of CS and CsA (21-26-160) responded to therapy.

In the eight relapsed patients in the CS group, CS was again tried as an initial re-induction therapy for six patients. CS alone was again tried as the initial re-induction therapy for two patients, and this treatment was successful (7-43-96, 14-44-27). Three patients responded to a combination of CS and CsA (8-45-67, 15-52-178) or CS and cyclophosphamide (17-46-124). One patient responded to CsA (9-42-132). The remaining two patients failed to respond to cyclophosphamide (4-53-93) or methylprednisolone followed by anabolic steroids (16-47-110), but responded to CS and CsA, respectively. As a result, 8/8 relapsed patients in CS responders achieved remission and CsA or cyclophosphamide was newly introduced in 4/8 patients as maintenance therapy. A combination of cyclophosphamide and CS was tried for one relapsed patient (18-57-49) in the cyclophosphamide group but this patient remained transfusion-dependent. Three patients were lost to the follow-up after successful re-induction (3-8-182 and 17-46-124) or during re-induction therapy (21-26-160).

Recurrent relapses

A second relapse occurred in 9/17 patients (Figure 4A). Three out of nine patients experienced a second relapse after discontinuation of CsA therapy (1-4-28, 2-5-60 and 3-8-182). One patient was lost to the follow-up (3-8-182). Seven out of the remaining eight patients were re-induced to a third remission (Figure 4B). One patient was treated by transfusion alone because of the presence of gastric carcinoma (1-4-28). CsA with or without concomitant CS was tried in 6/7 patients and induced remission in all six patients. One patient who had responded to CS achieved complete remission with a later on combination of cyclophosphamide (9-42-132). Thus, no patient treated with CS alone was present after the second relapse. A third relapse occurred in 4/7 patients (Figure 4B). One patient autonomously decided to discontinue CsA and relapsed (2-5-60). Two patients were lost to the follow-up after the third relapse (4-53-93 & 7-43-96). The remaining two patients were successfully re-induced into remission by CsA alone (Figure 4C) but have been experiencing frequent relapses up to the present due to self-discontinuation of CsA (2-5-60) and the limitation of dose escalation due to mild renal failure (5-6-50).

Mortality and overall survival (OS)

Six out of 62 patients (9.7%) died and the estimated 10-year OS after the onset of PRCA was 95%; the median OS

has not yet been reached. Two patients did not respond to remission induction therapy and died from infections (Figure 1D, 60-138 and 62-158). After the first relapse, three patients in the CsA-containing group died (Figure 4A, 22-25-179, 23-27-80 and 24-28-129). One patient (22-25-179) eventually developed aplastic anemia and died from a serious infection, one patient (23-27-80) died from renal failure associated with membranous nephropathy, and the other (24-28-129) died due to liver failure caused by cirrhosis of the liver after hepatitis B virus infection. After a second relapse, one patient (Figure 4B, 1-4-28) in the CsA-containing group, died; the cause of death was gastric carcinoma found 4 years after the onset of PRCA. All four of these patients were in the CsA-containing group who had experienced relapse at least once; however, the OS was not significantly different between patients in the CsA-containing group and those in the CS group (p=0.104).

Discussion

Primary idiopathic PRCA is a clinical disorder defined by the absence of any other disease and is pathogenetically heterogeneous. The most frequent disease underlying secondary PRCA is large granular lymphocyte leukemia (LGL),[6] also referred to as lymphoproliferative disease of granular lymphocytes[13] or granular lymphocyte proliferative disorders.[14] This often has unique clinical features such as autoimmune diseases including rheumatoid arthritis, aplastic anemia, PRCA, neutropenia and thrombocytopenia, and sustained remission may be achieved by treatment with CsA or cyclophosphamide, with or without prednisolone.[6,13,14] The diagnosis of LGL is somewhat difficult in patients without lymphocytosis. Although 14/185 patients were classified as having LGL and secondary PRCA in this study, it remains possible that some patients with LGL are included in this series of cases with supposedly primary idiopathic PRCA. In addition, the data of the current study are derived from a retrospective analysis and the responses cannot be attributed to CsA alone but must, more appropriately, be attributed to CsA-containing regimens, which include both CsA alone and CsA plus other drugs. In this study, we showed, for the first time, that the median RFS of patients in the CsA-containing group was 103 months, which is longer than that seen in the CS group (33 months) (p<0.01). In the CsA-containing group, the discontinuation of CsA was strongly correlated with relapse (p<0.001). Two patients have maintained remission after the discontinuation of CsA; however, the relapse-free periods after the discontinuation are only 1 and 5 months. Considering that a relapse can occur even 40 months after the discontinuation of CsA, these observation periods may be insufficient to conclude that some patients can be cured by CsA. In contrast, 88% of the relapses in the CS group occurred during maintenance prednisolone therapy. Therefore, CsA-containing therapy can sustain a longer duration of initial remission than CS and seems to be important to prevent relapse. Although vigorous and continuous immunosuppressive treatment is capable of inducing and maintaining remission in a majority of patients, it carries an increased risk of serious infections,[16] malignancy,[17,18] and sterility.[19] In our series, two patients died during remission induction due to opportunistic infections (*Pneumocystis jiroveci* pneumonia and bacterial meningitis), which suggests that adequate prevention and treatment of infection are requisites for successful management of patients. After achieving the first remission, four patients died and all of them were CsA responders who relapsed at least once. The causes of death were the development of aplastic anemia, renal failure with membranous nephropathy, liver failure associated with hepatitis B virus infection and gastric carcinoma. The relationship of CsA with the former three diseases is unclear because CsA is one of the effective treatments for aplastic anemia,[20] membranous nephropathy[21] and probably for hepatitis B virus infection as well.[22] Although immunosuppressive therapy enhances viral replication, it has been shown that CsA by itself impairs hepatitis B virus replication by blocking cytosolic calcium signaling.[22] Gastric carcinoma was found in one patient 4 years after the onset of PRCA, but the relationship of this neoplasm to the pathogenesis of PRCA or its treatment with CsA is not clear. Organ transplant experiences have shown that long-term immunosuppression is associated with post-transplant malignancies.[18,19] Therefore, continuous and careful follow-up is required for patients receiving long-term CsA therapy. In addition, the mean maintenance dosage of CsA in Japanese patients who are continuing in first remission for more than 24 months was 2.2±0.8 mg/kg, 40% of the initial dose, suggesting that it would be difficult to reduce the dose of CsA under this level while maintaining remission. One important question is whether or not the maintenance of patients in remission may have a beneficial influence on survival. In the era when CsA was not yet available, Clark *et al.* showed that the treatment of relapses was almost equally successful in 10/13 patients entering a second or third remission, and that the median survival of patients with primary PRCA was 14 years.[11] In our cohort the estimated 10-year OS was 95% and the median OS has not yet been reached; furthermore, we found that CsA-containing regimens can sustain remission for more than 10 years as continuous maintenance therapy. The decreased probability of relapse and the resulting decreased requirement of blood transfusions reduces the dangers of hemolysis, infections and iron overload with possible superoxide damage to body tissues. Although CsA-containing regimens are more expensive than prednisolone, CsA-containing regimens seem to be important to prevent relapse.

In conclusion, we have demonstrated for the first time that CsA-containing regimens, in comparison to CS, sustain a more prolonged RFS in patients with primary idiopathic PRCA. Furthermore, maintenance CsA-containing regimens seem to be important to prevent relapse. Nevertheless, an individualized approach to the manage-

ment of primary PRCA is suggested, and other therapeutic modalities may be required to cure primary PRCA. Prospective randomized studies are needed to identify agents and/or strategies that can cure primary idiopathic PRCA and to determine whether or not maintenance treatment is necessary. It should be appreciated that such studies must last decades considering the recurrent nature of this disorder.

Appendix

The following institutions participated in the Collaborative Study Group: Aichi Medical School, Akita University, Asahikawa Medical School, Chiba University, Dokkyo Medical School, Ehime University, Fujita Health University, Fukui University, Fukui National Hospital, Fukuoka University, Fukushima Medical University, Gifu University, Gunma University, Hamamatsu Medical School, Hirosaki University, Hiroshima University, Hokkaido University, Hyogo Medical University, Iwate Medical School, Jichi Medical School, Jikei University, Juntendo University, Kagawa Childrens' Hospital, Kagawa University, Kagoshima University, Kanazawa University, Kanazawa Medical School, Kansai Medical University, Kawasaki Medical School, Keio University, Kinki University, Kitazato University, Kobe University, Kochi University, Kumamoto University, Kurume University, Kyoto Prefectural University, Kyoto University, Kumamoto Medical Center, Kyushu University, Mie University, Nagasaki University, Nagoya City University, Nagoya Medical Center, Nagoya University, Nara Medical University, National Cancer Center, National Institute of Infectious Diseases, Niigata University, Nishi Sapporo National Hospital, Nippon Medical School, Nippon University, NTT Kanto Medical Center, Oita University, Okayama Medical Center, Okayama University, Osaka City University, Osaka Medical School, Osaka National Hospital, Osaka University, Ryukyu University, Saga University, Saitama Medical School, Sapporo Medical School, Sendai Medical Center, Shimane University, Shinsyu University, Showa University, St. Marianna University, Teikyo University, Toho University, Tohoku University, Tokai University, Tokushima University, Tottori University, Tokyo Medical Center, Tokyo Medical School, Tokyo Medical and Dental University, Tokyo University, Tokyo Women's Medical School, Tsukuba University, University of Occupational and Environmental Health, Wakayama Medical University, Waseda University, Yamagata University, Yamaguchi University, Yamanashi University, Yokohama City University.

Authors' Contributions

KS: designed the research, analyzed the data and wrote the paper. MH: analyzed data and contributed to writing the paper. NF: analyzed data. MT, MB, KD, HT, SN, AU, MO, and KO: designed the research and contributed to the organization of this collaborative study.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Dessypris EN. Pure red cell aplasia. Johns Hopkins Univ Press, 1988; Baltimore, USA.
2. Dessypris EN, Lipton JM. Red cell aplasia. In: Greer JP, Foerster J, Lukens JN, et al. Editors. Wintrobe's Clinical Hematology, 11th ed, Lippincott Williams & Wilkins, Philadelphia, USA. 2004. p. 1421-7,
3. Raghavachar A. Pure red cell aplasia: review of treatment and proposal for a treatment strategy. Blut 1990; 61:47-51.
4. Marmont AM. Therapy of pure red cell aplasia. Semin Hematol 1991; 28: 285-97.
5. Fisch P, Handgretinger R, Schaefer HE. Pure red cell aplasia. Br J Haematol 2000;111:1010-22.
6. Lacy MQ, Kurtin PJ, Tefferi A. Pure red cell aplasia: association with large granular lymphocyte leukemia and the prognostic value of cytogenetic abnormalities. Blood 1996; 87: 3000-6.
7. Mamiya S, Itoh T, Miura AB. Acquired pure red cell aplasia in Japan. Eur J Haematol 1997;59:199-205.
8. Zecca M, Stefano P, Nobili B, Locatelli F. Anti-CD20 monoclonal antibody for the treatment of severe, immune-mediated, pure red cell aplasia and hemolytic anemia. Blood 2001;97:3995-7.
9. Ghazal H. Successful treatment of pure red cell aplasia with rituximab in patients with chronic lymphocytic leukemia. Blood 2002;99:1092-4.
10. Ru X, Liebman HA. Successful treatment of refractory pure red cell aplasia associated with lymphoproliferative disorders with the anti-CD52 monoclonal antibody alemtuzumab (Campath-1H). Br J Haematol 2003; 123:278-81.
11. Clark AD, Dessypris EN, Krantz SB. Studies on pure red cell aplasia. XI. Results of immunosuppressive treatment of 37 patients. Blood 1984;63: 277-86.
12. Totterman TH, Nisell J, Killander A, Gahrton G, Lonqvist B. Successful treatment of pure red cell aplasia with cyclosporine. Lancet 1984;2: 694.
13. Go RS, Li CY, Tefferi A, Phyliky RL. Acquired pure red cell aplasia associated with lymphoproliferative disease of granular T lymphocytes. Blood 2001;98:483-5.
14. Oshimi K, Yamada O, Kaneko T, Nishinarita S, Iizuka Y, Urabe A, et al. Laboratory findings and clinical courses of 33 patients with granular lymphocyte-proliferative disorders. Leukemia 1993;7:782-8.
15. Means RT Jr, Dessypris EN, Krantz SB. Treatment of refractory pure red cell aplasia with cyclosporine A: disappearance of IgG inhibitor associated with clinical response. Br J Haematol 1991;78:114-9.
16. Yale SH, Limper AH. Pneumocystis carinii pneumonia in patients without acquired immunodeficiency syndrome: associated illness and prior corticosteroid therapy. Mayo Clin Proc 1996;71:5-13.
17. Opelz G, Dohler B. Lymphomas after solid organ transplantation: a collaborative transplant study report. Am J Transplant 2004;4:222-30.
18. Bustami RT, Ojo AO, Wolfe RA, Merion RM, Bennett WM, McDiarmid SV, et al. Immunosuppression and the risk of post-transplant malignancy among cadaveric first kidney transplant recipients. Am J Transplant 2004: 4:87-93
19. Pendes S, Ginsburg E, Singh AK. Strategies for preservation of ovarian and testicular function after immunosuppression. Am J Kidney Dis 2004;43:772-81.
20. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. Blood 2006;108:2509-19.
21. Cattran DC, Greenwood C, Ritchie S, Bernstein K, Churchill DN, Clark WF, et al. A controlled trial of cyclosporine in patients with progressive membranous nephropathy. Canadian Glomerulonephritis Study Group. Kidney Int 1995;47:1130-5.
22. Bouchard MJ, Puro RJ, Wang L, Schneider RJ. Activation and inhibition of cellular calcium and tyrosine kinase signaling pathways identify targets of the HBx protein involved in hepatitis B virus replication. J Virol 2003;77:7713-9.

Oligoclonal T cell expansion in blood but not in the thymus from a patient with thymoma-associated pure red cell aplasia

Despite the well-known association between thymoma and PRCA, the role of thymoma remains uncertain There is accumulating evidence that clonal T cells are involved in acquired PRCA We examined T cell receptor repertoires in blood and thymus from a patient with PRCA associated with thymoma and myasthenia gravis. Oligoclonal expansions of V?1- and V?1-expressing T cells were found in peripheral blood, whereas the repertoires of V?1+ and V?1+ T cells in thymoma were not skewed. Oligoclonal expansion of V?1-expressing T cells remained unchanged after thymectomy. Thymus may not be the site of clonal T cell expansion in thymoma-associated PRCA.

Haematologica 2006; 91:(10)e128-e131

Pure red cell aplasia (PRCA) is a syndrome characterized by selective inhibition of hematopoiesis for the erythroid lineage.[1] It is generally believed that acquired PRCA is principally mediated by the autoimmune mechanism There are several reports demonstrating the presence of clonal T cell expansions in PRCA.[2,3,4] Handgretinger *et al.* clearly demonstrated the association of PRCA with clonal expansion of granular lymphocytes expressing the V?1 T cell receptor (TCR).[5] They suggested that the killer-cell inhibitory receptors are involved in cytotoxicity against erythroid progenitors because of their low expression of HLA class I molecules Therefore, it is reasonable to assume that clonally expanded T cells are involved in the inhibition of erythropoiesis in acquired PRCA patients, although the antigen specificity of these T cell clones is largely unknown

Secondary acquired PRCA is complicated by various disorders including thymoma, hematologic malignancies, solid tumors, infections, or collagen diseases However, the role of thymus in the pathogenesis of thymoma-associated PRCA remains uncertain, since some patients develop PRCA after thymectomy or treatment of thymoma by radiation or chemotherapy.[6] We recently encountered a patient with thymoma-associated myasthenia gravis followed by the development of PRCA, in whom we were able to examine the clonality of T cells in blood and thymic tissues We found oligoclonal T cell expansion in the blood but not in the thymus.

Case report

A 52-year-old Japanese woman was diagnosed as having myasthenia gravis associated with thymoma in 1990 At the onset of myasthenia gravis, a hemoglobin level was 11.0 g per deciliter with a red cell count of 4.36 million per cubic millimeter, hematocrit of 33.2 percent, reticulocytes of 3.52 percent (0.153 million per cubic millimeter), a platelet count of 389,000 per cubic millimeter, and a white cell count of 7000 per cubic millimeter with 53 percent neutrophils and 29 percent lymphocytes She required the mechanical ventilation support for severe respiratory muscle paralysis Her critical condition responded to high dose steroid followed by oral prednisolone She refused thymectomy at that time Remaining blepharoptosis disappeared after additional daily 2 mg oral tacrolimus. She developed severe anemia in October 2004, and was referred to our department.

The hemoglobin level was 4.4 g per deciliter, with a red cell count of 1.15 million per cubic millimeter, hematocrit of 13.0 percent, and 0.28 percent (3200 per cubic millimeter) reticulocytes The platelet count was 363,000 per cubic millimeter and the white cell count was 4000 per cubic millimeter with 3480 per cubic millimeter neutrophils, 360 per cubic millimeter lymphocytes, 160 per cubic millimeter monocytes. Direct and indirect anti-Coombs tests were negative. Renal and liver function tests were normal. The serum anti-acetylcholine antibody level was 145 nmol per liter (normal range; <0.2) Anti-human parvovirus B19 IgM was not detected by enzyme-immunoassay Bone marrow was normocellular with 1.0 percent erythroblasts, without giant proerythroblasts, and the myeloid to erythroid ratio was 53.7 There was no proliferation of myeloblasts. Cytogenetic analysis of bone marrow cells showed a normal karyotype. Chest computed tomography demonstrated the presence of thymoma without invasion to surrounding tissues, and no remarkable change in the size as compared with those previously taken. She did not have a previous history of taking drugs that might cause PRCA such as anticonvulsants, antibiotics, anti-viral agents, or viral infections preceding this episode Hence, she was given a diagnosis of thymoma-associated PRCA with preceding myasthenia gravis She required regular red cell transfusion After obtaining informed consent, thymectomy was performed Histological diagnosis was type B2 thymoma according to the WHO classification She was still dependent on red cell transfusion for a while even after thymectomy Blood trough levels of tacrolimus were approximately 3 ng per milliliter on taking 2 mg each day Three months after thymectomy, she was given cyclosporine at a dose of 250 mg per day in place of tacrolimus, when blood trough levels were approximately 180 ng per milliliter Reticulocyte counts increased, and she became free of transfusion Hemoglobin levels normalized up to 12.3 g per deciliter with 58300 per cubic millimeter reticulocytes seven months after starting cyclosporine.

Material and methods

Flow cytometry

The experimental protocol was approved by the institutional review board and the written informed consent was obtained from the patient The monoclonal antibodies used in this study were as follows: anti-CD3 (Becton Dickinson, San Jose, CA, USA); anti-TCR-?/?-1 (Becton Dickinson, San Jose, CA, USA); anti-V? subfamilies (V?1, V?2, V?3; Immunotech, Marseille, France); anti-V? subfamilies (Immunotech, Marseille, France) and control mouse IgG (eBioscience, San Diego, CA, USA) Stained cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

CDR3 size spectratyping of TCRDV and TCRBV

The size of complementarity-determining region 3 (CDR3) of TCR ?- and ?-chains was determined as described elsewhere.[7,8] Total RNA was extracted from freshly isolated PBMCs and thymic tissues using an RNeasy Total RNA Kit (Qiagen, Hilden, Germany), and was used for first-strand cDNA synthesis with an oligo-dT primer (First-Strand cDNA Synthesis Kit, Amersham, Uppsala, Sweden) Aliquots of the cDNA were amplified with the V?-specific oligonucleotide and the C? primer for CDR3 size spectratyping of TCR ? chains, and with the V?-specific oligonucleotide and the C? primer for TCR ? chain analysis The sequences of primers have been described previously.[7-10] The unlabeled PCR products were subjected to one cycle of elongation with a FAM-

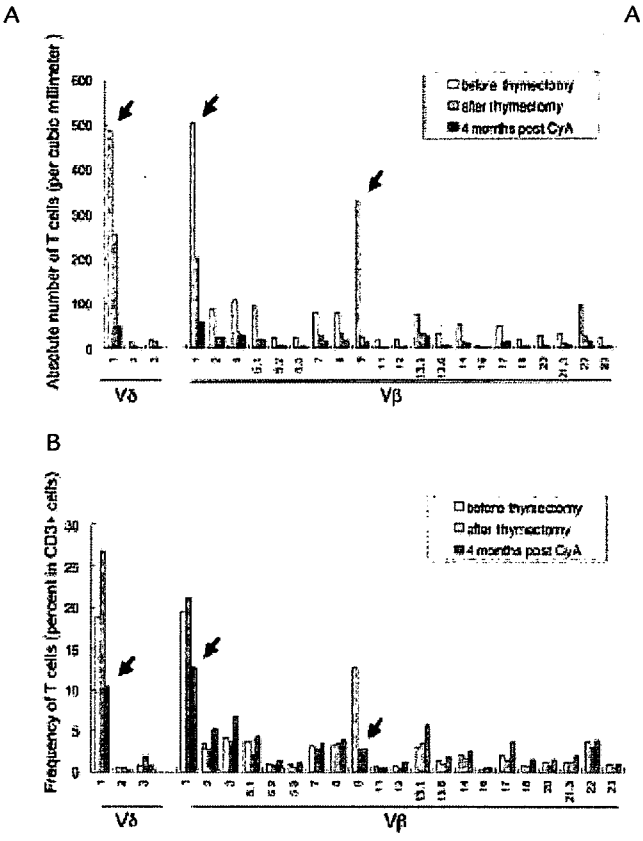

Figure 1. TCRBV and TCRDV repertoires before and after thymectomy and CyA therapy. T cell repertoires were analyzed by flow cytometry. V?1+??T cell, V?1+ and V?9+ ??T (arrows) in peripheral blood were expanded more than that in normal individuals at the onset of PRCA. Frequencies of V?1-, V?1- and V?9- expressing peripheral blood T cells in healthy individuals were 1.2 ± 0.5 percent (19 ± 14 per cubic millimeter), 3.9 ± 1.0 percent (64 ± 35 per cubic millimeter), and 3.4 ± 1.1 percent (51 ± 6 per cubic millimeter) (mean ± SD of 3 samples), respectively. The first flow cytometric analysis of TCR repertoires was performed one month after the onset of PRCA.

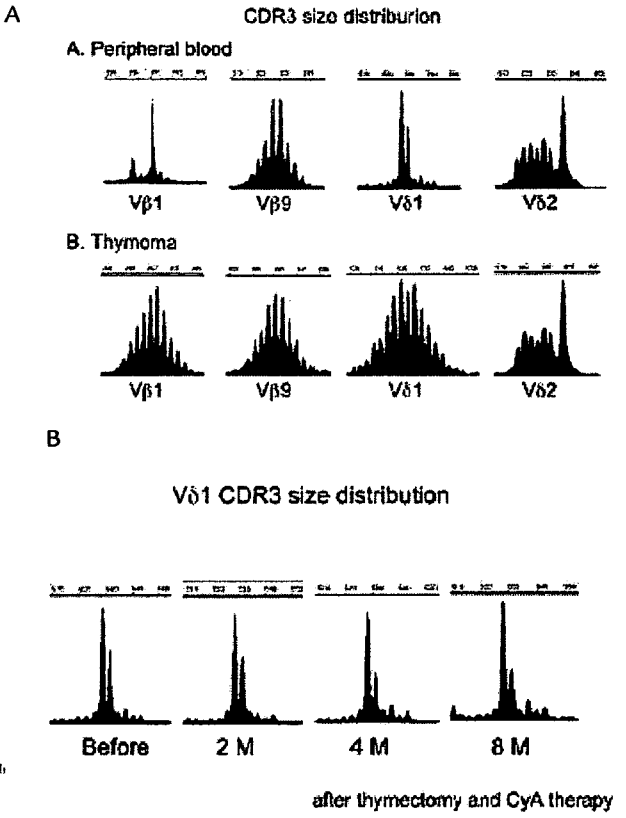

Figure 2. CDR3 size distribution patterns of T lymphocytes in peripheral blood and thymoma. V?1- and V?1- expressing T cells were skewed in blood but not in the thymoma (2A). Skewed pattern of the V?1 TCR repertoire were persisting even after successful treatment with thymectomy and cyclosporine (2B).

labeled C? or C? primer The labeled PCR products were electrophoresed on acrylamide sequencing gels in an automated DNA sequencer (ABI 377, Perkin-Elmer Applied Biosystems, Foster, CA, USA), followed by analysis using GeneScan software (Perkin-Elmer)

Sequencing of CDR3 regions

PCR products were cloned into the PCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) and were sequenced using a Big-Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) Sequence analysis was performed using an automated DNA sequencer.

Results

TCRBV and TCRDV repertoires of T lymphocytes in blood and thymoma

We first examined the usage of TCRBV and TCRDV subfamilies by peripheral blood T lymphocytes to determine whether there was a skew of TCR variable region repertoires As compared to those of healthy individuals, the usages of V?1, V?1 and V?9 segments were increased (Figure 1A and 1B)[11,12,13] V?1+ ??T cells accounted for 18.8 percent of the CD3+ T cell population with an absolute number of 487 per cubic millimeter Whereas V?2+ ??T cells, that are usually the major subtype of circulating ??T cells, accounted for only 0.5 percent with an absolute number of 15 per cubic millimeter V?1+ and V?9+ ??T cells accounted for 19.6 percent (506 per cubic millimeter), and 12.8 percent (332 per cubic millimeter) respectively CDR3 size distribution analysis demonstrated that V?1+ and V?1+ T cells were comprised of oligoclonally expanded clones (Figure 2A) In contrast, the diversity of V?1+ and V?1+ T cells in the thymus were polyclonal Oligoclonal expansion of T cells in blood but not in the thymoma was also confirmed by sequencing analysis of the CDR3 region of TCR ?- and ?-chains (Table 1).

Longitudinal analysis of TCR repertoires after thymectomy and CyA therapy

We studied the longitudinal kinetics of T cell repertoires before and after thymectomy and cyclosporine therapy The numbers of V?1+, V?1+ and V?9+ T cells, which were expanded in the present case, decreased along with treatment (Figure 1A) Although the absolute numbers of expanded V?1+ and V?1+ T cells were reduced after thymectomy and cyclosporine therapy, these subsets remained predominant in circulating blood (Figure 1B) Moreover, the skewed diversity of V?1+ T cells remained the same (Figure 2B), indicating that oligoclonally expanded T cells were still present during hematological remission.

Table 1. Oligoclonally expanded T cells in blood were not detected in the thymus.

TCR	V segment	n-D-n	J segment	ColonyFrequency Blood	Thymoma
TCRDV1	YFCALGE	LGPYGTGGS	YTDKLIFGKG	7/32	0/31
	YFCALGE	LGPGGYQIP	YTDKLIFGKG	3/32	0/31
	YFCALGE	LVGIPSPTGG	YTDKLIFGKG	3/32	0/31
TCRBV1	LYFCASSV	GGEA	GELFFGEG	11/13	0/13

Discussion

We presented a case of thymoma-associated PRCA with oligoclonal expansions of TCR?? and TCR?? T lymphocytes in blood but not in the thymus Mamiya *et al.* previously reported that 17 of 150 patients were complicated with thymoma, and that 10 patients developed PRCA after thymectomy or treatment of thymoma including radiation or chemotherapy.[6] Other groups also reported that 4 patients with myasthenia gravis developed PRCA after thymectomy.[14] These findings raise the question of whether thymoma is indeed involved in the pathogenesis of PRCA. To date, there are few reports describing the T cell repertoire in thymoma-associated PRCA We found a report describing the oligoclonal T cell expansion in thymoma but not in peripheral blood in contrast to the present case[15] Type B2 thymoma has been previously reported to be associated with myasthenia gravis, but PRCA is not a common complication.[16] Although we expected that oligoclonally expanded T cells would be also detected in the thymoma, our findings suggest that oligoclonal T cells in the present case were selected and expanded in peripheral tissues.

There are some reports suggesting the pathological roles of V?1+ ??T cells in GLPD-associated PRCA[4,5,17] Hara *et al.* have also demonstrated that ??T cells mediate an inhibition of erythropoiesis in type I autoimmune polyglandular syndrome.[18] This case also showed the oligoclonal expansion of V?1+ ??T cells in peripheral blood At present, we do not have direct evidence for this T cell subset in the inhibition of erythropoiesis No obvious improvement of anemia was observed during three months after thymectomy, while a global reduction of T lymphocytes in blood was seen after thymectomy and absolute numbers of oligoclonally expanded V?1 and V?1 T cells also decreased However, oligoclonal expansions of T cells are still present even after achieving complete hematological response Since cyclosporine is a functional inhibitor of T cells, cyclosporine can inhibit the function of pathogenic T lymphocytes but not eradicate those cells.

It would be an interesting to address why this patient developed PRCA during immunosuppressive therapy with tacrolimus and prednisolone, then showed amelioration after taking cyclosporine There is a report describing the patient developing PRCA on tacrolimus after liver transplantation.[19] However, there is also a report that tacrolimus was effective for PRCA.[20] In any case, it is possible that a sufficient dose of cyclosporine was given to control the disease in the present case but the dose of tacrolimus might not have been sufficient.

In summary, the present case implies that the thymus may not be the site of clonal T cell expansion in thymoma-associated PRCA, and further investigations are necessary to address this issue Clonal expansion of T cells in the marrow and erythroid suppressive effects of autologous T cells need to the studied in the future.

Naohito Fujishima, Makoto Hirokawa*, Masumi Fujishima*, Chizu Wada**, Itaru Toyoshima**, Sumio Watanabe** and Ken-ichi Sawada**

*Division of *Hematology and Oncology, and Division of **Neurology, Department of Medicine, Akita University School of Medicine*

Key words; V?1+??T cells, thymoma, pure red cell aplasia (PRCA)

Correspondence: Naohito Fujishima, M.D., Division of Hematology and Oncology, Department of Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita Japan 010-8543

Tel: +81-18-884-6116 Fax: +81-18-836-2613

E-mail: naofuji@doc.med.akita-u.ac.jp

References

1. Dessypris EN, et al: In: Greer JP, et al (eds): Wintrobe's Clinical Hematology, 11th ed, Lippincott Williams & Wilkins, Philadelphia, London, p1421-1427, 2004.
2. Masuda M, Arai Y, Okamura T, Mizoguchi H. Pure red cell aplasia with thymona: evidence of T-cell clonal disorder. Am J Hematol. 1997;54:324-328.
3. Masuda M, Saitoh H, Mizoguchi H. Clonality of acquired primary pure red cell aplasia. Am J Hematol. 1999;62:193-195.
4. Masuda M, Teramura M, Matsuda A, Bessho M, Shimamoto T, Ohyashiki K, et al. Clonal T cells of pure red-cell aplasia. Am J Hematol. 2005;79:332-333.
5. Handgretinger R, Geiselhart A, Moris A, Grau R, Teuffel O, Bethge W, et al. Pure red-cell aplasia associated with clonal expansion of granular lymphocytes expressing killer-cell inhibitory receptors. N Engl J Med. 1999;340:278-284.
6. Mamiya S, Itoh T, Miura AB. Acquired pure red cell aplasia in Japan. Eur J Haematol. 1997;59:199-205.
7. Horiuchi T, Hirokawa M, Satoh K, Kitabayashi A, Muira AB. Clonal expansion of gammadelta-T lymphocytes in an HTLV-I carrier, associated with chronic neutropenia and rheumatoid arthritis. Ann Hematol. 1999;78:101-104.
8. Hirokawa M, Horiuchi T, Kawabata Y, Kitabayashi A, Miura AB. Reconstitution of gammadelta T cell repertoire diversity after human allogeneic hematopoietic cell transplantation and the role of peripheral expansion of mature T cell population in the graft. Bone Marrow Transplant. 2000;26:177-185.
9. Saitoh H, Hirokawa M, Fujishima N, Ichikawa Y, Kawabata Y, Miura I, et al. The presence and longevity of peripherally expanded donor-derived TCRalphabeta+ mature T lymphocyte clones after allogeneic bone marrow transplantation for adult myeloid leukemias. Leukemia. 2003;17:1626-1635.
10. Hirokawa M, Horiuchi T, Kitabayashi A, Kawabata Y, Matsutani T, Suzuki R, et al. Delayed recovery of CDR3 complexity of the T-cell receptor-beta chain in recipients of allogeneic bone marrow transplants who had virus-associated interstitial pneumonia: monitor of T-cell function by CDR3 spectratyping. J Allergy Clin Immunol. 2000;106:S32-39.
11. Matsutani T, Yoshioka T, Tsuruta Y, Iwagami S, Toyosaki-Maeda T, Horiuchi T, et al. Restricted usage of T-cell receptor alpha-chain variable region (TCRAV) and T-cell receptor beta-chain variable region (TCRBV) repertoires after human allogeneic haematopoietic transplantation. Br J Haematol. 2000;109:759-769.
12. Parker CM, Groh V, Band H, Porcelli SA, Morita C, Fabbi M, et al. Evidence for extrathymic changes in the T cell receptor gamma/delta repertoire. J Exp Med. 1990;171:1597-1612.
13. Dechanet J, Merville P, Berge F, Bone-Mane G, Taupin JL, Michel P, et al. Major expansion of gammadelta T lymphocytes following cytomegalovirus infection in kidney allograft recipients. J Infect Dis. 1999;179:1-8.
14. Suzuki S, Nogawa S, Tanaka K, Koto A, Fukuuchi Y, Kuwana M. Initial predictors of development of pure red cell aplasia in myasthenia gravis after thymectomy. Clin Neurol Neurosurg. 2003;106:16-18.
15. Handa SI, Schofield KP, Sivakumaran M, Short M, Pumphrey RS. Pure red cell aplasia associated with malignant thymoma, myasthenia gravis, polyclonal large granular lymphocytosis and clonal thymic T cell expansion. J Clin Pathol. 1994;47:676-679.
16. Kuo T, Shih LY. Histologic types of thymoma associated with pure red cell aplasia: a study of five cases including a composite tumor of organoid thymoma associated with an unusual lipofibroadenoma. Int J Surg Pathol. 2001;9:29-35.
17. Kondo H, Uematsu M, Watanabe J, Takahashi Y, Hayashi K, Iwasaki H. CD3+, CD4-, CD8-, TCR alpha beta-, TCR gamma delta+ granular lymphocyte proliferative disorder without

lymphocytosis and clinical symptoms. Acta Haematol. 2000;104:54-56.
18. Hara T, Mizuno Y, Nagata M, Okabe Y, Taniguchi S, Harada M, et al. Human gamma delta T-cell receptor-positive cell-mediated inhibition of erythropoiesis in vitro in a patient with type I autoimmune polyglandular syndrome and pure red blood cell aplasia. Blood. 1990;75:941-950.
19. Misra S, Moore TB, Ament ME, Busuttil RW, McDiarmid SV. Red cell aplasia in children on tacrolimus after liver transplantation. Transplantation. 1998;65:575-577.
20. Yoshida S, Konishi T, Nishizawa T, Yoshida Y. Effect of tacrolimus in a patient with pure red-cell aplasia. Clin Lab Haematol. 2005;27:67-69.

Molecular basis of clonal expansion of hematopoiesis in 2 patients with paroxysmal nocturnal hemoglobinuria (PNH)

Norimitsu Inoue, Tomohisa Izui-Sarumaru, Yoshiko Murakami, Yuichi Endo, Jun-Ichi Nishimura, Ken Kurokawa, Maki Kuwayama, Hiroaki Shime, Takashi Machii, Yuzuru Kanakura, Gabrielle Meyers, Carl Wittwer, Zhong Chen, William Babcock, Debra Frei-Lahr, Charles J. Parker, and Taroh Kinoshita

Somatic mutation of *PIGA* in hematopoietic stem cells causes deficiency of glycosyl phosphatidylinositol–anchored proteins in paroxysmal nocturnal hemoglobinuria (PNH) that underlies the intravascular hemolysis but does not account for expansion of the PNH clone. Immune mechanisms may mediate clonal selection but appear insufficient to account for the clonal dominance necessary for PNH to become clinically apparent. Herein, we report 2 patients with PNH whose *PIGA*-mutant cells had a concurrent, acquired rearrangement of chromosome 12. In both cases, der(12) had a break within the 3′ untranslated region of *HMGA2*, the architectural transcription factor gene deregulated in many benign mesenchymal tumors, that caused ectopic expression of *HMGA2* in the bone marrow. These observations suggest that aberrant *HMGA2* expression, in concert with mutant *PIGA*, accounts for clonal hematopoiesis in these 2 patients and suggest the concept of PNH as a benign tumor of the bone marrow. (Blood. 2006;108:4232-4236)

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a consequence of nonmalignant clonal expansion of hematopoietic stem cells with somatic mutation of *PIGA*.[1] Mutant *PIGA*[2] explains the deficiency of glycosyl phosphatidylinositol–anchored proteins (GPI-APs) that underlies the intravascular hemolysis of PNH.[3] However, *PIGA*-mutant stem cells have no intrinsic proliferative advantage,[4,5] suggesting a 2-step model of pathogenesis.

Step 1 of this model, clonal selection,[6,7] is envisioned as a conditional survival advantage that depends on deficiency of 1 or more GPI-APs. The close association of PNH with aplastic anemia, suggests that the selection pressure is immune mediated.[6,7] But, although 60% to 70% of patients with aplastic anemia have small, subclinical populations of GPI-AP$^-$ hematopoietic cells at diagnosis,[8] only 10% to 15% subsequently develop clinically apparent PNH.[9] In the remainder, GPI-AP$^-$ cells persist subclinically or disappear,[8] suggesting that mutant *PIGA* (and the consequent deficiency of GPI-APs) is necessary for clonal selection but is insufficient to account for the clonal expansion required for clinical manifestations of PNH to become apparent.

Clonal expansion, step 2 of the PNH pathogenesis model, is envisioned as a consequence of clonal evolution in which a second somatic mutation bestows on the *PIGA*-mutant stem cell a proliferative advantage.[10] Herein, we present evidence supporting this 2-step model by showing a concurrent, acquired genetic abnormality in the *PIGA*-mutant cells of 2 patients that establishes a novel mechanism for the nonmalignant clonal hematopoieis characteristic of PNH.

Patients, materials, and methods

Patients

Informed consent was obtained from patients J20 and US1 according to protocols approved by the Institutional Review Boards of Osaka University Hospital (Osaka, Japan) and the University of Utah School of Medicine (Salt Lake City, UT), respectively.

Hybrid cell lines

Monocytes derived from J20 or US1 were fused with the hypoxanthine phosphoribosyltransferase–negative mouse myeloma cell line, P3-X63-Ag8.653, as previously described.[11] Lines carrying human chromosome 12 were selected by analyses of expression of both CD9 and polymorphic markers *D12S77* and *D12S78*. The B-lymphoblastoid cell line JY25[12] was used as a control in some experiments.

From the Department of Molecular Genetics, Osaka Medical Center for Cancer, Japan; the Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Japan; the Department of Immunology, Fukushima Medical University, Japan; the Department of Medicine, Duke University Medical Center, Durham, NC; the Genome Information Research Center, Osaka University, Japan; the Department of Hematology and Oncology, Osaka University School of Medicine, Japan; the Department of Medicine, Division of Hematology and Bone Marrow Transplant, University of Utah School of Medicine, Salt Lake City, UT; the Department of Medicine, Hematology/Oncology Section, George E. Whalen Veterans Administration (VA) Medical Center, Salt Lake City, UT; the Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT; the Division of Medical Genetics, Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, UT; the Department of Medicine, Division of Hematology/Oncology, Baptist Medical Center, Columbia, SC; the Department of Medicine, Division of Hematology, Medical University of South Carolina, Charleston, SC; and the Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan.

Submitted May 23, 2006; accepted August 8, 2006. Prepublished online as *Blood* First Edition Paper, August 29, 2006; DOI 10.1182/blood-2006-05-025148.

An Inside *Blood* analysis of this article appears at the front of this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Determination of chromosomal breakpoints

Initial mapping of breakpoints required a combination of polymerase chain reaction (PCR), inverse PCR, and Southern blotting. For fine mapping, sequence-tagged site markers were generated by PCR using primers based on sequences of ends of bacterial artificial chromosome (BAC) clones or on data in the human genome database of the National Center for Biotechnical Information. GenBank accession numbers of BAC clones (CHORI BACPAC Resource Center) are as follows: RP11-425I22 (425I22), AC074030; RP11-471G7 (471G7), AC024935; RP11-150C16 (150C16), AC046129; RP11-366L20 (366L20), AC090673; RP11-474P2, AC025031; RP11-221N13, AC090023; RP11-434C1, AC007450; RP11-438I19 (438I19), N0438I19.

PCR primers used to amplify breakpoints

PCR primers used to amplify junction sequences of the breakpoints in J20 were as follows: breakpoint (BP) 1, CTTATGTCTCACTTGGGCAC (108462-108443 in 150C16) and CCTTCACTTCACTTGTTAGC (113076-113057 in 425I22); BP2, TTCCTACAGAGCCAAAATGCCA (111355-111376 in 366L20) and ACTGCAACACCTCTCTAGCAG (109879-109899 in 425I22); BP3, TTGAACCCTTTGCCATTACGT (111047-111027 in 425I22) and TATTTAACACCTATCTGACTCC (97995-98016 in 150C16). Primers, GTGCCCAAGTGAGACATAAG (108443-108462 in 150C16) and TGTTGACTGAGCCCCATGAT (108598-108579 in 150C16), were used for positive control PCR.

Junction sequences of the breakpoints in US1 were confirmed by PCR with the following primer mixture to amplify both normal and abnormal alleles at the same time: BP5, CCAAAAGTGGGCTTACA-CATAAAA (44148-44125 in 474P2), TTCGCTCCTCCCACCTCATA (primer A, 111517-111498 in 366L20), and ACTCCCTGTAGTGAATC-CTCTGTTTAGA (primer B, 111043-111070 in 366L20); BP6, GC-CCGGGTTAATGTCGCTGAAT (37462-37483 in 474P2), GTT-GGGGGTGGGGACAAAATG (primer C, 30520-30540 in 434C1), and CGTTGGCAAAGCAGGGTTCCT (primer D, 31301-31281 in 434C1); BP7, GAAGCTCCAACTTTGCCCTCTG (88565-88586 in 221N13), primer B and primer A; and BP8, GGGCAGTTGGAATTGGGGAGAT (89202-89181 in 221N13), primer D and primer C.

Fluorescence in situ hybridization (FISH)

BAC clones 471G7, 150C16, and 366L20 were used as probes against hybrid cell lines derived from J20 and 438I19; 474P2 and 366L20 were used against bone marrow cells from US1. Signals were detected with biotinylated BAC probes and fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories, Burlingame, CA) and amplified by using biotinylated antiavidin antibody (Vector Laboratories) and FITC-conjugated avidin. Chromosomes were stained with propidium iodide and mounted with glycerol-based medium containing 1,4-diazabicyclo(2,2,2)octane antifade. The chromosomal localization was captured through a PlanApochromat objective lens (63×/1.4 NA oil objective) using a Zeiss laser-scanning LSM510 microscope (Carl Zeiss, Jena, Germany) for J20 samples and through a Leica HCS PL FLUOTAR objective lens (100×/0.6-1.3 NA oil objective) using a Leica fluoromicroscope DM RXA2 with a Leica DC350F digital camera (Leica Microsystems, Wetzlar, Germany) for US1. Images were analyzed using Zeiss LSM510 software or Leica QFluoro software, respectively, and were processed with Adobe Photoshop (Adobe Systems, San Jose, CA).

Real-time PCR

Marrow samples were collected from J20 and US1, and control samples from healthy donors were purchased from a commercial vendor (Cambrex, Walkersville, MD). $CD59^-$ cells were isolated from the J20 sample by cell sorting (Becton Dickinson, San Jose, CA). Random hexamer-primed RNA from these samples was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA).

On the basis of the sequence of *HMGA2*, the following PCR primers and TaqMan MGB probe were designed: forward primer, 5′-TTC-AGCCCAGGGACAACCT (located in exon 1); reverse primer, 5′-TCTT-GTTTTTGCTGCCTTTGG (located in exon 2); TaqMan MGB probe, 6-carboxy-fluorescein (FAM)–AGCAAGAACCAACCGGT–non-fluorescent quencher–Minor groove binder (MGB) (the sequence of the probe spanned the boundary between exons 1 and 2) (Applied Biosystems, Foster City, CA). The method for amplifying β-glucuronidase cDNA as an internal control has been published.[13] PCR was performed using QuantiTect Multiplex PCR Kit (QIAGEN, Valencia, CA) on an ABI 7900HT sequence detection system according to the manufacturer's instructions (Applied Biosystems).

Measurement of allele frequency

Real-time PCR was used to measure the ratio of der(12) to normal chromosome 12 in hematopoietic cells of US1. Primers were designed around one of the breakpoints for detection of der(12). For detection of the wild-type allele, a region deleted in der(12) was analyzed. To measure the frequency of mutant *PIGA*, exon 2 was amplified, and PCR products were cloned into pGEM-Teasy vector (Promega, Madison, WI) and sequenced.

Analysis of allele-specific expression of *HMGA2*

The previously described polymorphic marker in the 5′ UTR of *HMGA2* was used to determine allele-specific gene expression in bone marrow cells[14] using the following primers: 5′-GACCCTATCCCGGCGGAGTCTC and 5′-TTGAAATGTTAGGCGGGGAAAGAA. DNA from hybridoma cell lines carrying one chromosome 12 was used to determine the origin of each allele. PCR fragments were separated by 15% to 25% gradient polyacrylamide gel electrophoresis (PAGmini; Daiichi Pure Chemicals, Tokyo, Japan).

Results

Patients

At age 33, J20 presented with pancytopenia and a hypocellular marrow without karyotypic abnormalities.[15] Five months later, an abnormal karyotype, 46, XX, t(12;12)(q13;q15), was reported in 3 (14%) of 21 metaphase cells. One year after diagnosis, blood counts were essentially normal, and marrow analysis showed normal cellularity with mild erythroid dysplasia. Cytogenetic analysis indicated expansion of the mutant clone with 10 (50%) of 20 metaphase cells having the abnormal karyotype. Laboratory evidence of hemolysis was noted, and flow cytometry showed 55% to 60% $GPI\text{-}AP^-$ erythrocytes. A mutation in exon 2 of *PIGA* (G715A) was shown in patient neutrophils.[15] Marrow mononuclear cells were separated into $GPI\text{-}AP^+$ and $GPI\text{-}AP^-$ populations, and the abnormal karyotype was found only in $GPI\text{-}AP^-$ cells.[15] These findings established the somatic nature of both the *PIGA* mutation and the chromosome 12 rearrangement and suggested that both genetic abnormalities were involved in the pathogenesis of clonal hematopoiesis.

US1 was 31 years old at presentation with complaints of fatigue and dark urine. Her white blood cell count was 4.1×10^9/L (4100/μL), hemoglobin level was 38 g/L (3.8 g/dL), and platelet count was 171×10^9/L (171 000/μL). Laboratory studies indicated intravascular hemolysis, and flow cytometry showed 88% $GPI\text{-}AP^-$ neutrophils. Marrow analysis revealed normal cellularity with erythroid hyperplasia without dysplasia and a karyotype of 46, XX, ins(12)(p12~13q13q12) in 20 (100%) of 20 metaphases. FISH showed the insertion split the *TEL* locus at 12p13. The abnormal karyotype was identified in 23% of mitogen-stimulated lymphocytes. A 14-bp deletion in the 3′ end of exon 2 of *PIGA* (693-706) was identified in neutrophil DNA. These findings confirmed the somatic nature of both the der(12) and mutant *PIGA* in US1.

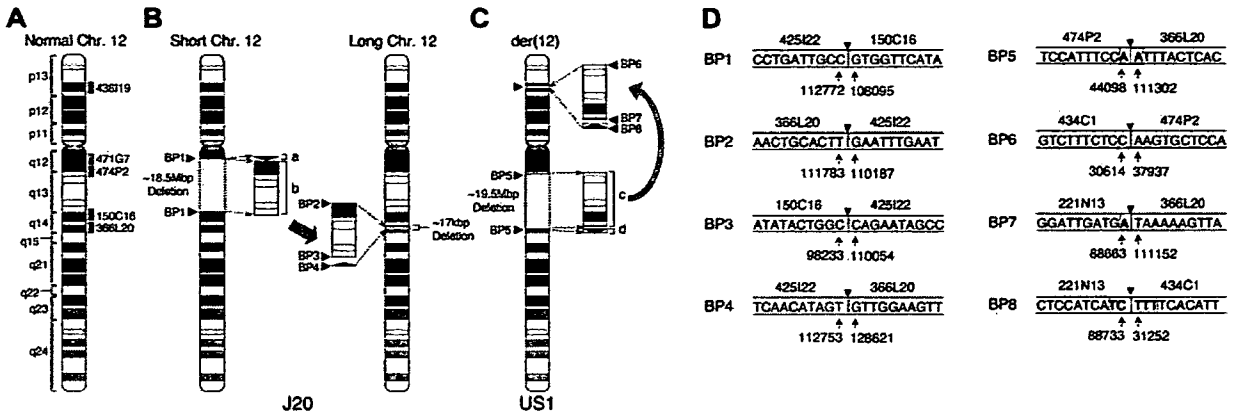

Figure 1. Chromosomal abnormalities in 2 patients with PNH. (A) Idiogram of normal chromosome 12 (Chr 12) modified from the NCBI Map viewer. Labeled gray boxes indicate the positions of the designated BAC clones used for FISH analysis. (B) J20. The karyotypic abnormality identified in the GPI-AP$^-$ bone marrow cells of J20 was defined as an interchromosomal insertion. An 18.5M-bp region from q12 to q14 (surrounded by broken lines) is deleted in short chromosome 12 as defined by characterization of BP1. The 2.7-kbp small fragment (bracketed arrowhead, labeled a) and the 18.5-Mbp large fragment (bracketed rectangle, labeled b) deleted from short chromosome 12 are inserted inversely and directly, respectively, into the 12q14 region of long chromosome 12 generating BP2, BP3, and BP4. The deleted region into which the 2 fragments are inserted lacks 17 kbp of sequence (broken lines). BP1, BP2, BP3, and BP4 indicate the breakpoint junctions generated by the chromosomal abnormality. (C) US1. The karyotypic abnormality identified in the bone marrow cells of US1 was defined as an intrachromosomal insertion. The large fragment (19.5 Mbp, labeled c) and the small fragment (300 kbp, labeled d) are inserted into the *TEL* locus (gray arrowhead) on 12p13. BP5 is generated by the deleted region. BP6, BP7, and BP8 are generated by rearranged fragments c and d. (D) Sequences of BP junctions in J20 and US1. The sequences around BP junctions 1 to 8 are shown. BAC clones containing the sequence are denoted above the lines. Arrows indicate the nucleotide numbers of the BAC clones. Arrowheads indicate one of the candidate breakpoints, and gray regions indicate ambiguous sequences shared between the 2 BAC clones at the site of the breakpoint.

Chromosomal abnormalities

Hybrid cell lines between patient monocytes and mouse myeloma cells were established. From J20, 2 lines (S1 and S2) carrying short chromosome 12 and 2 lines (L1 and L2) carrying long chromosome 12 (Figure 1B) were developed.[11] From US1, hybrid cells carrying normal chromosome 12 (US1W) or der(12) (US1M) were developed (Figure 1A,C). Chromosomal abnormalities (Figure 1) were delineated by using a combination of PCR analysis based on sequence-tagged site markers, FISH (Figure 2), Southern blotting, and inverse PCR (not shown).

For J20, the abnormality was defined as insertion of an 18.5-Mbp fragment derived from one chromosome 12 (short chromosome 12) into the other (long chromosome 12) (Figure 1B). The small fragment (a) and the large fragment (b) derived from the deleted region of short chromosome 12 were inversely and directly, respectively, inserted into 12q14 of long chromosome 12, generating BP2, BP3, and BP4 (Figure 1B).

For US1, the abnormality was an intrachromosomal insertion. The large fragment (19.5 Mbp, labeled c) and the small fragment (300 kbp, labeled d) derived from a region deleted from 12q13q14 are inserted inversely and directly, respectively, into the *TEL* locus on 12p13 (Figure 1C).

A combination of Southern blotting (not shown), PCR (Figure 2A-B), and FISH (Figure 2C-D) were used to confirm that breakpoints that defined the karyotypic abnormalities were present in peripheral blood and bone marrow of J20 and US1.

For J20, only GPI$^-$ cells had the chromosome 12 abnormality.[15] To investigate whether cells of US1 were also double mutants, the percentage of marrow cells with der(12) and mutant *PIGA* was quantitated. Mutant *PIGA* and der(12) were found in 91.8% and 93.9%, respectively, of bone marrow cells, indicating that the 2 mutations coexisted in the same cells. Together, these observations show that clonal hematopoiesis in these 2 patients is derived from a hematopoietic stem cell with somatic mutations of both *PIGA* and chromosome 12.

Effects of chromosome 12 abnormalities

Although the molecular details are different (Figure 1), the result of the chromosome 12 rearrangements is almost identical for the 2 patients, because in both cases, *HMGA2* is disrupted in the 3′ UTR of exon 5 (Figure 3A). No other effects of chromosome 12 rearrangement were detected for either J20 or US1. For US1, chimeric transcripts derived from *TEL* and *HMGA2* were not detected, and *TEL* transcripts appeared normal both quantitatively and qualitatively (not shown).

Real-time PCR showed that relative expression of *HMGA2* in bone marrow cells of both J20 and US1 was greater than normal (Figure 3B). In addition to rearranged *HMGA2*, both J20 and US1 have one intact *HMGA2* locus (Figure 1). To determine the allelic origin of *HMGA2* expression, a polymorphic region in the 5′ UTR[14] was analyzed. For both patients, *HMGA2* expression was derived almost exclusively from the rearranged locus (Figure 3C).

Discussion

These studies showed, in *PIGA*-mutant cells of 2 patients, rearrangement of chromosome 12 (Figures 1-2) that resulted in ectopic expression of *HMGA2* (Figure 3). The findings identify for the first time a molecular mechanism for clonal expansion of hematopoiesis in PNH.

HMGA2 is a member of the high-mobility group of proteins (HMGA1a, HMGA1b, HMGA2) that function as architectural transcription factors.[16-18] HMG members possess no intrinsic transcriptional activity. Instead, these nonhistone proteins orchestrate assembly of stereospecific transcriptional regulatory proteins into enhanceosomes.[18,19] The cellular targets of HMGA2 are incompletely defined but appear to include cyclin A.[19]

Molecular studies established a causal role for *HMGA2* in benign mesenchymal tumors.[17,20] Rearrangement of 12q13-15 is observed in these neoplasms, but tumorigenesis does not depend on generation of chimeric proteins derived from fusion of *HMGA2*

with specific translocation partners. Rather, clonal expansion induced by *HMGA2* appears to result from deregulated expression of a truncated version of the protein.[21-23] For the 2 patients with PNH, ectopic expression appears to be a consequence of gain-of-function mutational events (Figure 3B) caused by disruption of the 3′ UTR (Figure 3A) shown to contain elements that negatively regulate *HMGA2* transcription.[24] This hypothesis is supported by experiments showing that *HMGA2* transcripts from marrow of J20 and US1 are derived almost exclusively from the rearranged alleles (Figure 3C). Additional studies will be required to determine whether aberrant expression of *HMGA2* underlies clonal expansion in patients with PNH without structural abnormalities of 12q13-15.

PNH manifests many of the characteristics of a benign tumor because there is limited expansion of *PIGA* mutant clones (the peripheral blood of patients is a relatively stable mosaic of normal and abnormal cells), *PIGA*-mutant cells respect tissue boundaries

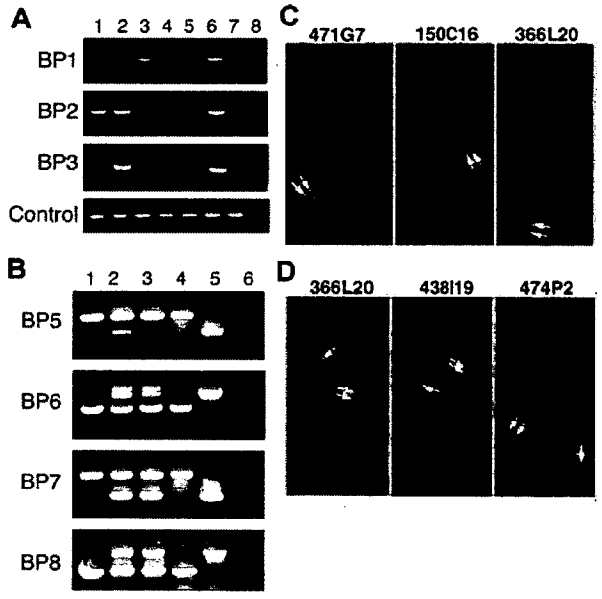

Figure 2. Confirmation of breakpoint junctions generated by the chromosomal abnormalities. (A) Confirmation of the breakpoints in J20 by PCR. Genomic DNA from L1 (lane 1), L2 (lane 2), S1 (lane 3), S2 (lane 4), JY25 (the wild-type control) (lane 5), white blood cells (WBCs) from J20 (lane 6), WBCs of a healthy volunteer (lane 7), or no DNA template (negative control, lane 8) was used for PCR analysis using primer sets designed according to the flanking regions of BP1, BP2, and BP3 (illustrated in Figure 1). Both the integrity and quantity of the DNA templates were confirmed by PCR using control primers (labeled Control). That appropriate-sized PCR products were amplified using DNA derived from the circulating WBCs of J20 (lane 6) shows that both the S1 and L1 versions of chromosome 12 were present in vivo. (B) Confirmation of the breakpoints in US1 by PCR. Genomic DNA from WBCs of a healthy volunteer (lane 1), bone marrow cells of US1 (lane 2), PMN of US1 (lane 3), US1W (the hybrid cell line containing wild-type chromosome 12) (lane 4), or US1M [the hybrid cell line containing (der(12)] (lane 5), or no DNA template (negative control, lane 6) were used for PCR analysis using primer sets designed according to the sequence of flanking regions of the BP5, BP6, BP7, and BP8 (illustrated in Figure 1). These experiments show that both wild-type chromosome 12 and der(12) were present in the peripheral blood and bone marrow of US1. (C) Metaphase FISH showing the chromosomal abnormality in J20. BAC probes 471G7, 150C16, and 366L20 were hybridized against chromosomal specimens derived from the cell line (L1) that contains only long chromosome 12. Two hybridization signals (arrows) were detected with all 3 BAC probes, confirming that long chromosome 12 contained the inserted material deleted from short chromosome 12. (D) Metaphase FISH showing the chromosomal abnormality in US1. BAC probes 366L20, 438I19, and 474P2 (illustrated in Figure 1) were hybridized with chromosomal specimens derived from bone marrow cells of US1. Each sample contained a der(12) (indicated by 2 hybridization signals on the same chromosome, double arrows) and a wild-type chromosome 12 (indicated by one hybridization signal, arrow). The intrachromosomal insertion splits the signal on 12p generated by hybridization of probe 438I19, whereas signals are generated on 12q and 12p when probes that overlap BP5 on the centromeric (474P2) and telomeric (366L20) ends are used.

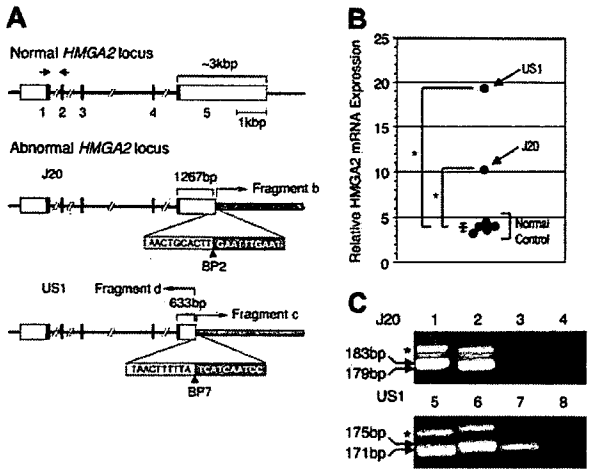

Figure 3. Effects of the chromosome 12 abnormalities in 2 patients with PNH. (A) Structure of normal and abnormal *HMGA2* locus in J20 and US1. White, black, and gray boxes indicate UTRs, coding regions, and abnormally fused fragments, respectively. The exon numbers of *HMGA2* are shown below the boxes. The nucleotide sequences on both sides of BP2 and BP7 (arrowheads) are shown in the white and gray boxes. The truncated *HMGA2* exon 5 of J20 and US1 are indicated by the brackets with the size (bp) shown above the brackets. The bent arrows indicate the fused fragments. The 3′ UTR of exon 5 of *HMGA2* on long chromosome 12 is disrupted as a result of insertion of fragment b (the 12q12q14 fragment from short chromosome 12; see Figure 1). In the case of US1, a similar disruption of exon 5 resulted from the rearrangement that occurred when material deleted from 12q (fragments c and d) was inserted into 12p (see Figure 1). (B) Real-time PCR analysis of HMGA2 transcripts in J20 and US1. The amount of *HMGA2* transcripts in bone marrow cells of 5 healthy individuals and of patients J20 and US1 were quantitated by using the TaqMan MGB PCR method. The positions of the forward (right-facing arrow) and reverse (left-facing arrow) PCR primers are indicated above the normal *HMGA2* locus shown in panel A. The relative expression of *HMGA2* transcripts is normalized to expression of β-glucuronidase transcripts. Each value of the relative expression indicates the average of triplicate measurements. Expression of HMGA2 was greater than normal (mean ± SD, 3.87 ± 0.45) for both J20 (mean, 10.23) and US1 (mean, 19.34) (*$P < .01$). The long and short horizontal bars indicate average and standard deviation (SD) in healthy individuals, respectively. (C) Allele-specific expression of HMGA2. A polymorphic region (based on TC repeats) in the 5′ UTR of *HMGA2* was amplified by PCR and analyzed by polyacrylamide gel electrophoresis. The products were also cloned and sequenced to characterize the polymorphisms. (Top panel) A 183-bp product (containing 29 TC repeats) was generated from the J20-derived hybrid cell line containing long chromosome 12 (lane 1), whereas a 179-bp product (containing 27 TC repeats) was generated from the cell line containing short chromosome 12 (lane 2). Analysis of the PCR product generated by amplification of cDNA derived from GPI-AP$^-$ bone marrow cells of J20 revealed only the 183-bp product (lane 3). No PCR products were visualized when the PCR template was prepared without reverse transcriptase (lane 4). (Bottom panel) A 171-bp product (containing 23 TC repeats) was generated from the US1 hybrid cell line containing the der(12) (lane 5), whereas a 175-bp product (containing 25 TC repeats) resulted from amplification of DNA from the cell line containing normal chromosome 12 (lane 6). Analysis of the PCR product generated by amplification of cDNA derived from unfractionated bone marrow cells of US1 revealed only the 171-bp product (lane 7). No PCR products were visualized when the PCR template was prepared without reverse transcriptase (lane 8). The asterisk (left of each panel) indicates the position of an uncharacterized PCR product. The abnormal allele-specific expression of HMGA2 in the bone marrow cells of US1 was confirmed by using a genetic analyzer (3100-Avant; Applied Biosystems) (not shown). For both J20 and US1, *HMGA2* expression appears to be derived exclusively from the mutant allele.

(there is no invasion of nonhematopoietic tissues), *PIGA*-mutant cells respond appropriately to signals that normally regulate hematopoiesis (function is not autonomous) and transformation into acute leukemia occurs rarely (PNH is not a premalignant condition).[25] Our studies suggest the concept of PNH as a benign tumor of the bone marrow with aberrant expression of *HMGA2* acting in concert with mutant *PIGA* (and the consequent deficiency of GPI-APs) to produce the proliferative phenotype that underlies clonal expansion. However, our studies neither establish the sequence of events that culminated in the clonal outgrowth of the

double mutant cells nor define how the aberrant expression of *HMGA2* works additively or synergistically with mutant *PIGA* to produce the proliferative phenotype. This latter issue will be the subject of future studies.

Findings reported herein provide new insights into the cause of the nonmalignant clonal hematopoiesis of PNH. Together with observations of others,[6,7] our studies support a 2-step process consisting of clonal immunoselection based on phenotype (ie, GPI-AP deficiency resulting from mutant *PIGA*) and clonal expansion as a consequence of a second somatic mutation that bestows the proliferative advantage. Clonal immunoselection may induce exit of *PIGA*-mutant stem cells from a dormant state,[4] thereby favoring acquisition of the mutation that underlies clonal expansion. But the benign nature of PNH suggests that genes involved in clonal expansion of *PIGA*-mutant stem cells are different from those that underlie malignant clonal diseases such as acute leukemia. Characterizing the molecular basis of benign clonal hematopoiesis is important not only for understanding the pathobiology of PNH but also for developing novel strategies for treatment of bone marrow failure and for enhancing stem cell function for both transplantation and gene therapy.

Acknowledgments

We thank Andrew Zinn (The University of Texas Southwestern Medical School at Dallas) and Kiran Chada (Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey) for helpful discussions, and Kiyo Kawata, Fumiko Ishii-Mori, and Keiko Kinoshita for technical assistance.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Ministry of Health, Labour, and Welfare of Japan (T.K. and N.I.); the Osaka Medical Research Foundation for Incurable Diseases (N.I.); the Mochida Memorial Foundation for Medical Pharmaceutical Research (N.I.); the Japan Health Sciences Foundation (J.-I.N.); and the National Institutes of Health (grant K23 RR020043) (G.M. and C.J.P.).

Authorship

Contribution: N.I. designed research, performed research, and wrote the paper; T.I.-S. designed and performed research; Y.M., Y.E., and J.-I.N. performed research; K.K. contributed bioinformatics expertise; M.K., H.S., T.M., and Y.K. collected data; G.M. performed research; C.W. designed research; Z.C. performed research; W.B. and D.F.-L. provided essential material; and C.J.P. and T.K. designed research and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

N.I. and T.I.-S. contributed equally to this study.

Correspondence: Taroh Kinoshita, Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan; e-mail: tkinoshi@biken.osaka-u.ac.jp.

References

1. Takeda J, Miyata T, Kawagoe K, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell. 1993;73:703-711.
2. Miyata T, Takeda J, Iida Y, et al. The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. Science. 1993;259:1318-1320.
3. Parker C, Omine M, Richards S, et al. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood. 2005;106:3699-3709.
4. Hu R, Mukhina GL, Piantadosi S, Barber JP, Jones RJ, Brodsky RA. PIG-A mutations in normal hematopoiesis. Blood. 2005;105:3848-3854.
5. Kawagoe K, Kitamura D, Okabe M, et al. Glycosylphosphatidylinositol-anchor-deficient mice: implications for clonal dominance of mutant cells in paroxysmal nocturnal hemoglobinuria. Blood. 1996;87:3600-3606.
6. Rotoli B, Luzzatto L. Paroxysmal nocturnal haemoglobinuria. Baillieres Clin Haematol. 1989;2:113-138.
7. Young NS, Maciejewski JP. Genetic and environmental effects in paroxysmal nocturnal hemoglobinuria: this little PIG-A goes "Why? Why? Why?" J Clin Invest. 2000;106:637-641.
8. Sugimori C, Chuhjo T, Feng X, et al. Minor population of CD55$^-$CD59$^-$ blood cells predicts response to immunosuppressive therapy and prognosis in patients with aplastic anemia. Blood. 2006;107:1308-1314.
9. Frickhofen N, Heimpel H, Kaltwasser JP, Schrezenmeier H. Antithymocyte globulin with or without cyclosporin A: 11-year follow-up of a randomized trial comparing treatments of aplastic anemia. Blood. 2003;101:1236-1242.
10. Inoue N, Murakami Y, Kinoshita T. Molecular genetics of paroxysmal nocturnal hemoglobinuria. Int J Hematol. 2003;77:107-112.
11. Inoue N, Izui T, Kuwayama M, et al. A possible intrinsic mechanism for clonal expansion of PNH abnormal cells. In: Omine T, Kinoshita T, eds. Paroxysmal Nocturnal Hemoglobinuria and Related Disorders. Tokyo, Japan: Springer-Verlag; 2003:117-126.
12. Hollander N, Selvaraj P, Springer TA. Biosynthesis and function of LFA-3 in human mutant cells deficient in phosphatidylinositol-anchored proteins. J Immunol. 1988;141:4283-4290.
13. Beillard E, Pallisgaard N, van der Velden VH, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR): a Europe against cancer program. Leukemia. 2003;17:2474-2486.
14. Ashar HR, Tkachenko A, Shah P, Chada K. HMGA2 is expressed in an allele-specific manner in human lipomas. Cancer Genet Cytogenet. 2003;143:160-168.
15. Nishimura JI, Inoue N, Azenishi Y, et al. Analysis of PIG-A gene in a patient who developed reciprocal translocation of chromosome 12 and paroxysmal nocturnal hemoglobinuria during follow-up of aplastic anemia. Am J Hematol. 1996;51:229-233.
16. Grosschedl R, Giese K, Pagel J. HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends Genet. 1994; 10:94-100.
17. Hess JL, Kossev P. Molecular genetics of benign tumors. Cancer Invest. 2002;20:362-372.
18. Reeves R. Molecular biology of HMGA proteins: hubs of nuclear function. Gene. 2001;277:63-81.
19. Tessari MA, Gostissa M, Altamura S, et al. Transcriptional activation of the cyclin A gene by the architectural transcription factor HMGA2. Mol Cell Biol. 2003;23:9104-9116.
20. Fedele M, Battista S, Manfioletti G, Croce CM, Giancotti V, Fusco A. Role of the high mobility group A proteins in human lipomas. Carcinogenesis. 2001;22:1583-1591.
21. Arlotta P, Tai AK, Manfioletti G, Clifford C, Jay G, Ono SJ. Transgenic mice expressing a truncated form of the high mobility group I-C protein develop adiposity and an abnormally high prevalence of lipomas. J Biol Chem. 2000;275:14394-14400.
22. Baldassarre G, Fedele M, Battista S, et al. Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway. Proc Natl Acad Sci U S A. 2001;98: 7970-7975.
23. Battista S, Fidanza V, Fedele M, et al. The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. Cancer Res. 1999;59:4793-4797.
24. Borrmann L, Wilkening S, Bullerdiek J. The expression of HMGA genes is regulated by their 3'UTR. Oncogene. 2001;20:4537-4541.
25. Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. N Engl J Med. 1995;333:1253-1258.