TABLE 2. Clinical characteristics of immune responses after reduced-intensity cord-blood transplantation

Type of immune responses	PIR	ES	GvHD
Number of patients with immune responses/number of evaluable patients	35/45	36/44	29/44
Fever (median, range)	39.8 (37.5–41.2)	39.0 (36.8–40.4)	39.2 (37.2–40.7)
Skin rash	16	15	21
Diarrhea	19	20	23
T-Bil >2.0 mg/dL	10	21	18
Body weight gain >10% of baseline	14	3	0
Central nervous system complications	0	5	0
Serum levels of C-reactive protein (mg/dL) (median, range)	14.1 (2.3–25.6)	6.5 (0.2–23)	8.3 (0.9–38.6)
Use of corticosteroid	23	25	25
Response to cortisosteroid (CR/PR/MR/NC)	14/5/3/1	4/4/1/16	15/7/1/2

PIR, preengraftment immune reactions; ES, engraftment syndrome; GvHD, graft-versus-host disease; CR, complete response; PR, partial response; MR, minimal response; NC, no change.

Among the 23 patients given corticosteroid, response was as follows: complete response (CR) (n=14), partial response (PR) (n=5), minimal response (MR) (n=3), and no change (NC) (n=1). PIR subsided spontaneously in the remaining 12 patients in whom corticosteroid had not been administered.

Engraftment Syndrome

Of the 45 patients who achieved engraftment, 44 were included in the analyses of ES, with the remaining patient being excluded because of documented *P. aeruginosa* septicemia. ES developed in 36 patients. Cumulative incidence of PIR or ES was 78% (Fig. 3). Clinical characteristics of ES are shown in Table 2. Five patients with ES developed central nervous system toxicity: cyclosporine neurotoxicity (n=1), limbic encephalopathy (n=2), and metabolic encephalopathy (n=2). No pathogens including bacteria, fungi, or viruses were cultured from cerebrospinal fluid in the five patients. Corticosteroid was given to 25 patients with ES, with the following response: CR (n=4), PR (n=4), MR (n=1), and NC (n=16). Corticosteroid was more frequently required in the patients with preceding PIR and ES than in those with de novo ES (21/27 vs. 4/9), and in the 14 patients with preceding PIR, ES was refractory to corticosteroid.

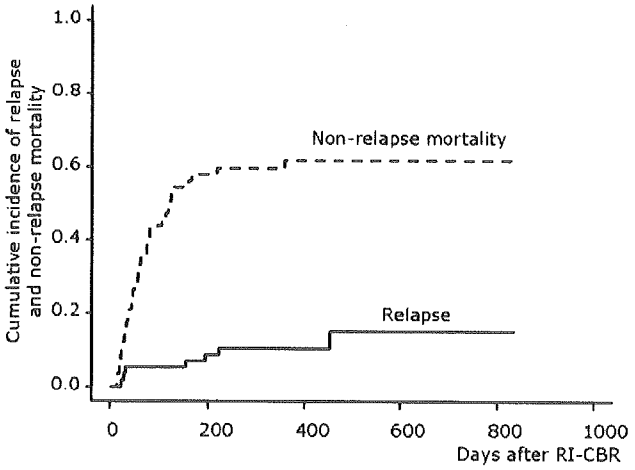

FIGURE 3. Cumulative incidences of relapse and nonrelapse mortality at day 180 were 62% and 15%, respectively.

Postengraftment Immune Reactions (Acute GvHD)

Of the 45 patients who survived longer than 6 days after engraftment, 44 patients were included in the analysis of GvHD. The other patient was excluded because of *E. fecalis* bacteremia. Thirty patients developed acute GvHD: grade I (n=1), II (n=9), III (n=13), and IV (n=7). Skin or gastrointestinal biopsy was conducted in 25 patients. GvHD was histopathologically confirmed in all of these patients. Histopathologic examination was not conducted in the remaining five patients. Cumulative incidence of grade I to IV acute GvHD, treating death without GvHD as a competing risk, was 51% (Fig. 4). It was not possible to differentiate GvHD from

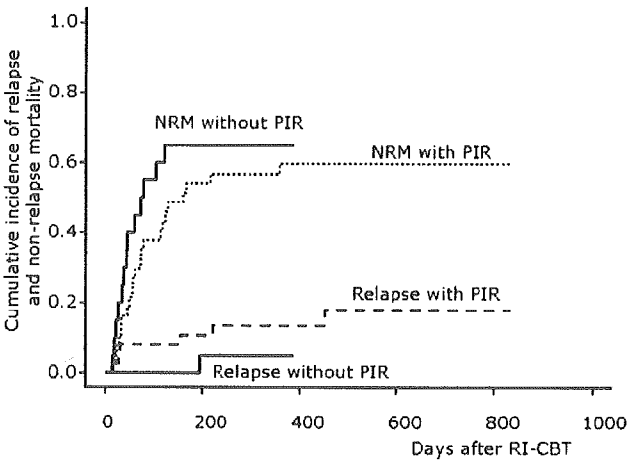

FIGURE 4. Cumulative incidences of relapse and nonrelapse mortality (NRM) for patients grouped by the presence or absence of PIR. Cumulative incidences of relapse were 18% in patients with PIR and 5% in those without it (P=0.32). Cumulative incidences of nonrelapse mortality were 60% in patients with PIR and 65% in those without it (P=0.35).

preceding PIR or ES in 15 patients who displayed continuous symptoms of immune reactions. Acute GvHD developed after resolution of PIR or ES in 12 patients. Cumulative incidences of grade I to IV acute GvHD grouped by the presence or absence of PIR were 57% and 40%, respectively (P=0.16).

Organ involvement was as follows: skin stage 1 (n=6), stage 2 (n=6), stage 3 (n=9), and stage 4 (n=1); liver stage 1 (n=6), stage 2 (n=5), stage 3 (n=8), and stage 4 (n=7); gut stage 1 (n=3), stage 2 (n=13), and stage 3 (n=5). Among the 25 patients given corticosteroid to treat GvHD, responses were CR (n=15), PR (n=7), MR (n=1), and NC (n=2).

Nonrelapse Mortality

Thirty-two patients died without disease progression. Cumulative incidences of relapse and NRM at day 180 were 15% and 62%, respectively. Causes of NRM comprised acute GvHD (n=5), interstitial pneumonitis (n=2), thrombotic microangiopathy (n=3), heart failure (n=2), cytomegalovirus infection (n=2), invasive aspergillosis (n=2), miliary tuberculosis (n=1), cerebral hemorrhage (n=2), bacteremia (n=7), pneumonia (n=3), multiple organ failure caused by PIR (n=1), alveolar hemorrhage (n=1), and gastrointestinal bleeding (n=1).

Effect of PIR, ES, and GvHD on Relapse and NRM

Cumulative incidences of relapse were 18% and 5% in patients with and without PIR, respectively (P=0.32). Cumulative incidences of NRM were 60% and 65% in patients with and without PIR, respectively (P=0.35). Because development of ES was closely associated with PIR, these two reactions could not be separated from each other in evaluation of their effect on relapse and NRM. Development of GvHD was not a significant prognostic factor for relapse or NRM when PIR was treated as a time-dependent covariate.

DISCUSSION

We have demonstrated that most patients exhibited some immune reactions, whereas a certain proportion of findings were accounted for by infection and regimen-related toxicity. In particular, it is likely that diarrhea was in some cases caused by melphalan, which has dose-limiting gastrointestinal toxicity (*31*). However, the development of similar reactions in most patients suggests that these reactions are characteristic of RI-CBT. PIR developed during posttransplant myelosuppression. When compared with ES and GvHD, the higher CRP levels and fever observed in PIR suggest that the inflammation occurring in this reaction is intense. Although optimal treatment remains unknown, corticosteroid was administered at the discretion of the primary physician. Most patients responded to corticosteroid, although PIR occasionally progressed and merged with ES and GvHD despite immunosuppressive treatments. Because cytokine storm associated with PIR might trigger the development of ES or GvHD, suppression of PIR could be effective in reducing NRM. This small-sized study failed to show a prognostic impact of PIR, and the clinical significance of this reaction awaits further investigation.

The mechanism of PIR remains unknown. Pathologic examination of the skin obtained from six patients showed edema and vascular dilatation without lymphocytic infiltration. Interestingly, PIR occurred in patients who had not achieved engraftment, suggesting that the mechanism of PIR differs from that of ES/GvHD. The reaction is probably related to the response of adult recipients to transplanted cord blood rather than to the cord-blood engraftment. Antithymocyte globulin (ATG) and corticosteroid, which have strong immunosuppressive properties, were commonly used in CBT (*6, 8–11, 14, 17, 32–34*), whereas neither was used in this study. Immune reactions after CBT might therefore have manifested more easily with the present regimen. PIR could be attributed to a cytokine storm induced by massive proliferation of cells with a unique cytokine profile. Another possibility is homeostasis-driven proliferation of naive T cells in highly immunosuppressed individuals, as demonstrated in murine models (*35*). This reaction is associated with cytotoxic cytokines (*35*). However, fever as a transient response to contamination with maternal blood or cells during cord-blood collection cannot be excluded (*36*), and reactivation of virus such as human herpesvirus 6 might be associated with PIR (*37*).

The reaction at engraftment was similar to the reaction known as ES after autologous transplantation (*27*). The inflammation occurring in ES was less intense than that observed in PIR, as evidenced by less marked fever, weight gain, and CRP elevation (Table 2). In this regard, corticosteroid, which was given for PIR and continued during the manifestation of ES, might have masked the inflammatory reaction of ES. Surprisingly, five patients with ES developed central nervous system complications, with two diagnosed as having limbic encephalopathy. This type of neurologic complication has not been emphasized in allo-SCT using marrow or peripheral blood and might be characteristic of CBT (*38*). Fluid accumulation during this period might have accentuated the tendency for brain edema. Engraftment processes may differ between CBT and conventional allo-SCT.

Postengraftment reaction was characterized by a higher incidence of jaundice and a lower incidence of edema when compared with PIR and ES. Clinical manifestation was consistent with the immune reaction conventionally known as acute GvHD. Although the incidence of GvHD after CBT for adult patients has been reported to be low, the incidence of grade II to IV acute GvHD varies from 25% to 72% (*9–12, 21–24, 39*) and has not been thoroughly investigated. In the present study, the incidence of grade I to IV acute GvHD was 51%. GvHD is a significant problem in RI-CBT as well as in conventional myeloablative CBT. Cord blood might have the potential to elicit an intense graft-versus-host effect, creating a niche for early engraftment and GvL effects.

Few studies have described the immune reaction after CBT, and none have characterized PIR and ES in CBT. In the present study, there are several possible reasons for these immune reactions being distinct. First, we only enrolled adult patients because children develop GvHD less frequently than do adults (*5, 6, 8*). Second, the median nucleated cell dose in our study (2.9×10^7 /kg) was greater than that reported in certain studies performed in Western countries (*9–12*). The low median body weight (53.8 kg) among the Japanese patients in this study might have favored engraftment and immune reactions. Third, 84% of our patients received cord blood from donors mismatched at tow to three HLA loci. The

association between HLA disparity and the risk of GvHD remains unclear in CBT. Although most studies have failed to show a significant relationship between HLA disparity and the risk of GvHD (*5, 8, 14, 33*), a recent multivariate analysis of the largest series showed a significant association between acute GvHD and HLA disparity (*40*). Fourth, our conditioning regimen, which did not include ATG and used cyclosporine alone for GvHD prophylaxis, was mild, allowing the manifestation of immune reactions.

Although the present study provides an important description of the immune reaction after RI-UCBT, it contains certain limitations. This was a small retrospective study, and unrecognized bias caused by heterogeneous patient background might have influenced the results. Furthermore, the diagnostic criteria for immune reactions based on clinical and pathologic findings could not exclude infection or toxicity from various drugs including conditioning regimens. Therefore, it is possible that incidence of immune reactions was overestimated, particularly for PIR developing during neutropenia. In contrast, immunosuppressive treatments (mostly corticosteroid) for preceding complications could have masked the incidence and severity of ES and GvHD. ES has some similarities to acute GvHD, and it is sometimes difficult to make an accurate diagnosis of these complications. Further investigations are warranted to reveal the mechanism of immune reactions after RI-CBT and to develop a strategy of their control without reducing GVL effects.

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Mycophenolate Mofetil Is Effective and Well Tolerated in the Treatment of Refractory Acute and Chronic Graft-versus-Host Disease

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Abstract

We enrolled 11 patients with refractory graft-versus-host disease (GVHD) in a prospective trial evaluating the efficacy of mycophenolate mofetil (MMF). Four (67%) of the 6 patients with acute GVHD and all 5 patients with chronic GVHD responded to MMF. Ten (91%) of the 11 patients were able to decrease steroid use (median decrease, 86%; range, 25%-100%). After a median follow-up of 18 months (range, 1-65 months), 7 patients (64%) remained alive. The adverse events were infectious complications (36%), diarrhea (27%), and neutropenia (18%); the only patient discontinuing MMF did so because of grade 4 neutropenia. This preliminary study suggests that MMF is a well-tolerated agent and has a beneficial effect in the treatment of refractory acute and chronic GVHD.
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Key words: Mycophenolate mofetil; Allogeneic stem cell transplantation; Mismatched donor; Graft-versus-host disease

1. Introduction

Graft-versus-host disease (GVHD) is a major cause of morbidity and mortality after allogeneic stem cell transplantation (SCT) [1]. Cyclosporin A (CSA), tacrolimus (FK506), and steroids are effective in the treatment of both acute GVHD and established chronic GVHD [1-3]. However, patients who fail to respond to standard therapy have a poor prognosis [4,5]. The therapeutic options for these patients are limited, and salvage therapies have produced disappointing results to date [6-11].

Mycophenolate mofetil (MMF; CellCept; Roche Diagnostics, Indianapolis, IN, USA) is an ester prodrug of the active immunosuppressant mycophenolic acid, which is a noncompetitive reversible inhibitor of inosine monophosphate dehydrogenase [12-14]. This inhibition blocks the de novo synthesis of guanosine nucleotides, necessary substrates for DNA and RNA synthesis. Lymphocytes depend on this pathway and do not possess the salvage pathways of other cells [14]. This drug has been successfully tested in multicenter randomized trials for preventing renal transplant rejection [15] and has been used in limited trials for the treatment of acute and chronic GVHD [12,16-23]. These retrospective reports have suggested that MMF is an effective agent in these settings. The toxicity profile of MMF, such as upper and lower enteritis, cytopenia, and lack of renal toxicity, is not cross-reactive with the toxicity profiles of CSA, tacrolimus, and steroids, making MMF an attractive candidate for combination therapy.

In February 2000, we began a prospective single-center study in which we analyzed the efficacy of MMF in combination with CSA, tacrolimus, or steroids in the treatment of acute and chronic GVHD in a series of 11 allograft recipients with refractory GVHD.

2. Patients and Methods

2.1. Patients

Eleven patients with refractory GVHD who had undergone allogeneic SCT between December 1997 and April 2004 were enrolled in this prospective trial. Eligibility criteria were the presence of refractory acute or chronic GVHD after treatment with steroids, CSA, and/or tacrolimus, and the absence of relapse at the time of study enrollment. The protocol received Institutional Review Board approval, and

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signed informed consent was obtained from every patient before study entry.

The patients' characteristics are shown in Table 1. The median age was 46 years (range, 28-66 years). The patients had undergone matched sibling (n = 7), related (n = 3), or unrelated (n = 1) allogeneic transplantation without T-cell depletion. GVHD prophylaxis included CSA and methotrexate for 6 patients, CSA alone for 2 patients, and tacrolimus and methotrexate for 2 patients. Antithymocyte globulin was administered in association with CSA and methotrexate to 1 patient who had received a related transplant mismatched at 2 loci.

2.2. GVHD Treatment

The assessment and grading of acute and chronic GVHD were primarily based on clinical findings and were carried out by following the commonly accepted diagnostic criteria [9,10,24,25]. Diagnosis was supported by skin, liver, or gut biopsies whenever indicated and clinically possible. The ocular involvement of chronic GVHD was diagnosed by the Schirmer test. Patient 1 developed skin and liver disease early after cord blood transplantation. The diagnosis of acute GVHD for this patient was based on skin and liver biopsy results, and the patient showed refractoriness to combination treatment with CSA and methylprednisolone (mPSE), suggesting a lower possibility of periengraftment syndrome after cord blood transplantation.

First-line treatment for acute GVHD of grade II or higher or for chronic GVHD consisted of a combination of CSA or tacrolimus with steroids. mPSE was initially administered to patients with acute GVHD of grade II to IV at a dosage of 2 mg/kg per day for 1 to 2 weeks; then the patients were switched to prednisolone (PSE). The tapering schedule for PSE was a dosage reduction of 0.1 to 0.2 mg/kg per week in the responsive cases. PSE was initially administered to patients with chronic GVHD at a dosage of 1 mg/kg and then tapered slowly. If partial or complete resolution of symptoms did not occur or if patients became dependent on steroids (defined as the need for >30 mg/day PSE for more than 6 weeks), they were considered refractory to treatment and were switched to MMF therapy. The blood levels of CSA and tacrolimus of all patients who had been given these drugs reached their target points before MMF treatment was initiated.

MMF was started at a dosage of 1500 mg/day except for 1 patient (no. 1), who received MMF at a dosage of 1000 mg/day because of low body weight (<50 kg) and coexisting pancytopenia. MMF was given orally, and the starting dose was maintained if it was tolerated. Patients were treated with MMF in addition to CSA and steroids (n = 2), tacrolimus and steroids (n = 6), or steroids alone (n = 3). The median time from GVHD onset to the initiation of MMF treatment was 17 days (range, 7-55 days) for acute GVHD and 82 days (range, 59-560 days) for chronic GVHD. The duration of therapy ranged from 30 days to more than 900 days (median, 133 days).

2.3. GVHD Monitoring

Response to MMF was assessed for each organ involved, as has been described previously [1,12,18,20]. A complete response was defined as complete resolution of clinical and/or biological signs (skin changes, digestive symptoms, bilirubin level, oral lesions, and joint, lung, and ocular clinical manifestations) that allowed a decrease in dosage or the discontinuation of steroid treatment. A partial response was defined as an improvement in but not a resolution of these clinical and/or biological signs. Stable disease was defined as stable organ involvement. An evaluation of no response referred to the progressive worsening of chronic GVHD. The patients were regularly monitored by full clinical and laboratory evaluations and by pathologic examinations in some cases. Adverse events attributed to MMF were graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0).

3. Results

3.1. Response to MMF in Refractory Acute GVHD

Response, complication, and survival data from the 6 patients who received MMF treatment for refractory acute GVHD are listed in Table 2. Four (67%) of the 6 patients responded to MMF treatment (Table 2). Although acute GVHD of the gut in patient 3 was resolved by MMF treatment, the patient was considered a nonresponder because of no response to the acute GVHD of the skin and liver. The median time for a patient to show initial signs of response to MMF was 13 days (range, 5-63 days). This interval was calculated as the time to the first objective signs of any improvement, not as the time to maximum response. The responses of these 6 patients according to the involved organs are shown in Figure 1. There was no preference for response according to involved organs.

3.2. Response to MMF in Refractory Chronic GVHD

All 5 patients with refractory chronic GVHD responded to MMF therapy and survived thereafter, allowing a decrease in the dosage or discontinuation of steroid treatment in 4 patients (Table 2). The median time to show initial signs of a response was 50 days (range, 27-180 days). Dosage reduction or discontinuation of steroid treatment was possible in 4 of the 5 patients.

3.3. Toxicity and Complications

The most common adverse event associated with MMF treatment was diarrhea, which occurred in 3 patients (27%). One patient (no. 9) had to discontinue MMF treatment because of grade 4 neutropenia that was attributed to MMF. Another patient (no. 2) also developed grade 4 neutropenia but required only a dosage adjustment. There were 6 infectious episodes during treatment (cytomegalovirus [CMV] antigenemia, n = 3; CMV pneumonia, n = 1; *Pseudomonas* septicemia, n = 2). The 2 patients with acute GVHD who did not respond to MMF therapy died of progressive acute GVHD and infection. Two other patients experienced relapse of disease while receiving MMF and died of disease progression.

Table 1.
Patient Characteristics*

Patient	Age, y/ Sex	Diagnosis	Donor (Sex)	Graft	Conditioning Regimen	GVHD Prophylaxis	Indication to MMF, GVHD Duration before MMF Tx, d	aGVHD Onset Posttransplantation, d/Grade/Sites	aGVHD Tx before MMF (Response)	cGVHD Onset Posttransplantation, d/Grade/Sites	cGVHD Tx before MMF	Concomitant Tx with MMF
1	66/M	ALL (CR)	2 Loci mis, UR (F)	CB	FL/L-PAM/ TBI4	CSA	aGVHD, 12	12/IV/skin, liver	CSA, mPSE (NR)	NE	—	CSA, mPSE
2	30/F	Marginal zone B-cell lymphoma (PR)	2 Loci mis, REL (F)	PB	CY/Ara-C/ TBI12	FK506/ MTX	aGVHD, 18	11/II/skin	FK506, mPSE, PUVA (NR)	No	—	FK506, mPSE
3	46/M	CML (CP)	Matched, REL (M)	PB	CY/TBI12	FK506/ MTX	aGVHD, 16	21/III/skin, liver, gut	FK506, mPSE (NR)	No	—	FK506, mPSE
4	28/M	CML (BC)†	2 Loci mis, REL (M)	PB	FL/BU	CSA/MTX/ ATG	aGVHD, 7	8/III/skin, liver	CSA, mPSE (skin, CR; liver, NR)	NE	—	CSA, mPSE
5	53/F	Diffuse large B-cell lymphoma (refractory)†	Id sibling (M)	PB	FL/BU	CSA	aGVHD, 44	13/III/skin, gut	FK506, mPSE (skin, NR; gut, CR)	No	—	FK506, mPSE
6	33/M	Nasal NK/T lymphoma (PR)	Id sibling (F)	PB	CY/TBI12	CSA/MTX	aGVHD, 55	36/III/skin, liver, gut	FK506, PSE (NR)	No	—	FK506, PSE
7	61/M	AML (CR)	Id sibling (M)	PB	FL/BU	CSA/MTX	cGVHD, 126	25/III/skin, liver, gut	CSA, mPSE (CR)	82/ext/liver, mouth, ocular	CSA, PSE	PSE
8	32/F	AML (CR)	Id sibling (M)	PB	CY/Ara-C/ TBI12	CSA/MTX	cGVHD, 285	35/II/skin, liver	CSA, PSE (CR)	560/ext/skin, liver	PSE	PSE
9	32/M	CML (CP)	Id sibling (M)	PB	CY/Ara-C/ TBI12	CSA/MTX	cGVHD, 5	25/III/skin, liver, gut	FK506, mPSE (skin/liver, NR‡; gut, CR)	59/ext/skin	FK506, PSE	FK506, PSE
10	52/M	AML from RAEB	Id sibling (F)	PB	CY/Ara-C/ TBI12	CSA/MTX	cGVHD, 1776	No	—	79/ext/liver, mouth, ocular	FK506, PSE	FK506, PSE
11	59/M	ALL (CR)	Id sibling (M)	PB	FL/BU	CSA/MTX	cGVHD, 235	No	—	126/ext/liver	PSE	PSE

*GVHD indicates graft-versus-host disease; MMF, mycophenolate mofetil; Tx, therapy; aGVHD, acute GVHD; cGVHD, chronic GVHD; ALL, acute lymphoblastic leukemia; CR, complete response; mis, mismatched; UR, unrelated; CB, cord blood; FL, fludarabine; L-PAM, melphalan; TBI, total body irradiation; CSA, cyclosporin A; mPSE, methylprednisolone; NR, no response; NE, nonevaluable; PR, partial response; REL, relative; PB, peripheral blood stem cell; CY, cyclophosphamide; Ara-C, cytarabine; FK506, tacrolimus; MTX, methotrexate; PUVA, psoralen and ultraviolet A irradiation; CML, chronic myeloid leukemia; CP, chronic phase; BC, blast crisis; BU, busulfan; ATG, antithymocyte globulin; Id, identical; NK/T, natural killer/T-cell; PSE, prednisolone; AML, acute myeloid leukemia; ext, extensive; RAEB, refractory anemia with excess of blasts.

†Patient 4 had a history of allogeneic stem cell transplantation, and patient 5 had a history of autologous stem cell transplantation.

‡Progressive type of chronic GVHD of the skin and liver developed subsequently.

Table 2.
Response and Toxicity*

Patient	Response to MMF (Time from MMF Initiation to Response, d)					Reduction in Steroid Dosage†	MMF Tx Duration, (Cause of Disruption)	Infections during MMF Tx	Adverse Events (Grade)	Outcome (Cause of Death), Time Posttransplantion
	Skin	Liver	Gut	Joints	Ocular	Mouth				
1	NR	NR	—	—	—	—	63%	30 d (death)	*Pseudomonas* septicemia, CMV-Ag	No	Dead (aGVHD, *Pseudomonas* septicemia), 54 d
2	CR (63)	—	—	—	—	—	100%	384 d (efficacy)	CMV-Ag	Neutropenia (4)‡	Alive CR, 26+ mo
3	NR	NR	CR (7)	—	—	—	80%	111 d (death)	*Pseudomonas* septicemia, CMV pneumonia	No	Dead (aGVHD, CMV pneumonia), 147 d
4	—	CR (5)	—	—	—	—	87%	204 d (relapse)	CMV-Ag	No	Dead (relapse), 209 d
5	CR (10)	—	—	—	—	—	90%	89 d (relapse)	No	No	Dead (relapse), 145 d
6	CR (15)	CR (15)	CR (15)	—	—	—	80%	133 d (efficacy)	No	diarrhea (3)‡	Alive CR, 68+ mo
7	—	CR (70)	—	—	CR (70)	CR (360)	100%	825 d (efficacy)	No	No	Alive CR, 25+ mo
8	PR (57)	SD	—	—	—	—	25%	110 d (efficacy)	No	Diarrhea (1)‡	Alive CR, 66+ mo
9	SD	PR (27)	—	—	—	—	85%	56 d (neutropenia)	No	Neutropenia (4)‡	Alive CR, 64+ mo
10	—	PR (180)	—	—	PR (180)	PR (180)	0%	30+ mo	No	Diarrhea (1)‡	Alive CR, 91+ mo
11	—	CR (31)	—	—	—	—	100%	16+ mo	No	No	Alive CR, 27+ mo

*CMV-Ag indicates cytomegalovirus antigenemia; SD, stable disease. Other abbreviations are expanded in the first footnote to Table 1.
†Percent reduction in steroid dosage at the end of MMF treatment or at last follow-up.
‡Neutropenia in patients 2 and 9 was resolved with a reduction in MMF dosage and MMF discontinuation, respectively. Diarrhea in patients 6, 8, and 10 was resolved with supportive medication.

4. Discussion

Even with the best immunosuppressive regimens using CSA, tacrolimus, and steroids, many patients still succumb to acute and chronic GVHD. These patients are likely to die of GVHD itself or from infectious complications secondary to prolonged immunosuppression, as well as to the depression of their immune system by GVHD [1-11]. We attempted to improve the prognosis of such patients by combining MMF with other commonly used immunosuppressive agents. Four (67%) of 6 patients with refractory acute GVHD responded with no subsequent development of chronic GVHD, and MMF therapy was eventually stopped in 2 of these patients because of successful outcomes. Additionally, all 5 patients with refractory chronic GVHD who were treated with MMF showed improvements of clinical symptoms, and MMF was discontinued in 2 patients. These results seemed comparable to the outcomes reported for previous studies on treatment of acute GVHD (response rates, 31%-71%) and chronic GVHD (response rates, 46%-77%) [12,16-23]. In addition, the administration of MMF allowed a dosage reduction of steroids in 10 of the 11 patients. The remaining patient (no. 10), who had been treated with a combination of 7.5 mg PSE daily and the maximum dose of tacrolimus before the initiation of MMF therapy, became free of tacrolimus treatment despite continuing the same PSE dosage thereafter. These findings suggest that MMF may be an effective salvage treatment for refractory GVHD.

Although all 5 patients with chronic GVHD in the current study have maintained good clinical conditions after the initiation of MMF treatment, only 2 patients (33%) with acute GVHD have survived. The difference between the 2 groups in the rate of response to MMF may partly account for this observation. Another explanation is that 4 of the 6 patients with acute GVHD had advanced disease at the time of transplantation, whereas only 1 of the 5 patients with chronic GVHD had advanced disease.

Several reports have shown that the response to MMF developed within 2 months after MMF introduction, irrespective of whether acute or chronic GVHD was targeted [20,22,23]. The median time for a patient to show initial signs of response to MMF treatment was 31 days (range, 5-180 days) in the present study. This interval was the time to the start of any improvement and not the time to maximum response. Of note is that 3 (33%) of 9 responders began to show improvements in GVHD more than 2 months after MMF initiation (at 63, 70, and 180 days). These findings suggest that MMF should be continued for at least 3 months to provide an opportunity for late responses to develop.

MMF was generally well tolerated. Of note is that treatment with MMF was not discontinued for adverse events except in a single patient who responded to MMF but experienced grade 4 neutropenia that required the discontinuation of MMF therapy. Other adverse events were resolved with supportive medication or by reducing the MMF dosage. Our findings may serve to strengthen the advantage of MMF, which causes a relatively small number of adverse events including nephrotoxicity and liver toxicity compared with other new immunosuppressive drugs [19].

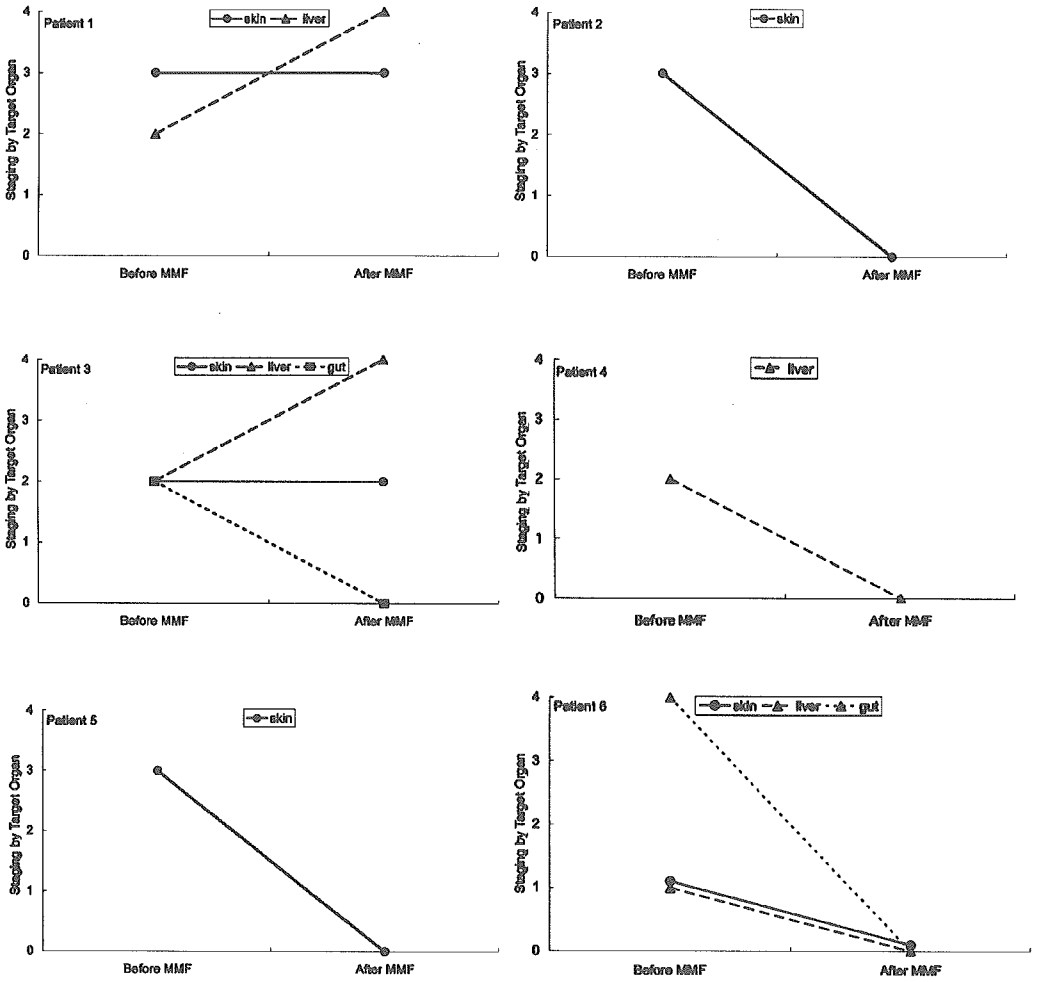

Figure 1. Response to mycophenolate mofetil (MMF) by target organ in 6 patients with acute graft-versus-host disease.

Six opportunistic viral or bacterial infections occurred in 4 of the patients. Two patients died from infection (*Pseudomonas* septicemia in one patient and CMV pneumonia in the other) coinciding with progressive acute GVHD, which developed while these patients received MMF. These findings may be consistent with previous reports that the use of MMF in allogeneic SCT was an independent risk factor for CMV infection [26] and was associated with a high risk of serious viral or bacterial infections [20,22]. However, it is difficult to accurately ascertain the negative impact of MMF on infectious complications in such a small retrospective study that lacks a comparison group in terms of salvage GVHD treatment.

In the current study, 2 patients relapsed during MMF therapy, although both patients were at high risk of relapse at the time of transplantation (Tables 1 and 2). Recently, Shapira et al [27] reported that MMF does not impair graft-versus-leukemia (GVL) effects or reduce lymphokine-activated killer cell activity in mice, whereas CSA had already been shown in mice [28] and in clinical practice [29] to suppress the GVL effects inducible by allogeneic donor lymphocytes. A study that compared tacrolimus with CSA for GVHD prophylaxis has shown that the relapse rate among recipients of HLA-matched transplants from siblings was significantly higher in the tacrolimus group than in the CSA group [30], indicating that tacrolimus may compromise the GVL effects more significantly than CSA. However, whether MMF treatment is irrelevant to disease relapse is still unknown.

No patients in the current study developed thrombotic microangiopathy (TMA) during treatment with MMF. TMA is a syndrome of microangiopathic hemolytic anemia, thrombocytopenia, and renal dysfunction [31]. The association of TMA with immunosuppressive agents given after SCT, such as CSA, tacrolimus, and sirolimus, is well established [31,32]. Despite the unknown etiology of TMA, the pathologic finding of endothelial injury is commonly seen in patients with TMA. Of note is that no literature review has reported that MMF induces endothelial toxicity. These findings suggest that MMF, if used instead of CSA and tacrolimus, could have a benefit in decreasing the risk of TMA after SCT.

These preliminary results support the hypothesis that MMF can be used safely and has encouraging efficacy in the treatment of patients with GVHD who fail to benefit from conventional therapy. We emphasize that our results may have been influenced by the small number of patients in this study, and it is difficult to draw a final conclusion. In addition, MMF reduced the requirement for steroids, thereby reducing the risk of complications due to iatrogenic immunosuppression. A prospective randomized clinical trial is warranted to assess the impact of MMF in the treatment of refractory GVHD. The early combination of MMF with other treatment strategies may further improve the response rate and survival of these patients. Additional studies are also needed to test this hypothesis.

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Stem Cell Transplantation

High incidence of cytomegalovirus reactivation in adult recipients of an unrelated cord blood transplant

This retrospective analysis for cytomegalovirus (CMV)-seropositive adult transplant recipients showed that CMV antigenemia occurred after transplantation in 10/10 (100%) recipients of unrelated cord blood, 17/39 (43%) recipients of a related matched donor graft, 16/23 (79%) recipients of an unrelated matched donor graft, and 8/12 (67%) recipients of a mismatched related donor graft. These results suggest that unrelated cord blood transplantation itself may be correlated with a high incidence of CMV reactivation.

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Cytomegalovirus (CMV) infection is still a major concern following allogeneic hematopoietic transplantation because CMV pneumonia is fatal in 70% of patients, even when treated with a combination of antiviral therapies and CMV hyperimmune immunoglobulin.[1] Allogeneic cord blood transplantation, especially from unrelated donors, has progressively gained favor as treatment for patients with both malignant and non-malignant disorders.[2-4] As compared to allogeneic bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT), advantages of unrelated cord blood transplantation (UCBT) include ease and safety of cell collection, low risk of transmitting viral infections, prompt availability of stem cells, and reduced incidence and severity of graft-versus-host disease (GVHD).[2-4] The reduction of GVHD after UCBT is likely due to the naïve state of cord blood lymphocytes and the low cytotoxic capacity of cord blood T cells.[5] However, such immunological immaturity after UCBT can place a patient at risk of early infectious complications, accounting for most transplant-related deaths, especially in adults.[1,6] We have observed that patients undergoing UCBT appear to be at increased risk of CMV infection. Ninety-one consecutive adult patients who were CMV-seropositive and received non-T-cell-depleted allogeneic transplants at the Kanazawa University Hospital between April 1999 and April 2004 were eligible for inclusion. Written informed consent was obtained from all patients. Six patients died of regimen-related toxicities before engraftment and one developed primary graft rejection followed by autologous hematopoietic recovery. The remaining 84 patients had

Table 1. Patient characteristic.

Characteristics	Stem cell donor: HLA identical sibling	HLA matched unrelated donor	HLA mismatched relative	Unrelated CB
No. of patients	39	23	12	10
Sex, male/female	23/16	10/13	5/7	6/4
Median age (range), years	53 (14-69)	36 (17-54)	43 (15-58)	61 (15-69)
Disease				
Acute myelogenous leukemia	9	6	0	2
Acute lymphoblastic leukemia	4	6	6	2
Chronic myeloid leukemia	3	4	2	0
Myelodysplastic syndrome	6	2	0	1
Non-Hodgkin's lymphoma	7	3	3	1
Sever aplastic anemia	4	2	1	1
Myelofibrosis	1	0	0	0
Renal cell carcinoma	4	0	0	3
Osteosarcoma	1	0	0	0
Standard risk/advanced risk*	18/21	14/9	3/9	1/9
Stem cell source				
PBSC/BM	33/6	0/23	10/2	0/0
HLA disparity				
0/1/2/3	39/0/0/0	23/0/0/0	0/2/6/3/1	3/1/6/0
CMV-seropositive donor	35	22	11	0
Prior transplantation	4	2	1	5
Conditioning regimen				
Myeloablative/Reduced-intensity	15/24	18/5	7/5	1/9
GVHD prophylaxis				
CSP-based/FK506-based	38/1	11/11	7/5	7/3
Use of ATG	5	3	2	1
Use of steroids	13	4	8	6
Use of MMF	3	0	5	5
Survival >100 days, %	92	87	75	78
Survival >365 days, %	82	83	40	56

*PBSC, peripheral blood stem cell; BM, bone marrow; CB, cord blood; CSP, cyclosporine; FK506, tacrolimus; MMF, mycophenolate mofetil. *Acute leukemia in first remission, chronic myeloid leukemia in the first chronic phase, myelodysplastic syndrome with refractory anemia or refractory anemia with ringed sideroblasts, malignant lymphoma in any remission, and aplastic anemia were defined as standard-risk diseases. All other patients were classified as having advanced disease.*

Table 2. Acute GVHD and CMV infection according to stem cell donor.

	Stem cell donor			
	HLA-identical sibling	*HLA-matched unrelated donor*	*HLA-mismatched relativedonor*	*Unrelated CB*
II-IV acute GVHD	14/39 (36)	8/23 (35)	8/12 (67)	5/10 (50)
CMV antigenemia (%)	17/39 (44)	16/23 (70)	8/12 (67)	10/10 (100)
Days between transplantation and first antigenemia, median (range)	43 (20-99)	29 (18-47)	38.5 (5-95)	32.5 (0-42)
Days between final and first antigenemia, median (range)	14 (1-117)	21.5 (0-80)	94 (0-161)	60 (7-104)
Peak no. of CMV-positive cells among $5x10^4$ leukocytes, median (range)	10 (1-395)	8 (1-714)	15 (4-250)	46 (7-543)
CMV disease (%)	1/39 (3)	1/23 (4)	0/12 (0)	1/10 (10)
Late CMV antigenemia (%)	3/36 (9)	1/19 (5)	5/9 (56)	3/7 (43)

successful initial engraftment and were included in the analysis. The patients' characteristics are given in Table 1. CMV antigenemia assays were carried out as previously described.[7,8] In brief, heparinized blood samples were fractionated by dextran sedimentation. Slides were prepared in duplicate after cytocentrifugation; 1.5 x 105 leukocytes were fixed with formaldehyde and stained with HRP-C7 monoclonal antibodies that specifically bind the pp65 antigen of CMV (Teijin, Tokyo, Japan). The degree of CMV antigenemia was expressed as the number of CMV antigen-positive cells per $5x10^4$ leukocytes. For the evaluation of CMV antigenemia, $5x10^4$ leukocytes were always analyzed, because the detection limit was one CMV antigen-positive cell per $5x10^4$ leukocytes in this assay.[7,8] CMV antigenemia was defined as ≥1 antigen-positive cell.[7,8] For the diagnosis of CMV disease, such as pneumonia, gastroenteritis, retinitis, and hepatitis, the CMV antigenemia had to be accompanied by clinical symptoms, signs, and histologic confirmation.[9] Late CMV antigenemia was defined as that occurring after day 100. Ganciclovir or foscarnet was used as pre-emptive therapy to prevent CMV disease. The decision to use preemptive therapy was based entirely on a positive antigenemia test (≥3 antigen-positive cells/$5x10^4$ leukocytes).[7,8] Ganciclovir was administered as an intravenous infusion at the dose of 5 mg/kg/b.i.d. Neutropenic patients (absolute neutrophil count, less than 750/μL) were given foscarnet instead of ganciclovir; the induction dose of foscarnet was 60 mg/kg intravenously every 12 hours, followed by maintenance doses of 90 mg/kg once daily.[10] Treatment was stopped if two consecutive CMV antigenemia assays were negative. Granulocyte colony-stimulating factor was administered when the absolute neutrophil count was <500/μL. Previous reports demonstrated the high sensitivity of the HRP-C7 assay and validated the analyzed cell count and the cut-off we relied on in our study.[7,8]

All UCBT recipients developed CMV antigenemia whereas 44% of the recipients of related matched donor grafts, 70% of the recipients of unrelated matched donor grafts, and 67% of those receiving mismatched related donor transplants did so (Table 2). CMV-associated disease occurred in three patients (4%), gastroenteritis in two and interstitial pneumonia in one. Of these three patients only one patient, who developed interstitial pneumonia after UCBT, died of CMV disease. Forty-one patients (80%) received antiviral therapy; ganciclovir was used in 20 patients, foscarnet in 5, and the combination of both in 16. In the remaining 10 patients, CMV antigenemia remained below the detection level and disappeared without antiviral therapy.

Although our data still require confirmation in a larger prospective study, the impact of UCBT on the development of CMV antigenemia might be considered when designing future transplant strategies, at least until more effective methods for prophylaxis of CMV reactivation become available.

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Stem Cell Transplantation

Two allogeneic hematopoietic stem cell transplantations without the use of blood-product support

We successfully performed two allogeneic hematopoietic stem cell transplantations from matched unrelated donors without the use of blood-product support after treosulfan-based conditioning in two women with acute myeloid leukemia who were Jehovah's witnesses and refused transfusions of blood products.

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In the last two years we were confronted with a mother and her daughter with acute myelogenous leukemia (AML) who were both members of the community of Jehovah's witnesses, a religious group that refuses transfusion of any major blood product.

Despite their religious objection to blood products we offered both induction chemotherapy and allogeneic hematopoietic stem cell transplantation as consolidation therapy, which they accepted. We felt able to propose this strategy for two reasons: (i) based on our experience with a stringent therapeutic platelet transfusion protocol that we have developed during the last years, we know that severe thrombocytopenia can be managed without prophylactic platelet transfusion. In more than 200 patients (during induction chemotherapy for AML or after autologous peripheral stem cell transplantation) we have shown that a therapeutic transfusion strategy is safe. In one third of our patients autologous transplantation could be performed without any platelet transfusions. Bleeding complications among patients transfused on demand were completely comparable to those among our former patients who received prohylactic platelet transfusions at a trigger platelet count of 10×10^9/L;[1,2] (ii) we used allogeneic stem cell transplantation after a reduced toxicity conditioning regimen as consolidation treatment since hematologic regeneration could be expected to be significantly quicker than after repeated cycles of high-dose cytosine arabinoside as consolidation. The same is true for autologous transplantation because stem cells should be collected only after a minimum of two intensive courses of chemotherapy as *in vivo* purging. The risks of graft-versus-host disease (GVHD) after allogeneic transplantation and its higher probability of cure had to be weighed against the greater hematologic and non-hematologic toxicity of the alternative procedures.

Table 1. Patients' characteristics and follow-up.

	Mother	*Daughter*
Diagnosis	AML-M1	AML-M2
Karyotype	Normal female	Normal female
Status of remission before transplantation	CR	CR
Age at transplantation	48	21
Conditioning regimen	TBI/Fludarabine/Treosulfan	TBI/Fludarabine/Treosulfan
Donor	Matched unrelated male	Matched unrelated male
Blood group P/D	A Rh+/A Rh+	A Rh+/A Rh+
CMV	P^-/D^-	P^-/D^-
Source of stem cells	Bone marrow	Peripheral blood
No. of transplanted cells ($CD34\times10^6$/kg)	1. 64	8.4
Immunosuppression (oral)	CSA/MMF(4×500mg)	CSA/MMF(4×500mg)
Hematological toxicity		
Leukocytes $<1.0\times10^9$/L (days)	6	14
Neutrophils $<0.5\times10^9$/L (days)	12	18
Platelets $<20\times10^9$/L[$<10\times10^9$/L]	2 [0]	3 [0]
Minimal hemoglobin (g/dL)	11	9
Chimerism analysis (FISH)	> 90% donor, day +15, ongoing	> 90% donor, day +12, ongoing

TBI: total body irradiation; P: patient; D: donor; CSA: cyclosporine A; MMF: mycophenolatmofetil; FISH: fluorescent in situ hybridization.

In the daughter we favored allogeneic transplantation despite normal cytogenetics because her AML was diagnosed as a first relapse after a chemotherapy-treated AML as a child more than 10 years previously. The mother was informed that allogeneic transplantation from a matched unrelated donor is not standard therapy in AML in first remission without high-risk cytogenetics. Both patients were informed on the extraordinary risks of refusing blood transfusions during the treatment of AML. Both patients gave their written informed consent.

The characteristics of the patients, their treatment and the follow-up are shown in Table 1. A complete remission was achieved after dose-reduced induction chemotherapy with daunorubicin (50 mg/m^2×2) and cytosine arabinoside (100 mg/m^2 for 5 days as a continuous infusion). Once HLA-identical unrelated donors had been identified for each patient we started conditioning in both with a combination of a marrow ablative dose of treosulfan (3×10 g/m^2)

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

Chiharu Sugimori, Tatsuya Chuhjo, Xingmin Feng, Hirohito Yamazaki, Akiyoshi Takami, Masanao Teramura, Hideaki Mizoguchi, Mitsuhiro Omine, and Shinji Nakao

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

Introduction

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

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

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

Patients, materials, and methods

Patients

We evaluated PNH-type cells in peripheral-blood samples from 122 Japanese patients (55 men and 67 women; median age, 56 years) with idiopathic AA (75 severe and 47 moderate AA) before they received IST. The patients were diagnosed with AA at Kanazawa University Hospital, hospitals participating in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions. The severity of AA was classified according to the criteria proposed by Camitta et al.[20] All patients were treated with ATG Lymphoglobuline (Aventis Behring, King of Prussia, PA) 15 mg/kg/d, 5 days; plus CsA (Novartis, Basel, Switzerland) 6 mg/kg/d; within 1 year of diagnosis between April 1999 and December 2004. The dose of CsA was adjusted to maintain trough levels between 150 and 250 ng/mL, and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (G-CSF; filgrastim, 300 $\mu g/m^2$ or lenograstim, 5 $\mu g/kg$) was administered to some patients. Response to IST was evaluated according to the response criteria described by Camitta.[21] Complete response (CR) was defined as hemoglobin normal for age, neutrophil count more than 1.5×10^9/L, and platelet count more than 150×10^9/L. Partial response (PR) was defined as transfusion independent and no longer meeting criteria for severe disease in patients with severe AA, and it was defined as transfusion independence (if previously dependent) or doubling or normalization of at least one cell line or increase in baseline hemoglobin of more than 30 g/L (if initially less than 60 g/L), neutrophil count of more than 0.5×10^9/L (if initially less than 0.5×10^9/L), and platelet count of more than 10×10^9/L (if initially less than 20×10^9/L) in patients with moderate AA. The patients provided written, informed consent to participate in all procedures associated with the study, which was reviewed and approved by the ethical committee of Kanazawa University Hospital (study no. 46). The study also conforms to the recently revised tenets of the Helsinki protocol.

High-resolution 2-color flow cytometry

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

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

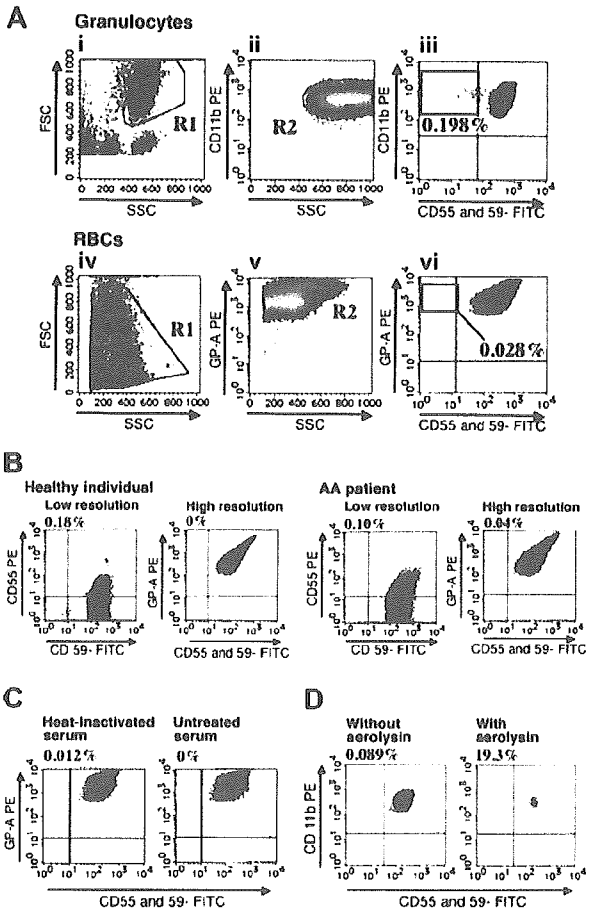

Figure 1. Validity of high-resolution flow cytometry. (A) An example of analysis on a patient with PNH^+ AA is shown. Gates were set up to exclude SSC^{dim} (i) and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs (ii,v). Cells within rectangles showing horizontal distribution represent PNH-type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

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

Modified Ham test

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

Aerolysin treatment of granulocytes

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

Statistics

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

Results

Validity of high-resolution flow cytometry

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

Proportions of PNH-type cells in patients with AA

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

Response to ATG and CsA therapy

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

Prognosis after IST

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

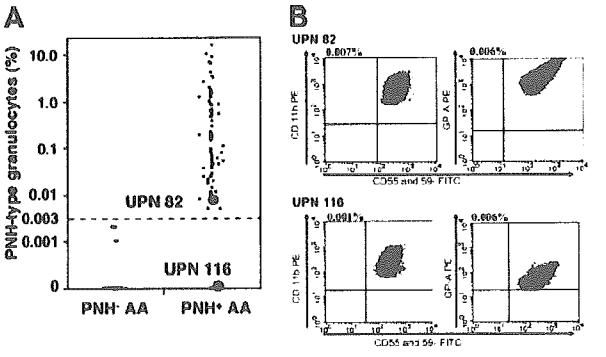

Figure 2. Proportions of PNH-type granulocytes. (A) Proportions of $CD55^-CD59^-$ granulocytes in each patient. (B) Histograms from one patient with PNH^+ (UPN 82) with minimal PNH-type cells and from another patient with increased PNH-type cells only in RBCs (UPN 116).

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

	PNH⁺	PNH⁻	*P*
No. of patients	83	39	NA
Median age, y (range)	57 (13-83)	54 (12-83)	.16
Sex, M/F	36/47	19/20	.58
Severity, severe/moderate	53/30	22/17	.43
Chromosome abnormality, no. of patients	7	3	.88
−7	0	1	
+8	2	1	
−Y	3	0	
Others	2	1	
Median WBC count, × 10^9 (range)	2.1 (0.5-4.3)	1.9 (0.7-3.2)	.15
Median neutrophil count, × 10^9/L (range)	0.53 (0.02-2.2)	0.49 (0.01-2.7)	.65
Median hemoglobin level, g/L (range)	67 (32-140)	67 (40-108)	.92
Mean corpuscular volume, fL (range)	101.5 (84.2-123.5)	98.5 (77.2-118.0)	.13
Median platelet count, × 10^9/L (range)	14.0 (2.0-60.0)	16.0 (1.0-87.0)	.65
Median reticulocyte count, × 10^9/L (range)	19.0 (3.0-90.0)	24.0 (2.0-106.0)	.50
Median time from diagnosis to IST, d (range)	30 (1-334)	33 (2-268)	.46
No. of patients who received G-CSF during IST	25	12	.94

NA indicates not applicable.

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

Changes in PNH-type granulocytes after IST

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

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

Discussion

An increase in the proportion of PNH-type cells in peripheral blood has been implicated in the immune pathophysiology of bone marrow failure.[10] Several studies including our previous investigation found a correlation between an increase in the proportion of PNH-type cells and a favorable response to IST among patients with MDS[11,12,15] and with AA.[16,26] However, the clinical application of these findings has been hampered. Small patient cohorts and the relatively low prevalence of an increased number of PNH-type cells in these studies have led to concerns about unreliability of the correlation. The present study based on a larger number of patients with recently diagnosed AA conclusively demonstrated that a minor population of PNH-type cells predicts a good response to IST as well as good prognosis for patients with AA after IST.

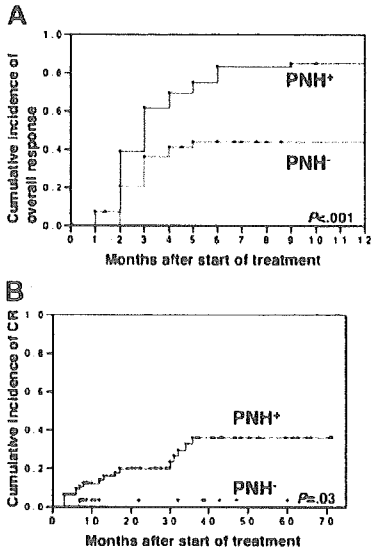

Figure 3. Response to immunosuppressive therapy. Incidence of overall (A) and complete (B) responses in patients with PNH⁺ and PNH⁻.

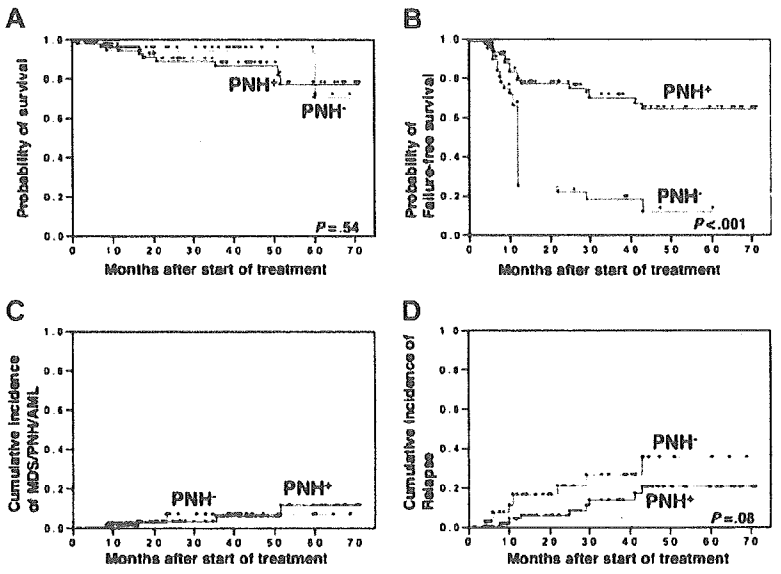

Figure 4. Prognosis after IST compared between patients with PNH$^+$ and with PNH$^-$. (A) Overall survival; (B) failure-free survival; (C) incidence of clonal hematologic disorders, including PNH, myelodysplastic syndrome, and acute myelogenous leukemia; and (D) incidence of relapse.

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

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

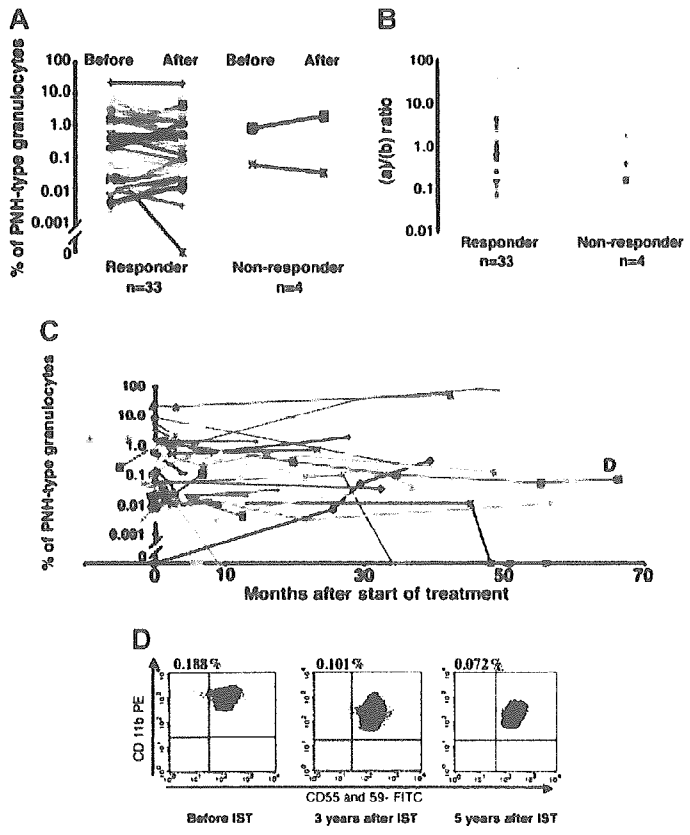

Figure 5. Changes in proportions of PNH-type granulocytes associated with responses to IST. (A) Change in responders and nonresponders. (B) Proportions of granulocyte counts after and before IST determined for PNH-type (a) and normal-type (b) granulocytes and ratios of PNH-type granulocytes (a) to normal-type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

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

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

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

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

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