Kimura, H., Morii, E., Ikeda, J.I., Ezoe, S., Xu, JX., Nakamichi, N., Tomita, Y., Shibayama, H., Kanakura, Y., and Aozasa, K.	Role of DNA methylation for expression of novel stem cell marker CDCP1 in hematopoietic cells.	Leukemia	20	1551-1556	2006
Yamanishi, H., Imai, N., Ohmine, T., Nishiyama, M., Suehisa, E., Kanakura, Y., and Iwatani, Y.	Urine flow cytometer quantification of leukocytes in samples containing a large proportion of lymphocytes.	Clin. Biochem.	39	857-859	2006
Yu, M., Luo, J., Yang, W., Wang, Y., Mizuki, M., Kanakura, Y., Besmer, P., Neel, BG., and Gu, H.	The scaffolding adapter Gab2, via Shp-2, regulates kit-evoked mast cell proliferation by activating the Rac/JNK pathway.	J. Biol. Chem.	281	28615-28626	2006
Tanaka, H., Matsumura, I., Itoh, K., Hatsuyama, A., Shikamura, M., Satoh, Y., Heike, T., Nakahata, T., and Kanakura, Y.	HOX decoy peptide enhances the ex vivo expansion of human umbilical cord blood CD34+ hematopoietic stem cells/hematopoietic progenitor cells.	Stem Cells	24	2592-2602	2006
Yoshida, H., Maeda, T., Ishikawa, J., Inoue, S., Matsunaga, H., Kosugi, S., Shiraga, M., Oritani, K., Kanakura, Y., and Tomiyama, Y.	Expression of CD27 on peripheral CD4+ T-lymphocytes correlates with the development of severe acute graft-versus-host disease after allogeneic bone marrow transplantation.	Int. J. Hematol.	84	367-376	2006
Inoue, N., Izui-Sarumaru, T., Murakami, Y., Endo, Y., Nishimura, J., Kurokawa, K., Kuwayama, M., Shime, H., Machii, T., Kanakura, Y., Meyers, G., Wittwer, C., Chen, Z., Babcock, W., Frei-Lahr, D., Parker, CJ., and Kinoshita, T.	Molecular basis of clonal expansion of hematopoiesis in 2 patients with paroxysmal nocturnal hemoglobinuria (PNH).	Blood	108	4232-4236	2006
Saito, K., Hirokawa, M., Inaba, K., Fukaya, H., Kawabata, Y., Komatsuda, A., Yamashita, J., and Sawada, K.	Phagocytosis of co-developing megakaryocytic progenitors by dendritic cells in culture with thrombopoietin and tumor necrosis factor-{alpha} and its possible role in hemophagocytic syndrome.	Blood	107	1366-1374	2006

Hirokawa, M., Sato, H., Kawabata, Y., and Sawada, K.	Vaginal outflow tract obstruction associated with chronic graft-versus-host disease following allogeneic peripheral blood stem cell transplantation.	Int. J. Hematol.	83	181-182	2006
Kawabata, Y., Hirokawa, M., Saitoh, Y., Kosugi, S., Yoshioka, T., Fujishima, M., Fujishima, N., Kameoka, Y., Saitoh, H., Kume, M., Takahashi, N., and Sawada, K.	Late-Onset fatal epstein-barr virus-associated hemophagocytic syndrome following cord blood cell transplantation for A dult acute lymphoblastic leukemia.	Int. J. Hematol.	84	445-448	2006
Komastuda, A., Kawabata, Y., Horiuchi, T., Motegi, M., Ozawa, M., Fijishima, N., Kume, M., Hirokawa, M., Wakui, H., Yamaguchi, A., and Sawada, K.	Successful autologous peripheral blood stem cell transplantation using thiotepa in a patient with systemic sclerosis and cardiac involvement.	Tohoku J. Exp. Med.	209	61-67	2006
Taguchi, J., Miyazaki, Y., Tsutsumi, C., Sawayama, Y., Ando, K., Tsushima, H., Fukushima, T., Hata, T., Yoshida, S., Kuriyama, K., Honda, S., Jinnai, I., Mano, H., and Tomonaga, M.	Expression of the myeloperoxidase gene in AC133 positive leukemia cells relates to the prognosis of acute myeloid leukemia.	Leuk. Res.	30(9)	1105-1112	2006
Nakao, S., Sugimori, C., and Yamazaki, H.	Clinical significance of a small population of paroxysmal nocturnal hemoglobinuria-type cells in the management of bone marrow failure.	Int. J. Hematol.	84	118-122	2006
Takami, A., Takamatsu, H., Yamazaki, H., Ishiyama, K., Okumura, H., Ohata, K., Konaka, H., Asakura, H., Namiki, M., and Nakao, S.	Reduced-intensity unrelated cord blood transplantation for treatment of metastatic renal cell carcinoma: first evidence of cord-blood-versus-solid-tumor effect.	Bone Marrow Transplant.	38	729-732	2006

Umeda, K., Heike, T., Nakata-Hizume, M., Niwa, A., Arai, M., Shinoda, G., Ma, F., Suemori, H., Luo, H.Y., Chui, D.H.K., Torii, R., Shibuya, M., Nakatsuji, N., and Nakahata., T.	Sequential analysis of alpha- and beta-globin gene expression during erythropoietic differentiation from primate embryonic stem cells.	Stem Cells	24	2627-2636	2006
Kitawaki, T., Kadowaki, N., Sugimoto, N., Kambe, N., Hori, T., Miyachi, Y., Nakahata, T., and Uchiyama, T.	IgE-activated mast cells in combination with pro-inflammatory factors induce T(h)2-promoting dendritic cells.	Int .Immunol.	1812	1789-1799	2006
Suzuki, T., Yokoyama, Y., Kumano, K.I., Takanashi, M., Kozuma, S., Takato, T., Nakahata, T., Nishikawa, M., Sakano, S., Kurokawa, M., Ogawa, S., and Chiba, S.	Highly efficient ex vivo expansion of human hematopoietic stem cells using Delta1-Fc chimeric protein.	Stem Cells	24	2456-2465	2006
Kobayashi, R., Yabe, H., Hara, J., Morimoto, A., Tsuchida, M., Mugishima, H., Ohara, A., Tsukimoto, I., Kato, K., Kigasawa, H., Tabuchi, K., Nakahata, T., Ohga, S., and Kojima, S.	Preceding immunosuppressive therapy with antithymocyte globulin and ciclosporin increases the incidence of graft rejection in children with aplastic anaemia who underwent allogeneic bone marrow transplantation from HLA-identical siblings.	Brit. J. Haematol.	135	693-696	2006
Umeda, K., Heike, T; Yoshimoto, M; Shinoda, G., Shiota, M., Suemori, H., Luo, HY., Chui, DHK., Torii, R., Shibuya., Nakatsuji, N., Nakahata, T.	Identification and characterization of hemoangiogenic progenitors during cynomolgus monkey embryonic stem cell differentiation.	Stem Cells	24	1348-1358	2006
Matsubara, H., Kobayashi, M., Tokumasu, M., Nakanishi, H., Miyazaki, M., Mizushima, Y., Hiramatsu, H., Adachi, S., Nakayama, T., Onishi, E., and Nakahata, T.	Salmonella enteritidis septic arthritis after allogeneic peripheral blood stem cell transplantation.	Leuk. Lymphoma	47	1435-1437	2006

Nagayama, J., Tomizawa, D., Koh, K., Nagatoshi, Y., Hotta, N., Kishimoto, T., Takahashi, Y., Kuno, T., Sugita, K., Sato, T., Kato, K., Ogawa, A., Nakahata, T., Mizutani, S., Horibe, K., and Ishii, E.	Infants with acute lymphoblastic leukemia and a germline MLL gene are highly curable with use of chemotherapy alone: results from the Japan Infant Leukemia Study Group.	Blood	107	4663-4665	2006
Kato, T., Heike, T., Okawa, K., Haruyama, M., Shiraishi, K., Yoshimoto, M., Nagato, M., Shibata, M., Kumada, T., Yamanaka, Y., Hattori, H., and Nakahata, T.	A neurosphere-derived factor, cystatin C, supports differentiation of ES cells into neural stem cells.	Proc. Natl. Acad. Sci. USA.	103	6019-6024	2006
Sobue, S., Iwasaki, T., Sugisaki, C., Nagata, K ,Kikuchi, R., Murakami, M., Takagi, A., Kojima, T., Banno, Y., Akao, Y., Nozawa, Y., Kannagi, R., Suzuki, M., Abe, A., Naoe, T., and Murate, T.	Quantitative RT-PCR analysis of sphingolipid metabolic enzymes in acuteleukemia and myelodys- plastic syndromes	Leukemia	20	2042-2046	2006
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	91	ECR47	2006
Kato, T., Heike, T., Okawa, K., Haruyama, M., Shiraishi, K., Yoshimoto, M., Nagato, M., Shibata, M., Kumada, T., Yamanaka, Y., Hattori, H., and Nakahata, T.	A neurosphere-derived factor, cystatin C, supports differentiation of ES cells into neural stem cells.	Proc. Natl. Acad. Sci. USA.	103	6019-6024	2006

Sobue, S., Iwasaki, T., Sugisaki, C., Nagata, K ,Kikuchi, R., Murakami, M., Takagi, A., Kojima, T., Banno, Y., Akao, Y., Nozawa, Y., Kannagi, R., Suzuki, M., Abe, A., Naoe, T., and Murate, T.	Quantitative RT-PCR analysis of sphingolipid metabolic enzymes in acuteleukemia and myelodys-plastic syndromes	Leukemia	20	2042-2046	2006
Nagashima, T., Muroi, K., Kawano- Yamamoto, C., Miyoshi, T., Ohmine, K., Toshima, M., Miyazato, A., Takatoku, M., Nagai, T., Mori, M., Komatsu, N., and Ozawa, K.	Autologous gamete cryopreservation before hemopoietic stem cell transplantation.	Med. Sci. Monit	11	CR91-94	2005
Sasaki, K., Nagao, Y., Kitano, Y., Hasegawa, H., Shibata, H., Takatoku, M., Hayashi, H., Ozawa, K., and Hanazono, Y	Hematopoietic microchimerism in sheep after in utero transplantation of cultured cynomolgus embryonic stem cells.	Transplantation	79	32-37	2005
Sasaki, K., Inoue, M., Shibata, H., Ueda, Y., Muramatsu, S., Okada, T., Hasegawa, M., Ozawa, K., and Hanazono, Y.	Efficient and stable Sendai virus-mediated gene transfer into primate embryonic stem cells with pluripotency preserved.	Gene Ther.	12	203-210	2005
Ito, Y., Ohyashiki, K., Hirai, H.,Ogawa, S., Mitani, K., Hotta, T., Bessho, M., Naoe, T., Mizoguchi, H., Uchiyama, T., Omine, M.	Assessment of the international prognostic scoring system for determining chemotherapeutic indications in myelodysplastic syndrome: Japanese retrospective multicenter study.	Int. J. Hematol.	82	236-242	2005
Aisa, Y., Mori, T., Kudo, M., Yashima, T., Kondo, S., Yokkoyama, A., Ikeda, Y., and Okamoto, S.	Oral cryotherapy for the prevention of high-dose melphalan-induced stomatitis in allogeneic hematopoietic stem cell transplant recipients.	Supporr Care Cancer	13	266-269	2005

Kashiwagi, H., Shiraga, M., Kato, H., Kamae, T., Yamamoto, N., Tadokoro, S., Kurata, Y., Tomiyama, Y., and Kanakura, Y.	Negative regulation of platelet function by a secreted cell repulsive protein, semaphorin 3A.	Blood	106	913-921	2005
Shiraga, M., Miyata, S., Kato, H., Kashiwagi, H., Honda, S., Kurata, Y., Tomiyama, Y., and Kanakura, Y.	Impaired platelet function in a patient with P2Y12 deficiency caused by a mutation in the translation initiation codon.	J. Thromb. Haemost.	3	2315-2323	2005
Ishida, N., Oritani, K., Shiraga, M., Yoshida, H., Kawamoto, S., Ujiie, H., Masaie, H., Ichii, M., Tomiyama, Y., and Kanakura, Y.	Differential effects of a novel IFN-zeta/limitin and IFN-alpha on signals for Daxx induction and Crk phosphorylation that couple with growth control of megakaryocytes.	Exp. Hematol.	33	495-503	2005
Tanaka, H., Matsumura, I., and Kanakura, Y.	Cell cycle regulation in hematopoietic stem/progenitor cells.	J. Biol. Sci.	5	50-60	2005
Iwanaga,M., Soda,M., Koba,T., Yamamura,M., Atogami,S., Momita,S., Joh,T., Yoshida, Y., and Tomonaga,M.	Myelodysplastic Syndromes in Atomic Bomb Survivors in Nagasaki : A Preliminary Analysis.	Acta Med. Nagasaki	50(3)	97-100	2005
Matsuda, A., Ulrich Germing, Jinnai, I., Misumi, M., Andrea Kuendgen, Sabine Knipp, Manuel Aivado, Iwanaga, M., Miyazaki, Y., Tsushima, H., Sakai, M., Bessho, M., and Tomonaga, M.	Difference in clinical features between Japanese and German patients with refractory anemia in myelodysplastic syndromes.	Blood	106(8)	2633-2640	2005
Nakao,S., Feng,X., and Sugimori,C	Immune pathophysiology of aplastic anemia	Int. J. Hematol	82	196-200	2005
Takami, A., Asakura, H., Takamatsu,H., Yamazaki, H., Arahata, M., Hayashi, T., Shibayama, M., Orito, M., Yoshida, T., Namiki, M., and Nakao, S.	Isolated hyperkalemia associated with cyclosporine administration in allogeneic stem cell transplantation forrenal cell carcinoma	Int. J. Hematol	81	159-161	2005
Yoshimoto, M., Heike, T., Shiota, M., Kobayashi, H., Umeda, K., and Nakahata, T.	Two different role of purified CD45+ hematopoietic stem cells after transplantation in muscles.	Stem Cells	23	610-618	2005

Ishikawa, F., Yasukawa, M., Lyons, B., Yoshida, S., Miyamoto, T., Yoshimoto, G., Watanabe, T., Akashi, K., Shultz, LD., and Harada, M.	Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ hainnuU mice.	Blood	106	1565-15732	2005
Kakumitsu, H., Kamezaki, K., Shimoda, K., Karube, K., Haro, T., Numata, A., Shide, K., Matsuda, T., Oshima, K., and Harada, M.	Transgenic mice overexpressing murine thrombopoietin develop myelofibrosis and osteosclerosis.	Leuk. Res	29	761-769	2005
Koda, M., Murate, T., Wang, S., Ohguchi, K., Sobue, S., Ikeda, M., Koizumi-T, K., Igarashi, Y., Nozawa, Y., and Banno, Y.	Sphingosine kinase 1 is involved in dibutyryl cyclic AMP-induced granulocytic differentiation through the upregulation of extracelllular signal-regulated kinase, but not p38 kinase, in HL60 cells.	Biochim. Biophys. Acta	1733	101-110	2005
Mitsuma, A., Asano, H., Kinoshita, T., Murate, T., Saito, H., Stamatoyannnopoulos, G., and Naoe, T.	Transcirptional regulation of FKLF-2 (KLF13) gene in erythroid cells	Biochim. Biophys. Acta	1727	125-133	2005

Ⅵ. 研究成果の刊行物・別刷

（主なもの）

Danazol therapy for aplastic anemia refractory to immunosuppressive therapy

Tatsuya Chuhjo,[1] Hirohito Yamazaki,[2] Mitsuhiro Omine,[3] and Shinji Nakao[2*]

Although there are anecdotal reports of the efficacy of danazol in the treatment of aplastic anemia (AA), there has been no systematic study to clarify its efficacy and toxicity. Therefore, we assessed the efficacy of danazol for treatment of patients with AA refractory to immunosuppressive therapy (IST) and those who relapsed after IST, in a prospective clinical trial. Sixteen patients (12 males and four females; six severe cases and 10 moderate cases) were treated with 300 mg of danazol daily for 12 weeks. All patients completed the treatment period without occurrence of severe toxicity. Three female patients achieved partial remission, whereas only two of the 12 male patients did so. None of the responders had shown a response to previous IST or an increase in the percentage of paroxysmal nocturnal hemoglobinuria (PNH)-type cells which are known to be a marker for a good response to IST. These findings indicate that danazol is effective for a subset of AA patients, and particularly for female patients with AA refractory to IST. Am. J. Hematol. 00:000–000, 2007. © 2007 Wiley-Liss, Inc.

Introduction

Acquired aplastic anemia (AA) is a syndrome characterized by pancytopenia and bone marrow hypoplasia. Although AA is an intractable hematopoietic disorder, recent advances in immunosuppressive therapy (IST) have greatly improved prognosis of the disease. Approximately 70% of AA patients respond to IST and achieve transfusion-independence. However, for the remaining 30% of patients who fail to respond to IST and are ineligible for allogeneic stem cell transplantation, therapeutic options are limited.

Danazol, a type of synthetic anabolic steroid [1], has unique properties similar to those of corticosteroids [2,3] such as inhibition [4] of both interleukin-1 and TNF-α production, and has been successfully used for treatments of ITP (immune thrombocytopenic purpura) [5,6], AIHA (autoimmune hemolytic anemia) [7,8], pancytopenia associated with SLE (systemic lupus erythematosus) [9], and pure red cell aplasia [10]. Several case reports have documented the efficacy of danazol in the treatment of AA refractory to IST. Although anabolic steroids are effective in restoring hematopoietic function in a subset of AA patients [11,12], the role of danazol in the treatment of AA has not been studied in a designed clinical trial. Hence, we conducted a prospective study to clarify the efficacy of danazol in the treatment of AA refractory to IST and determined the characteristics of responders to danazol.

Results

Patients

A total of 16 patients (12 males and four females), age ranging from 20 to 68 years, (median age: 45) were registered from five different facilities from December, 2000 through March, 2004. Patient characteristics are shown in Table I. Seven males and three females had moderate disease, and the rest of the patients had severe disease. Thirteen patients were refractory to CsA or ATG, and three had relapsed after successful IST. One of the patients in the study suffered abnormality in cytogenetics, and no one showed apparent dysplasia or fibrosis in bone marrow before this study.

Toxicity

After 13 weeks of danazol therapy, one patient died of fungal pneumonia, a complication thought to be due to severe leucopenia not connected to administration of danazol. Grade 2 liver toxicity according to WHO criteria developed in one patient, but the patient completed the protocol without dose reduction or cessation of danazol. The liver function of this patient normalized soon after the completion of danazol therapy. No toxicities were seen in the other 15 patients during the treatment period.

Response

Among the 16 patients who completed the 12-week treatment period, 5 (31.3%) showed a partial response. Table II summarizes changes in blood cell counts after danazol treatment in these responders. Anemia markedly improved in the three female patients who had moderate AA, and was further improved by continuation of danazol after the 12-week therapy period. In the other two patients, pancytopenia ameliorated and the severity of AA changed from a severe to a moderate state. However, neither of these patients achieved transfusion independence, and therefore, danazol was discontinued at completion of the study.

PNH-type cells and response to danazol

A small population of PNH-type cells were detected in four patients out of 14 patients examined. All four patients failed to respond to danazol, and none of the five res-

[1]Department of Internal Medicine, NTT Kanazawa Hospital, Kanazawa, Ishikawa, Japan; [2]Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan; [3]Division of Hematology, Internal Medicine, Showa University Fujigaoka Hospital, Yokohama, Kanagawa, Japan

*Correspondence to: Shinji Nakao, MD, PhD, Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan.
E-mail: snakao@med3.m.kanazawa-u.ac.jp

Received for publication 4 March 2007; Revised 29 October 2007; Accepted 31 October 2007

Am. J. Hematol. 00:000–000, 2007.

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/ajh.21118

TABLE I. Patient Characteristics

Age	Gender	Immnosuppressive therapy/outcome	Grade of AA at the start of danazol treatment	Cytogenetics
74	Male	ATG+CsA/relapse	moderate	normal
42	Female	CsA/ineffective	moderate	normal
48	Male	CsA/ineffective	severe	normal
28	Male	ATG+CsA/ relapse→ATG/CsA ineffective	severe	normal
22	Male	ATG+CsA/ineffective	severe	normal
28	Male	CsA/ineffective	moderate	normal
69	Male	CsA/ineffective	moderate	45,X
70	Male	ATG+CsA/relapse	moderate	normal
74	Male	ATG+CsA/relapse	moderate	normal
26	Male	ATG+CsA/ineffective	severe	normal
28	Female	CsA/ineffective	moderate	normal
72	Male	CsA/ineffective	moderate	normal
26	Female	ATG+CsA/ineffective	moderate	normal
62	Female	ATG+CsA/ineffective	severe	normal
40	Male	ATG+CsA/ineffective	moderate	normal
62	Male	ATG+CsA/ineffective	severe	normal

ATG, antitymocyte globulin; CsA, cyclosporine.

ponders showed an increase in the number of PNH-type cells before danazol therapy. Among 13 patients for whom HLA-DR alleles were determined, six patients possessed the HLA-DR15 allele and only one of the five responders possessed this DR allele.

Discussion

In this prospective study, all patients tolerated 12-week administration of danazol, at 300 mg/day, showing the relative safety of the low dose of danazol. None of the four females showed apparent signs of virilization, which is common with anabolic steroids, and liver toxicity, the most frequent side effect of anabolic steroids, was seen in only one patient. Thus, it appears that 300 mg of danazol can be safely administered to AA patients for at least 12 weeks.

There was a difference in the rate of response to danazol between males (17%) and females (75%), which was significant in the result of the chi-square test ($P = 0.03$). Such a difference in the response rate to danazol has not been observed in other immune-mediated diseases such as ITP [5,6] and AIHA [7,8]. However, Bacigalupo et al. demonstrated that the addition of an anabolic steroid to ATG treatment was only of benefit for female AA patients [13], and it is possible that the antagonistic effects of danazol on female hormones may lead to augmentation of hematopoiesis in AA. Therefore, the influence of gender on the effect of danazol needs to be examined in a larger number of AA patients.

The presence of PNH-type cells and HLA-DRB1*1501 in AA patients represents good markers for response to IST [14,15]. In the present study, all responders lacked these markers. Moreover, all responders had been refractory to CsA at the initiation of danazol therapy. These findings suggest that mechanisms other than immunomodulatory effects may be responsible for stimulation of hematopoiesis in AA by danazol.

Danazol has been shown to improve thrombocytopenia in some patients with MDS [16–18]. Stadtmauer et al. demonstrated that patients with MDS who responded to danazol tended to show higher levels of platelet-associated IgG or platelet-bindable IgG in plasma, compared to non-responders [17], and further showed that administration of danazol inhibits expression of Fc gamma receptors by monocytes, thereby sparing destruction of platelets bound by IgG. In the present study, a beneficial effect of danazol was seen mainly on anemia, and not on thrombocytopenia. All responders showed not only improvement of anemia but also an increase in the number of reticulocytes. Therefore, administration of danazol appears to stimulate erythropoiesis, rather than inhibit degradation of mature blood cells.

Treatment options are limited for AA patients who fail to respond to IST. The second use of ATG for patients refractory to the first administration of ATG is not approved by the Japanese government. Although some patients improve with anabolic steroids such as metenolone acetate, virilization is a serious side effect for female patients. Danazol at a dose of 300 mg/day did not cause virilization in this study. Although toxicities associated with long-term administration remain to be determined, the results of our study suggest that danazol is a reasonable treatment of choice for AA patients who do not respond to IST, and warrant further clinical study.

Material and Methods

The subjects of the study included patients with severe or moderate AA refractory to antithymocyte globulin (ATG) and/or cyclosporine A (CsA) or those who relapsed with AA after successful IST. All the patients with severe AA were not eligible for bone marrow transplantation due to the lack of an appropriate related donor or high age. Patients with Fanconi anemia or those with hepatitis-associated AA were excluded from the study. The cases in which the patients met two of the three following criteria, neutrophils $< 500\ \mu l^{-1}$, platelets $< 20{,}000\ \mu l^{-1}$, and reticutocytes $< 20{,}000\ \mu l^{-1}$, the disease was defined as severe. The patients whose disease was defined as moderate show two of the three following criteria: neutrophils $< 1{,}000\ \mu l^{-1}$, platelets $< 50{,}000\ \mu l^{-1}$, and reticutocytes $< 60{,}000\ \mu l^{-1}$. All other cases were defined as mild disease.

Patients who met these criteria were enrolled in an open trial, in which danazol, 100 mg, was given orally three times a day (300 mg/day), for 12 weeks. Response to the treatment was assessed at the completion of the 12-week treatment period according to the criteria proposed by Camitta [19] shown in Table III.

Three to five milliliters of anticoagulated blood was taken from each patients and examined for the presence of PNH-type cells using flow cytometry as reported previously [14]. HLA-DRB1 alleles were determined using the polymerase chain reaction (PCR)–RFLP method.

TABLE II. Changes in Hematologic Parameters in Patients Who Responded to Danazol

Patient no.	Granulocytes (μl^{-1})		Hemoglobin (g/dl)		Reticulocytes (μl^{-1})		Platelets (μl^{-1})		Response	Summary of effect	Blood cells which increased first, as a sign of response (time)
	Pre	Post	Pre	Post	Pre	Post	Pre	Post			
2	1,500	1,200	TD	6.4	32,000	48,000	21,000	22,000	PR	TD→independent	Red blood cell after 4 weeks
4	700	900	TD	TD	12,000	23,000	17,000	27,000	PR	severe→moderate	Platelet after 6 weeks
10	300	1,200	TD	TD	8,000	66,000	8,000	16,000	PR	severe→moderate	Reticulocytes after 6 weeks
t11	200	1,100	TD	13.6	71,000	108,000	5,000	10,000	PR	TD→independent	Reticulocytes after 2 weeks
13	1,800	1,200	TD	9.2	26,000	53,000	17,000	40,000	PR	TD→independent	Platelet after 6 weeks

TD, transfusion-dependent.

TABLE III. Response Criteria

Severe aplastic anemia

NR Still severe

PR Transfusion independent or no longer meeting criteria for severe disease

CR Hemoglobin, normal for age
Granulocytes > 1500 μl^{-1}
Platelets > 150,000 μl^{-1}

Moderate aplastic anemia

NR Worse or not meeting criteria below

PR Transfusion independence (if previously required) or doubling or normalization of at least one cell line or increase above baseline
Hemoglobin 3 g/dl (if initially <6)
Granulocytes 500 μl^{-1} (if initially <500)
Platelets 20,000 μl^{-1} (if initially <20,000)

CR Same criteria as for severe disease

Acknowledgments

The following is a list of additional investigators who participated in this study: Shintaro Shiobara (Kanazawa university Hospital), Kazuo Hatanaka and Toshiharu Tamaki (Rinku General Medical Center), Tetsuya Otsuki (Jichi Medical School), Yasunori Kawachi (Takamatsu Red Cross Hospital), Norihumi Tsukamoto (Gunma University Graduate School of Medicine), and Kazuhisa Fujikawa (Chibaken Saiseikai Narasino Hospital).

References

1. Madanes AE, Farber M. Danazol. Ann Intern Med 1982;141:453–463.
2. Beutler B, Krochin N, Milsark W, et al. Control of cachectin (tumor necrosis factor) synthesis: Mechanisms of endotoxin resistance. Science 1986;232: 977–980.
3. Kern JA, Lamb RJ, Reed JC, et al. Dexamethasone inhibition of interleukin 1 beta production by human monocytes. J Clin Invest 1988;81:237–244.
4. Mori H, Nakagawa M, Itoh N, et al. Danazol suppresses the production of interleukin-1 beta and tumor necrosis factor by human monocytes. Am J Reprod Immunol 1990;24:45–50.
5. Ahn YS, Harrington WJ, Simon SR, et al. Danazol for treatment of idiopathic thrombocytopenic purpura. N Engl J Med 1983;308:1396–1399.
6. Ahn YS. Efficacy of danazol in hematologic disorders. Acta Haematol 1990; 84:122–129.
7. Ahn YS, Harrington WJ, Mylvaganam R, et al. Danazol therapy for autoimmune hemolytic anemia. Ann Intern Med 1985;102:298–301.
8. Ahn YS, Rocha R, Harrington WJ. Long-term danazol therapy in autoimmune thrombocytopenia (abstract). Blood 1989;74:110.
9. Cervera H, Jara LJ, Pizarro S, et al. Danazol for systemic lupus erythematosus with refractory autoimmune thrombocytopenia or Evans' syndrome. J Rheumatol 1995;22:1867–1871.
10. Lippman SM, Durie BGM, Garewal HS, et al. Efficacy of danazol in pure red cell aplasia. Am J Hematol 1986;23:373–379.
11. Najean Y, Pecking A, Danvic ML. Androgen therapy of aplastic anaemia—A prospective study of 352 cases. Scand J Haematol 1979;22:343–356.
12. Najean Y. Long-term follow-up in patients with aplastic anemia, a study of 137 androgen-treated patients surviving more than two years. Am J Med 1981;71:543–551.
13. Bacigalupo A, Chaple M, Hows J, et al. Treatment of aplastic anaemia (AA) with antilymphocyte globulin (ALG) and methylprednisolone (MPred) with or without androgens: A randomized trial from the EBMT SAA working party. Br J Haematol 1993;83:145–151.
14. 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.
15. Nakao S, Takamatsu H, Chuhjo T, et al. Identification of a specific HLA class II haplotype strongly associated with susceptibility to cyclosporine-dependent aplastic anemia. Blood. 1994;84:4257–4261.
16. Buzaid AC, Garewal HS, Lippman SM, et al. Danazol in the treatment of myelodysplastic syndromes. Eur J Haematol 1987;39:346–348.
17. Stadtmauer EA, Cassileth PA, Edelstein M, et al. Danazol treatment of myelodysplastic syndromes. Br J Haematol 1991;77:502–508.
18. Chan G, DiVenuti G, Miller K. Danazol for the treatment of thrombocytopenia in patients with myelodysplastic syndrome. Am J Hematol 2002;71:166–171.
19. Camitta BM. What is the definition of cure for aplastic anemia? Acta Haematol 2000;103:16–18.

International Journal of
HEMATOLOGY

Cyclosporine Therapy for Acquired Aplastic Anemia: Predictive Factors for the Response and Long-term Prognosis

Hirohito Yamazaki,[a] Chiharu Sugimori,[a] Tatsuya Chuhjo,[b] Shinji Nakao[a]

[a]*Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan;* [b]*Division of Internal Medicine, NTT West Kanazawa Hospital, Kanazawa, Japan*

Received July 26, 2006; received in revised form November 24, 2006; accepted December 14, 2006

Abstract

Although cyclosporine (CsA) is a key drug in the treatment of acquired aplastic anemia (AA), the role of single-agent therapy with CsA remains unclear. To determine the efficacy of CsA in the treatment of AA, we treated 38 AA patients with CsA alone and followed up the patients for 6 months to 16 years. Twenty patients (53%) achieved either a partial or complete remission within 1 year of starting CsA therapy. Thirteen (81%) of 16 patients who showed an increase in the reticulocyte count of $>20 \times 10^9$/L within 2 months achieved remission, whereas the response rate was only 32% in patients who failed to show such an increase in the reticulocyte count. The actuarial overall survival and failure-free survival rates at 5 years were 91% and 37%, respectively. These data indicate that CsA alone can achieve a sustained remission in approximately 40% of AA patients, with a low probability of inducing secondary clonal diseases. Given its low toxicity and because the effectiveness of CsA can be judged within 2 months of therapy, CsA may be the first drug of choice at outpatient clinics for AA patients not requiring transfusions.

Int J Hematol. 2007;85:186-190. doi: 10.1532/IJH97.06156

Key words: Aplastic anemia; Cyclosporine; Monotherapy; Reticulocytes; Predictive factor

1. Introduction

Cyclosporine (CsA) has been successfully used for the treatment of acquired aplastic anemia (AA) as a single agent [1-3] or in combination with antithymocyte globulin (ATG) [4-6]. A randomized controlled study conducted by a French group clarified that CsA as single-agent therapy and CsA therapy with ATG are equally effective in restoring hematopoietic function in patients with severe AA [3]. CsA has not been used alone for the treatment of severe AA, however, because a subsequent controlled study demonstrated the superiority of combination therapy with ATG plus CsA compared with ATG alone in the treatment of severe AA [4]. Another controlled study by the European Group for Blood and Marrow Transplantation (EBMT) showed that therapy with ATG plus CsA led to a higher rate of remission than with CsA alone in patients with nonsevere AA [7]. CsA is therefore considered a supplemental drug rather than a main drug for the treatment of AA, and the role of CsA as single-agent therapy has not attracted much attention.

Monotherapy with CsA has several advantages over therapy with ATG and androgen. Because its toxicity is lower than with ATG, CsA can be administered safely at outpatient clinics, and its use does not necessitate platelet transfusions, which are often necessary when ATG is administered. CsA does not cause virilism as androgen does. Therefore, CsA may be the treatment of choice for AA patients who do not require transfusions but do show progressive pancytopenia. We have treated AA patients with CsA alone since the late 1980s because of the unavailability of ATG in Japan until 1995; thereafter, we used CsA only in cases not requiring transfusions. To determine the role of CsA monotherapy in the treatment of AA, we analyzed its effectiveness and the long-term prognosis of patients treated with this therapy.

Correspondence and reprint requests: Shinji Nakao, MD, PhD, Professor, Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan; 81-76-265-2274; fax: 81-76-234-4252 (e-mail: snakao@med3.m.kanazawa-u.ac.jp).

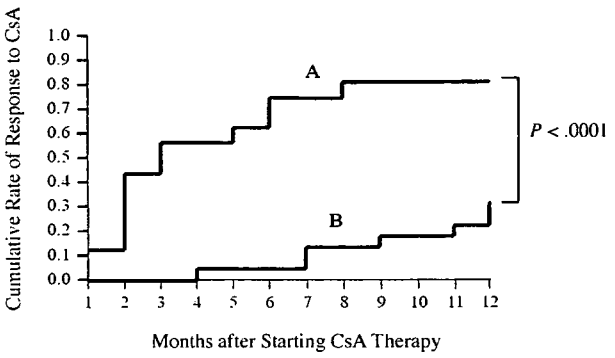

Figure 1. Cumulative rate of response to cyclosporine (CsA). Patients were divided into 2 groups according to the reticulocyte response, which was defined as an increase of $\geq 20 \times 10^9$/L in the reticulocyte count from the pretreatment value, and cumulative rates of response to CsA for responders (A) and nonresponders (B) were compared with respect to reticulocyte numbers.

2. Patients and Methods

2.1. Patients

We treated 38 patients with acquired idiopathic AA (9 with severe disease, 29 with moderate disease) ranging in age from 16 to 82 years (median, 53 years) from October 1986 to December 2005 [8]. The severity of AA was graded according to the criteria proposed by Camitta et al [9]. The male-female ratio was 21:17. All patients had a neutrophil count $>0.2 \times 10^9$/L. The time from AA diagnosis to CsA therapy ranged from 0 to 271 months (median, 6 months). Fourteen patients (37%) were dependent on red blood cell transfusions at the initiation of therapy. The reasons for not receiving ATG as first-line therapy were the unavailability of ATG in Japan at the time of diagnosis in 18 patients and the absence of a transfusion requirement in 17 patients.

2.2. Treatment with CsA

All patients received 3 to 5 mg/kg of CsA for at least 3 months. Patients who did not show any signs of improvement at the completion of 3 months of therapy were changed to either ATG or androgen therapy in combination with CsA therapy. The response to therapy was assessed by the criteria proposed by Camitta [10]. A complete response (CR) was defined as a hemoglobin concentration normal for the patient's age, a neutrophil count $>1.5 \times 10^9$/L, and a platelet count $>150 \times 10^9$/L. A partial response (PR) was defined for patients with severe AA as being transfusion independent and no longer meeting the criteria for severe disease. In patients with nonsevere AA, a PR was defined as transfusion independence (if the patient previously had been transfusion dependent), the doubling or normalization of at least 1 cell line, or an increase in the baseline hemoglobin level by >3 g/dL (if initially <6 g/dL), in the neutrophil count to $>0.5 \times 10^9$/L (if initially $<0.5 \times 10^9$/L), and in the platelet count to $>20 \times 10^9$/L (if initially $<20 \times 10^9$/L).

2.3. Detection of Paroxysmal Nocturnal Hemoglobinuria–Type Cells

To detect paroxysmal nocturnal hemoglobinuria (PNH)-type granulocytes, we used phycoerythrin-labeled anti-CD11b monoclonal antibodies (MoAbs) (BD Medical Systems, Mountain View, CA, USA), fluorescein isothiocyanate (FITC)-labeled anti-CD55 (clone IA10, mouse immunoglobulin G2a [IgG2a]; BD Pharmingen, San Diego, CA, USA), and FITC-labeled anti-CD59 (clone p282, mouse IgG2a; BD Pharmingen) in combination with isotype-matched control antibodies, as described in our previous report [11]. For the analysis of PNH-type red blood cells, we used phycoerythrin-labeled antiglycophorin A MoAb (clone JC159; Dako, Glostrup, Denmark) instead of using antiglycophorin A MoAb, FITC-labeled anti-CD55, and anti-CD59 MoAb on ice for 25 minutes. We analyzed at least 105 $CD11B^+$ granulocytes and glycophorin A–positive red blood cells within each corresponding gate by means of FACScan flow cytometry (BD Medical Systems).

2.4. Determination of DRB1 Alleles

The DRB1 alleles of the 38 AA patients were determined by means of polymerase chain reaction analysis with sequence-specific primers (Micro SSP HLA DNA typing trays; One Lambda, Canoga Park, CA, USA). Genomic DNA was prepared from blood samples with a DNA-extraction kit (Generation Capture Column Kit; Gentra Systems, Minneapolis, MN, USA).

2.5. Statistical Analysis

Logistic regression was used in both univariate and multivariate analyses to assess factors that predict the response to CsA. The following clinical parameters were analyzed to determine their relationship with the response to CsA: age, time to CsA therapy from diagnosis, the presence of HLA-DRB1*1501, the presence of PNH-type cells, an increase in the reticulocyte count, and disease severity. The Kaplan-Meier method graphically compared the cumulative incidences of the response to CsA therapy and the times to event, and any differences in reticulocytes between responders and nonresponders were assessed by the log-rank test. All statistical analyses were performed with the JMP software package, version 5.0.1J (SAS Institute, Cary, NC, USA).

3. Results

3.1. Response Rate

The patients were followed up from 6 months to 16 years (median, 6.5 years) after CsA therapy. The rate of CR plus PR responses to CsA was 34% at 6 months and 53% at 12 months. Only 1 patient achieved a CR by the completion of 12 months of treatment. The response rates in severe AA and nonsevere AA patients were 44% and 55%, respectively. The reticulocyte count increased by $>20 \times 10^9$/L from the pretreatment value in 42% of the patients, and the increase in reticulocytes occurred within 2 months of CsA

Table 1.
Relationship between Clinical Parameters and Response to Cyclosporine (CsA)*

	Relative Risk (95% CI)	P
Age	0.036 (0.0001-4.67)	.18
Time from diagnosis to CsA therapy	1.43 (0.108-20.5)	.77
HLA-DRB1*1501	1.84 (0.223-17.6)	.56
PNH-type cells	6.24 (0.875-77.88)	.09
Increase of reticulocytes	9.07 (1.327-113.3)	.04
Dependency on transfusion	1.00 (0.09-9.03)	.99

*In this multivariate analysis, AA severity was classified according to the dependency on transfusions. CI indicates confidence interval; PNH, paroxysmal nocturnal hemoglobinuria.

therapy in these responders. The cumulative rate of achieving a PR or CR in the reticulocyte-count responders by 1 year of therapy was 81%, which was significantly higher than the 32% rate for nonresponders regarding the reticulocyte count (Figure 1).

The administration of CsA was continued for more than 2 years in 14 (70%) of 20 responders. Eleven patients (55%) achieved an unmaintained remission after ceasing CsA treatment, and 6 patients (30%) required a low CsA dose (40-150 mg/day) because of the dependency of their hematopoietic function on CsA. AA relapsed in 5 patients (25%) between 3 and 6.5 years after the cessation of CsA therapy. Two of the patients responded to a reinitiation of CsA therapy and thereafter developed CsA dependency.

3.2. Factors Affecting the Response to Therapy

Several parameters, such as the presence of PNH-type cells [12] and HLA-DRB1*1501 [13], have been suggested to demonstrate a good response to immunosuppressive therapy. Recent reports revealed a higher prevalence of increases in PNH-type cells in AA patients than had previous reports [12,14]. When we examined these parameters in our patients, 19 (50%) of 38 patients had small populations of PNH-type cells, whereas 11 (29%) of 38 patients possessed HLA-DRB1*1501. The response rates to CsA therapy were 68% in patients with a small population of PNH-type cells and 37% in patients without PNH-type cells (P = .051); the response rates were 55% with DRB1*1501 and 52% without DRB1*1501 (P = .88). Table 1 summarizes the relationship between the clinical parameters, including dependency on transfusions, and the response to CsA. A multivariate analysis revealed that only an increase in the reticulocyte count within 2 months of CsA therapy predicted a good response.

3.3. Prognosis

The disease of 1 patient (2.6%) developed into PNH after 5 years of CsA therapy, and none of the patients later developed myelodysplastic syndrome or acute myeloid leukemia. Gastric cancer and cholangiocarcinoma in 1 patient each, and these 2 patients died 13 years and 7 years, respectively, after starting CsA therapy. The actuarial overall survival and failure-free survival rates at 5 years were 91% and 37%, respectively (Figure 2). The time to treatment failure was defined as the time from the first day of treatment until salvage treatment for nonresponse, the occurrence of relapse, the development of a clonal hematologic disease or solid tumor, or disease-related or treatment-related death, whichever came first.

4. Discussion

This retrospective analysis revealed that treatment with CsA alone can achieve a remission in approximately 50% of AA patients, regardless of disease severity. The response rate is similar to that reported by Hinterberger-Fischer et al [1] but is slightly higher than the rates reported by Leonard et al and Gluckman et al [2,3]. The better response rate in our study is probably because all patients received CsA as the first therapy and no patient in this study had very severe AA, as defined by a neutrophil count of $<0.2 \times 10^9$/L.

Among several factors that may affect the response to CsA, only an increase in the reticulocyte count within 2 months of therapy was associated with a good response to CsA. Although we previously reported that the presence of HLA-DRB1*1501 predicts a favorable response to CsA [13], the current study could not confirm this finding. This discrepancy may be due to the inclusion of patients who had a long history of AA. Illness duration is known to negatively affect the response to immunosuppressive therapy [15]. Although some patients carried HLA-DRB1*1501, the long disease duration before they started to receive CsA may have limited their responsiveness to CsA. We recently demonstrated that the presence of small populations of PNH-type cells is associated with a good response to therapy with ATG plus CsA in AA patients [12]; however, the present study failed to show such a role for the presence of PNH-type cells in predicting a good response to CsA. CsA alone may not be potent enough to eradicate the immune mechanisms and restore hematopoiesis in AA patients.

Clonal hematopoietic diseases such as PNH and myelodysplastic syndrome have been reported to eventually develop in 10% to 15% of AA patients treated with immunosuppressive

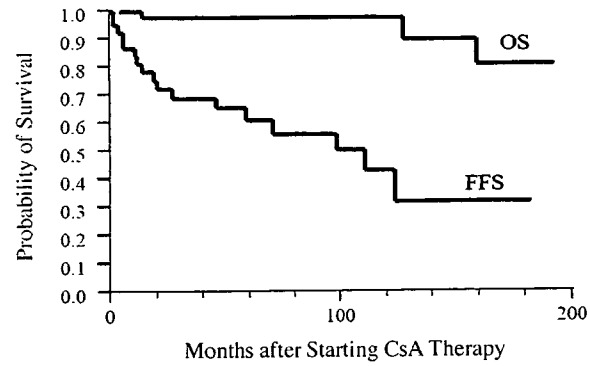

Figure 2. Prognosis after cyclosporine (CsA) therapy. OS indicates overall survival; FFS, failure-free survival.

therapy [16,17]. A study of pediatric AA patients by a Japanese group found that the risk of developing myelodysplastic syndrome in AA patients who received CsA and granulocyte colony-stimulating factor was significantly higher than in patients who did not receive these drugs [18]. In the present study, PNH developed in only 1 patient, and myelodysplastic syndrome or acute myeloid leukemia developed in none of the patients. Although the study population was relatively small and the follow-up period was short, our findings suggest that monotherapy with CsA may not be associated with an increased risk of developing clonal disorders in AA patients. On the other hand, solid tumors later developed in 2 patients who had received CsA for many years (13 years and 7 years). A recent follow-up study of AA patients who had undergone immunosuppressive therapy revealed a relatively high risk of developing solid tumors [17]. It therefore seems necessary to carefully observe AA patients on CsA therapy to identify any occurrence of malignancies.

Responders to CsA are believed to often require continuous drug administration to maintain remission [13]. This dependency has been thought to be one of the drawbacks of CsA therapy. A recent study by the EBMT group reported that only 38% of responders to therapy with ATG plus CsA no longer needed CsA at 5 years after therapy [19]. In our patients treated with CsA alone, however, the cumulative rate for patients no longer needing CsA at 5 years was 55%. Therefore, the rate of developing CsA dependency in responders to CsA therapy may not be as high as originally expected. The long-term administration of CsA without any apparent necessity thus should be avoided in order to prevent chronic nephrotoxicity and secondary malignancies that may occur as a result of CsA-induced immunodeficiency. The results of our study warrant further attempts to taper the CsA dosage in all responders to CsA.

The results of this analysis have important implications for the management of patients with moderate or severe AA who do not require transfusions. ATG plus CsA is the standard therapy for AA. All AA patients who need transfusions and are ineligible for allogeneic stem cell transplantation should undergo this immunosuppressive therapy as soon as possible. However, many patients with moderate AA and some with severe AA do not need transfusions at the time of AA diagnosis, and because of an absence of symptoms, these patients usually do not want to be hospitalized. CsA treatment on an outpatient basis is the therapy of choice for such patients, because a response rate of approximately 40% can be expected without causing any significant toxicity. A change of therapy should be considered unless the reticulocyte count increases within 2 months. The effectiveness of CsA for the treatment of AA patients not requiring transfusions still needs to be confirmed by prospective studies.

Acknowledgments

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

References

1. Hinterberger-Fischer M, Hocker P, Lechner K, Seewann H, Hinterberger W. Oral cyclosporin-A is effective treatment for untreated and also for previously immunosuppressed patients with severe bone marrow failure. *Eur J Haematol.* 1989;43:136-142.
2. Leonard EM, Raefsky E, Griffith P, Kimball J, Nienhuis AW, Young NS. Cyclosporine therapy of aplastic anaemia, congenital and acquired red cell aplasia. *Br J Haematol.* 1989;72:278-284.
3. Gluckman E, Esperou-Bourdeau H, Baruchel A, et al. Multicenter randomized study comparing cyclosporine-A alone and antithymocyte globulin with prednisone for treatment of severe aplastic anemia. *Blood.* 1992;79:2540-2546.
4. Frickhofen N, Kaltwasser JP, Schrezenmeier H, et al. Treatment of aplastic anemia with antilymphocyte globulin and methylprednisolone with or without cyclosporine. The German Aplastic Anemia Study Group. *N Engl J Med.* 1991;324:1297-1304.
5. Bacigalupo A, Broccia G, Corda G, et al. Antilymphocyte globulin, cyclosporin, and granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): a pilot study of the EBMT SAA Working Party. *Blood.* 1995;85:1348-1353.
6. Rosenfeld SJ, Kimball J, Vining D, Young NS. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. *Blood.* 1995;85:3058-3065.
7. Marsh J, Schrezenmeier H, Marin P, et al, on behalf of the EBMT Severe Aplastic Anaemia Working Party. Prospective randomized multicenter study comparing cyclosporin alone versus the combination of antithymocyte globulin and cyclosporin for treatment of patients with nonsevere aplastic anemia: a report from the European Blood and Marrow Transplant (EBMT) Severe Aplastic Anaemia Working Party. *Blood.* 1999;93:2191-2195.
8. Nakao S, Urabe A, Bessho M, et al. Clinical guide to the diagnosis and treatment of aplastic anemia [in Japanese]. *Rinsho Ketsueki.* 2005;47:27-46.
9. Camitta BM, Thomas ED, Nathan DG, et al. Severe aplastic anemia: a prospective study of the effect of early marrow transplantation on acute mortality. *Blood.* 1976;48:63-70.
10. Camitta BM. What is the definition of cure for aplastic anemia? *Acta Haematol.* 2000;103:16-18.
11. Wang H, Chuhjo T, Yasue S, Omine M, Nakao S. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. *Blood.* 2002;100:3897-3902.
12. 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.
13. Nakao S, Takamatsu H, Chuhjo T, et al. Identification of a specific HLA class II haplotype strongly associated with susceptibility to cyclosporine-dependent aplastic anemia. *Blood.* 1994;84:4257-4261.
14. Okamoto M, Shichishima T, Noji H, et al. High frequency of several PIG-A mutations in patients with aplastic anemia and myelodysplastic syndrome. *Leukemia.* 2006;20:627-634.
15. Marsh JC, Gordon-Smith EC. Treatment of aplastic anaemia with antilymphocyte globulin and cyclosporin. *Int J Hematol.* 1995;62:133-144.
16. de Planque MM, Bacigalupo A, Wursch A, et al. Long-term follow-up of severe aplastic anaemia patients treated with antithymocyte globulin. Severe Aplastic Anaemia Working Party of the European Cooperative Group for Bone Marrow Transplantation (EBMT). *Br J Haematol.* 1989;73:121-126.
17. 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.
18. Ohara A, Kojima S, Hamajima N, et al. Myelodysplastic syndrome and acute myelogenous leukemia as a late clonal complication in children with acquired aplastic anemia. *Blood.* 1997;90:1009-1013.

19. Bacigalupo A, Bruno B, Saracco P, et al, for the European Group for Blood and Marrow Transplantation (EBMT) Working Party on Severe Aplastic Anemia and the Gruppo Italiano Trapianti di Midolio Osseo (GITMO). Antilymphocyte globulin, cyclosporine, prednisolone, and granulocyte colony-stimulating factor for severe aplastic anemia: an update of the GITMO/EBMT study on 100 patients. *Blood.* 2000;95:1931-1934.

Treatment of severe aplastic anemia with antithymocyte globulin and cyclosporin A with or without G-CSF in adults: a multicenter randomized study in Japan

Masanao Teramura,[1] Akiro Kimura,[2] Satsuki Iwase,[3] Yuji Yonemura,[4] Shinji Nakao,[5] Akio Urabe,[6] Mitsuhiro Omine,[7] and Hideaki Mizoguchi[1]

[1]Department of Hematology, Tokyo Women's Medical University, Tokyo; [2]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima; [3]Department of Hematology/Oncology, Aoto Hospital, Tokyo; [4]Blood Transfusion Service, Kumamoto University School of Medicine, Kumamoto; [5]Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, Kanazawa; [6]Division of Hematology, Nippon Telegraph and Telephone (NTT) Kanto Medical Center, Tokyo; [7]Division of Hematology, Internal Medicine, Showa University Fujigaoka Hospital, Yokohama, Japan

We report the results of a randomized study to elucidate whether addition of granulocyte colony-stimulating factor (G-CSF) to immunosuppressive therapy is valuable for the treatment of severe aplastic anemia (SAA) in adults. A total of 101 previously untreated patients (median age, 54 years; range, 19 to 75 years) were randomized to receive antithymocyte globulin (ATG) and cyclosporin A (CyA) (G-CSF− group) or ATG, CyA, and G-CSF (G-CSF+ group). In the G-CSF+ group, the hematologic response rate at 6 months was higher (77% vs 57%; $P = .03$) than in the G-CSF− group. No differences were observed between the groups in terms of the incidence of infections and febrile episodes. There were no differences between the G-CSF− group and the G-CSF+ group in terms of survival (88% vs 94% at 4 years), and the development of myelodysplastic syndrome (MDS)/acute leukemia (AL) (1 patient vs 2 patients). However, the relapse rate was lower in the G-CSF+ group compared with the G-CSF− group (42% vs 15% at 4 years; $P = .01$). Further follow-up is required to elucidate the role of G-CSF in immunosuppressive therapy for adult SAA. (Blood. 2007;110:1756-1761)

Introduction

Acquired aplastic anemia (AA) is a serious hematologic disorder characterized by peripheral blood pancytopenia and hypocellular bone marrow. Bone marrow transplantation (BMT) and immunosuppressive therapy (IST) are standard treatment strategies for severe AA (SAA), and the decision of initial treatment depends largely on an availability of a human leukocyte antigen (HLA)-identical sibling donor and patient age. Antithymocyte globulin (ATG) and cyclosporine A (CyA) are immunosuppressive drugs generally used for AA and had an equivalent efficacy in terms of hematologic response rate and a survival rate.[1] It has been also demonstrated that the combination of ATG and CyA is superior to ATG or CyA alone in terms of hematologic response.[2,3]

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that mainly stimulates the proliferation and differentiation of granulocyte precursors; however, a stimulatory effect of G-CSF on multipotential hematopoietic stem cells has also been demonstrated.[4] Clinically, G-CSF can induce a short-term increase in the neutrophil count in most patients with AA.[5] In addition, multilineage recovery of hematopoiesis in some patients with AA by G-CSF has been reported.[6,7] Therefore, addition of G-CSF to IST may not only decrease the risk for infection but also increase the hematologic response rate. In 1995, a European group showed promising results that ATG, CyA, and G-CSF therapy produced a high response rate (82% at a median follow-up period of 115 days), a high actuarial survival rate (92% with a median follow-up of 428 days), and a relatively low number of early deaths (8%) from infection.[8] This encouraging result formed the basis of our prospective randomized trial.

Evolution of AA to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) is a major problem in patients undergoing IST.[9-11] Because G-CSF can stimulate the growth of leukemic clones, combined use of G-CSF with IST may facilitate the progression of AA to MDS/AML.[12,13]

To elucidate whether the addition of G-CSF to IST increases the response rate, prevents infections during the treatment, improves the survival or relapse rate, and increases the risk for MDS/AML, we have started the prospective randomized controlled study comparing ATG and CyA therapy with or without G-CSF in adult patients with AA. During the period in which our study has been ongoing, 2 groups have reported the results of similar prospective randomized studies.[14,15] However, 1 study focuses on childhood AA,[14] and another includes both childhood and adult patients with AA.[15] To our knowledge, this is the first prospective randomized study to investigate the role of combined use of G-CSF and IST focusing on adult patients with AA.

Patients and methods

Patients

From June 1996 to June 2000, a total of 101 patients with acquired AA from 43 centers were enrolled. Patients with acquired AA were eligible if they

Submitted November 28, 2006; accepted May 9, 2007. Prepublished online as *Blood* First Edition paper, May 25, 2007; DOI 10.1182/blood-2006-11-050526.

The online version of this article contains a data supplement.

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.

met the following criteria: aged 18 to 75 years, newly diagnosed patients, no specific prior treatment for the disease, and severe AA. The disease was considered severe if at least 2 of the following were fulfilled: a neutrophil count of less than 0.5×10^9/L, a platelet count of less than 20×10^9/L, and a reticulocyte count of less than 20×10^9/L with hypocellular bone marrow.[16] Patients were excluded if they had been diagnosed as having Fanconi anemia or dyskeratosis congenita, severe uncontrolled infection, or malignancies. Cytogenetic studies were performed for all patients. We estimated a 30% difference in response rate between the G-CSF− group (ATG + CyA) and the G-CSF+ group (ATG + CyA + G-CSF). To detect a 30% difference, 45 patients per treatment group were required. Compensating for an estimated nonevaluability rate of 10%, it was considered reasonable to enroll at least 100 patients.

Informed written consent was obtained from all patients prior to study entry with Institutional Review Board approval at each of the participating centers and in accordance with the Declaration of Helsinki.

Treatment protocol

Patients were randomized to receive either ATG and CyA or ATG, CyA, and G-CSF. Horse ATG (Lymphoglobuline; Merieux, Lyon, France) was administered at a dose of 15 mg/kg per day for 5 days as a slow intravenous infusion over 12 hours. For the prevention of serum sickness, prednisolone was given orally at a dose of 1 mg/kg per day from day 1 to day 9, 0.5 mg/kg per day from day 10 to day 15, and 0.2 mg/kg per day from day 16 to day 21. CyA, given orally at a dose of 6 mg/kg per day, was started on day 1 and continued for at least 12 weeks. The dose was adjusted to achieve a whole-blood trough level of 150 to 250 ng/mL. In responders, CyA was continued for at least 28 weeks. In patients with a stable hematologic status for at least 4 weeks, gradual tapering of CyA (1 mg/kg every 2 weeks) was permitted if hematologic data remained stable during the course of tapering. In patients randomized to receive G-CSF, filgrastim (Gran; Kirin-Sankyo, Tokyo, Japan) or lenograstim (Neutrogin; Chugai, Tokyo, Japan) was given intravenously at a dose of 400 $\mu g/m^2$ per day and 50 $\mu g/kg$ per day, respectively, every other day until day 28, and then once or twice a week until day 84. The daily doses of filgrastim and lenograstim were those proved to be effective in clinical studies performed in Japan and approved by the Japanese Ministry for Health, Labor, and Welfare.[17,18] The primary end point of the study was the hematologic response at 12 weeks, 3 months, and 1 year after IST, and the secondary end points included the incidence of infections and febrile episodes during the first 12 weeks, survival rate, relapse rate, and incidence of the development of MDS /AL.

Evaluation of response and toxicity

Complete response (CR) was defined as a neutrophil count greater than 1.5×10^9/L, a platelet count greater than 150×10^9/L, and a hemoglobin level of greater than 110 g/L (11.0 g/dL). Partial response (PR) was defined by transfusion independence and no longer meeting criteria for severe disease.[19] Relapse was indicated by the requirement for blood transfusion. Toxicity of treatment was evaluated for the first 12 weeks and was graded according to the criteria of the World Health Organization.[20]

The Fisher Exact test was used to compare categoric variables, and the Mann-Whitney *U* test or the Student *t* test was used to compare continuous variables. The probability of survival and relapse was analyzed using the Kaplan-Meier method.[21] All statistical analyses were performed using SPSS 15.0 software (SPSS Japan, Tokyo, Japan).

Results

Patient characteristics

A total of 50 patients were randomized to receive ATG and CyA (G-CSF− group), and 51 patients were randomized to receive ATG, CyA, and G-CSF (G-CSF+ group). A total of 6 patients were excluded from analysis because of a diagnosis of lymphoma after randomization (1 patient), or treatment without ATG (5 patients) according to the patient's wishes after enrollment. Patient characteristics of the G-CSF+ and G-CSF− groups are summarized in Table 1. All patients, except 3 with hepatitis-associated AA, had no identifiable cause of AA (idiopathic AA). There were no significant differences between 2 groups in age, sex, hemoglobin level, neutrophil count, platelet count, reticulocyte count, number of patients with a neutrophil count of less than 0.2×10^9/L (ie, very severe AA [vSAA]), and interval between diagnosis and treatment. A total of 8 and 11 patients had a neutrophil count of more than 0.5×10^9/L in the G-CSF− group and in the G-CSF+ group, respectively.

Response

At 12 weeks, CR was observed in 2 (4%) patients, and PR was observed in 22 (47%) patients in the G-CSF− group, for an overall response rate of 51% (24 of 47 patients). In the G-CSF+ group, no patients had a CR and 28 (58%) patients had a PR for an overall response rate of 58% (28 of 48) (Table 2). There were no statistically significant differences in overall response rates at 12 weeks between the 2 groups ($P = .31$). At 6 months, the overall response rate increased from 51% to 57% in the G-CSF− group, and from 58% to 77% in the G-CSF+ group. The difference in overall response rates at 6 months between the 2 groups was statistically significant ($P = .03$). At 1 year, the overall response rate increased from 57% to 76% in the G-CSF− group, but did not change (from 77% to 79%) in the G-CSF+ group. There was no statistically significant difference in overall response rate at 1 year between the 2 groups ($P = .46$). In the G-CSF+ group, there were

Table 1. Patient characteristics

Characteristic	ATG + CyA	ATG + CyA + G-CSF	*P*
No. of patients randomized	50	51	—
No. of patients evaluable	47	48	—
Age, median y (range)	54 (19-75)	53 (19-74)	.55
Sex, male/female	21/26	23/25	.75
Cause of AA, no. patients			
Idiopathic	46	46	.51
Hepatisis	1	2	—
Hemoglobin, median g/L (range)	60 (35-82)	60 (31-84)	.67
Neutrophil count, median $\times 10^9$/L (range)	0.32 (0.02-1.01)	0.30 (0.01-1.21)	.45
Platelet count, median $\times 10^9$/L (range)	9 (1-38)	9 (1-31)	.62
Reticulocyte count, median $\times 10^9$/L (range)	11 (0-65)	9 (0-35)	.08
No. of patients with a neutrophil count less than 0.2×10^9/L	11	19	.07
Interval between diagnosis and treatment, median d (range)	20 (3-152)	18 (1-112)	.89

— indicates not applicable.

Table 2. Response to treatment at 12 weeks, 3 months, and 1 year after treatment

Time after treatment	ATG + CyA, no. (%)	ATG + CyA + G-CSF, no. (%)	P
12 weeks			
No. of patients evaluable	47	48	—
CR	2	0	—
PR	22	28	—
Total response, CR+PR	24 (51)	28 (58)	.31
Death	0	2	—
6 months			
No. of patients evaluable	46	47	—
CR	3	2	—
PR	23	34	—
Total response, CR+PR	26 (57)	36 (77)	.03
Death	0	2	—
1 year			
No. of patients evaluable	41	47	—
CR	1	3	—
PR	30	34	—
Total response, CR+PR	31 (76)	37 (79)	.46
Death	1	2	—

— indicates not applicable.

no differences in overall response rate between the filgrastim-treated group and lenograstim-treated group (data not shown).

When the overall response rate was analyzed focusing on the vSAA patients (ie, a neutrophil count of less than 0.2×10^9/L), it was 27% (3 of 11 patients) at 12 weeks, 20% (2 of 10 patients) at 6 months, and 63% (5 of 8 patients) at 1 year in the G-CSF− group, and was 42% (8 of 19 patients) at 12 weeks, 63% (12 of 19 patients) at 6 months, and 63% (12 of 19 patients) at 1 year in the G-CSF+ group. Similar to the result obtained in total patients, overall response rate in patients with vSAA at 6 months but not at 12 weeks and 1 year was significantly higher in the G-CSF+ group compared with the G-CSF− group (12 weeks, $P = .34$; 6 months, $P = .03$; 1 year; $P = .68$).

The overall response rate for patients with a neutrophil count of more than 0.5×10^9/L at 12 weeks, 6 months, and 1 year was 50% (4 of 8 patients), 63% (5 of 8 patients), and 86% (6 of 7 patients) in the G-CSF− group, and 64% (7 of 11 patients), 64% (7 of 11 patients), and 91% (10 of 11 patients) in the G-CSF+ group, respectively. There was no significant difference in overall response between the 2 groups (12 weeks, $P = .45$; 6 months, $P = .51$; 1 year, $P = .64$). A total of 7 patients had chromosomal abnormalities at diagnosis, and 4 patients responded to IST.

When patients who responded to immunotherapy but needed continuous administration of CyA to maintain hematologic response were defined as CyA dependent, 8 patients in the G-CSF− group and 6 in the G-CSF+ group were included.

The median neutrophil counts at 4 weeks, 12 weeks, 6 months, and 1 year was 0.36×10^9/L, 1.32×10^9/L, 1.20×10^9/L, and 1.35×10^9/L in the G-CSF− group and 1.03×10^9/L, 1.19×10^9/L, 1.61×10^9/L, and 1.57×10^9/L in the G-CSF+ group, respectively. At 4 weeks but not 12 weeks, 6 months, and 1 year, the median neutrophil count in the G-CSF+ group was significantly higher compared with the G-CSF− group ($P = .004$).

A total of 3 patients (1 in the G-CSF− group and 2 in the G-CSF+ group) who failed to respond to initial therapy received a second course of IST; however, no patients responded. A total of 2 patients (both patients were in the G-CSF− group) who failed to respond to initial therapy received a BMT. One patient who received a transplant from an HLA-matched sibling is alive at 44 months after transplantation, but another patient who received a transplant from an HLA-matched unrelated donor died of pulmonary bleeding 2 months after transplantation.

Infectious complications

During the first 12 weeks, infections developed in 19 (40%) patients in the G-CSF− group and in 28 (58%) patients in the G-CSF+ group (Table 3). There was no significant difference in the proportion of patients with documented infection between the 2 groups ($P = .07$). Severe infections (grade 3 or 4) developed in 5 patients in the G-CSF− group and 8 patients in the G-CSF+ group, and the difference in the proportion of patients who contracted severe infections between the 2 groups was not statistically significant ($P = .29$). There were 31 infectious events in the G-CSF− group, including 6 severe infectious events such as bacteremia (5 events) and pneumonia (1 event), and 39 infectious events in the G-CSF+ group, including 11 severe infections such as bacteremia (7 events), pneumonia (3 events), and fungemia (1 event). There was no difference in the incidence of infectious events between the 2 groups ($P = .07$). Among 13 infectious events, including bacteremia, pneumonia, and fungal infection, observed in the G-CSF+ group, 7 (54%) occurred during the period of a neutrophil count less than 0.5×10^9/L.

To investigate the correlation between infection and the degree of neutropenia, the morbidity of infection in patients with vSAA and non-vSAA was compared, and was higher in patients with vSAA than in those with non-vSAA (61% in vSAA, 45% in non-vSAA); however, the difference was not statistically significant ($P = .07$).

The median number of febrile days (38°C or higher) was 4 days in both groups. Deaths due to infection during the first 12 weeks occurred in 2 patients (bacteremia and fungemia) in the G-CSF+ group.

Survival

The overall probability of survival at 4 years is 88% for the G-CSF− group and 94% for the G-CSF+ group, with a median follow-up period of 52 months (range, 1 to 78 months) and 54 months (range, 1 to 86 months), respectively (Figure 1). There was no significant difference in the overall probability of survival between the 2 groups ($P = .44$). In the G-CSF+ group, there were no differences in survival rate between the filgrastim-treated group and lenograstim-treated group (data not shown). There were 6 deaths in the G-CSF− group, and 4 in the G-CSF+ group. Causes of death were bacteremia (1 patient each in the G-CSF− and G-CSF+ groups), pneumonia (1 patient in the G-CSF− group), fungal infection (1 in the G-CSF+ group), intracranial hemorrhage (1 patient each in the G-CSF− and G-CSF+ groups), BMT-related toxicity (1 patient each in the G-CSF− and G-CSF+ groups), renal failure (1 in the G-CSF− group) and metastatic brain tumor (1 in the G-CSF− group).

Cytogenetic analysis and clonal disease

Before therapy, a clonal cytogenetic abnormality was detected in 7 (8%) of 91 evaluable patients (4 patients with −Y, 2 patients with deletion 13, and 1 patient with trisomy 8). Among 7 patients with cytogenetic abnormalities, 1 patient (−Y) randomized to receive G-CSF developed refractory anemia with ringed sideroblasts (RARS) at 14 months after treatment, and 2 patients (1 with −Y, 1 with trisomy 8) not randomized to receive G-CSF experienced the disappearance of clonal abnormalities during the follow-up period.

Table 3. Documented infections and febrile episodes during 12 weeks after initiation of treatment

	G-CSF⁻ group	G-CSF⁺ group	*P*
n	47	48	—
No. of patients with documented infections*; severe infection, grade 3 or 4	19; 5	28; 8	.07; .30
Accumulated number of infectious events†	31	39	.07
Bacteremia	5	7	—
Pneumonia	1	3	—
Upper respiratory tract infection	5	6	—
Intestinal infection	2	4	—
Urinary tract infection	1	0	—
Genital infection	0	2	—
Cellulitis	1	2	—
Herpes zoster	1	0	—
Herpes simplex	2	4	—
Fungal infection	0	3	—
Gingivitis	1	0	—
Fever of unknown origin	12	8	—
Febrile days over 38°C, median no. (range)	4 (1-17)	4 (1-38)	.83
Death due to infection, no. patients	0	2	.25

— indicates not applicable.
*Patients who had fever of unknown origin were included.
†Events of fever of unknown origin were included

In the G-CSF− group, chromosomal abnormalities appeared after treatment in 2 (6%) of 33 evaluable patients (1 with −Y and 1 with trisomy 8), with a subsequent disappearance of the chromosomal abnormality in 1 patient with −Y. In the G-CSF+ group, chromosomal abnormalities appeared after treatment in 6 (15%) of 41 evaluable patients (1 with −Y, 2 with monosomy 7, 1 with inv 7, 1 with trisomy 8, and 1 with monosomy 19), with a subsequent disappearance of the chromosomal abnormalities in 3 patients (1 with −Y, 1 with inv 7, and 1 with monosomy 19). All patients who developed chromosomal abnormalities after IST had revealed normal cytogenetics before IST.

There was no significant difference in the incidence of development of chromosomal abnormalities between the 2 groups ($P = .21$). In 8 patients in whom chromosomal abnormalities appeared after treatment, 2 patients (1 with −Y in the G-CSF− group and 1 with monosomy 7 in the G-CSF+ group) developed refractory anemia (RA).

As for the development of the clonal diseases, including MDS, AML, and paroxysmal nocturnal hemoglobulinemia (PNH), 1 patient developed RA at 38 months and 1 patient developed PNH at 25 months after treatment in the G-CSF− group, 2 patients developed MDS (RA and RARS) at 14 and 40 months, and 1 patient developed PNH at 41 months after treatment in the G-CSF+ group. No patients had definitive PNH before therapy. The presence of PNH was defined by a positive Ham test or loss of expression of CD55 and CD59 on red blood cells by flow cytometry. The overall risk for MDS/AML at 4 years is 3% for the G-CSF− group and 5% for the G-CSF+ group. There were no significant differences in the overall risk for MDS/AML between the 2 groups ($P = .63$). One patient who developed RA received peripheral blood stem-cell transplantation from an HLA-matched sibling and died of chronic graft-versus-host disease 5 months after transplantation.

Relapse

A total of 21 patients (15 in the G-CSF− group and 6 in the G-CSF+ group) relapsed after IST. In relapsed patients, all patients had received CyA at the time of relapse or had a history of CyA administration for at least for 6 months. The risk for relapse at 4 years was 42% in the G-CSF− group, and 15% in the G-CSF+ group (Figure 2). There was a significant difference in relapse rate between the 2 groups ($P = .01$). A total of 6 patients who relapsed after initial responses received a second course of IST, of whom 4 were in the G-CSF− group and 2 in the G-CSF+ group. Of the 6 patients 2 (33%) responded to the second therapy. Both responders belonged to the G-CSF+ group. A total of 2 patients (both in the G-CSF+ group) who relapsed after initial responses received BMT (1 from a HLA-matched sibling and another from an HLA-matched unrelated donor) and were alive at 41 months and 32 months after transplantation.

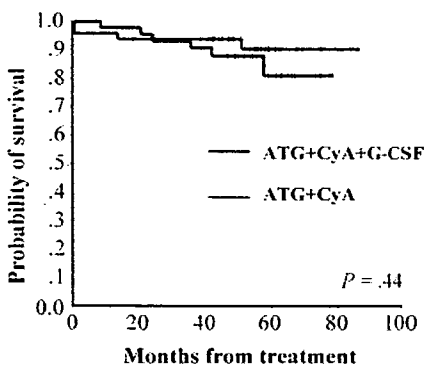

Figure 1. Actuarial survival of adult patients with SAA in the G-CSF− and G-CSF+ groups.

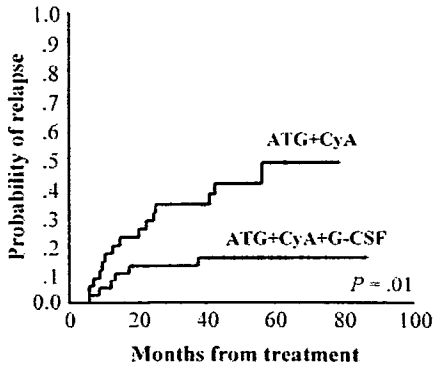

Figure 2. Cumulative incidence of relapse in adult patients with SAA in the G-CSF− and G-CSF+ groups.