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ORIGINAL ARTICLE

Reduced-intensity unrelated cord blood transplantation for treatment of metastatic renal cell carcinoma: first evidence of cord-blood-versus-solid-tumor effect

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We report a 69-year-old man with cytokine-resistant metastatic renal cell carcinoma treated with reduced-intensity unrelated cord blood transplantation. The patient achieved durable donor engraftment with minimal graft-versus-host disease. The patient showed regression of metastatic disease, providing the first evidence of a graft-versus-tumor effect on a solid tumor resulting from cord blood graft.
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Keywords: reduced-intensity unrelated cord blood transplantation; metastatic renal cell carcinoma; graft-versus-tumor effect

Introduction

Metastatic renal cell carcinoma (RCC) is resistant to standard radiotherapy or chemotherapy, and patients with this disease have a poor outlook.[1] Although immunotherapy with cytokines such as interleukin 2 and interferon alpha can lead to regression of RCC in some patients, the response rate for these treatments remains around 10–20%, and response is usually temporary.[2] Recently, allogeneic stem cell transplantation utilizing mobilized peripheral blood from a matched donor has been investigated as an alternative immunotherapeutic strategy for the treatment of advanced RCC. The results of pilot reduced-intensity transplant trials for metastatic RCC are encouraging and show that responses can occur in patients with advanced metastatic disease that has failed to respond to conventional cytokine-based therapy.[1,3–15]

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Unrelated cord blood (UCB) is considered an alternative hematopoietic stem cell source for transplantation, and its use in adult patients with hematologic disorders is increasing.[16–21] Thus far, UCB transfer has not been attempted in patients with a solid-organ malignancy such as RCC. Here, we report a patient with metastatic RCC treated with reduced-intensity unrelated cord blood transplantation (RI-UCBT).

A 56-year-old man with clear cell RCC of his right kidney underwent a right nephrectomy in March 1991. Six years later, metastatic diseases were found in the right upper jaw and pancreas and were partially removed. The remaining metastases grew and new metastases developed in the left lung, left kidney, retroperitoneal space and subcutaneous space. The patient was treated with a 12-week course of combination therapy of subcutaneous interferon alpha $2\,MU/m^2$ and interferon gamma $2\,MU/m^2$ five times per week. However, these metastases showed a progressive increase in the size. Because of the low probability of response to further conventional treatment for metastatic RCC, the patient was referred to our institute in February 2004 at the age of 69 years. Then, serum LDH level was 286 IU/l (normal range, 0–250), hemoglobin level 10.6 g/dl, serum calcium level 9.3 mg/dl and erythrocyte sedimentation rate 38 mm/h. Reduced-intensity allogeneic stem cell transplantation was considered in order to decrease regimen-related toxicity, but because of the lack of a suitable donor candidate among his family members, unrelated RI-UCBT was planned. The patient gave written informed consent to participate in an institutional review board-approved investigational protocol designed to evaluate graft-versus-tumor (GVT) effects in metastatic RCC after nonmyeloablative allogeneic transplantation. The preparative regimen, which was based on a previous report,[22] consisted of cyclophosphamide, 50 mg/kg, on day −6, fludarabine, $40\,mg/m^2$, daily on days −6 to −2, and a single dose of 200 cGy of total body irradiation on day −1. UCB, phenotypically mismatched at one HLA-B antigen and one DRB1 antigen, was obtained through the Japanese Cord Blood Bank Network (J-CBBN). The patient received a UCB graft at a dose of 2.0×10^7 nucleated cells/kg of recipient body weight in

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March 2004. To prevent rejection of the graft and graft-versus-host disease (GVHD), intravenous cyclosporine A (1.5 mg/kg b.i.d.) and oral mycophenolate mofetil (15 mg/kg b.i.d. until neutrophil engraftment) were started 3 days before transplantation. Granulocyte colony-stimulating factor (G-CSF) was initiated on day 1. The patient developed poor engraftment with at most 50% of peripheral blood granulocytes of donor origin. This resulted in graft rejection, with complete autologous recovery on day 41. One hundred and six days after first transplant, the patient received a second UCB graft from J-CBBN containing 2.2×10^7 nucleated cells/kg of recipient body weight, which was phenotypically mismatched at one HLA-B antigen and one DRB1 antigen. Conditioning therapy consisted of fludarabine, 25 mg/m^2, daily on days −7 to −3, melphalan, 80 mg/m^2, on day −2 and a single dose of 400 cGy of total body irradiation on day −1, as previously reported.[18] A continuous infusion of tacrolimus, 0.03 mg/kg, was started from 3 days before transplant for prophylaxis of GVHD and graft rejection. G-CSF was started on day 1. The patient tolerated the conditioning regimen well and exhibited rapid engraftment, with neutrophil rising above 5×10^8/l by day 15. Chimerism analysis of blood on day 20 after second transplant revealed 100% donor origin in both myeloid and T-lymphoid lineages. On day 47, grade II acute GVHD of the skin and gut developed. Acute GVHD improved rapidly after increasing doses of tacrolimus without corticosteroid therapy, but it became dependent on the treatment of tacrolimus. The tacrolimus was finally tapered off at 11 months, and thereafter no GVHD developed. Treatment response was evaluated monthly after transplantation according to the Response Evaluation Criteria in Solid Tumors (RECIST).[23] A computed tomography (CT) scan at 2 months showed substantial regression of metastasis in the left kidney and retroperitoneal space (Figure 1), and the patient was determined as partial remission (PR). The PR had lasted for 3 months until new metastatic lesions in the liver and pancreas appeared at 5 months after second transplantation, defined as progressive disease (PD). At the onset of PD, he had active GVHD of the gut, which was treated with oral tacrolimus alone. Metastatic lesions progressed in size very slowly until 18 months after second transplantation, but since then, they have been unchanged until the time of this writing. The association of the onset of GVHD with the development of PR as well as that of discontinuation of the immunosuppression with no further progression of disease is suggestive of a GVT effect in this patient. The patient continues to show a good performance 26 months after second transplantation without active GVHD.

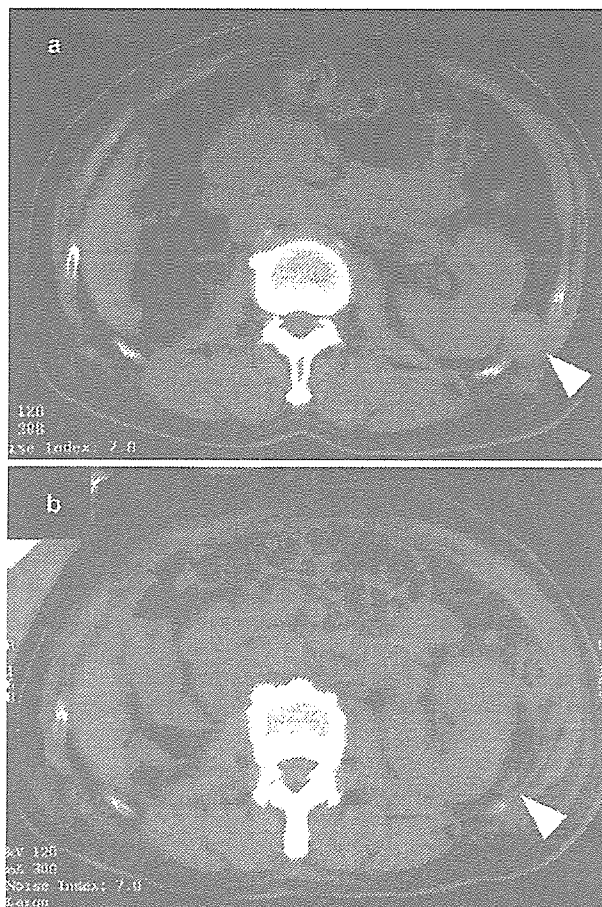

Figure 1 CT images of retroperitoneal metastasis (arrowheads) in a patient before second transplantation (a) and 2 months after second transplantation (b). Regression in the patient was concordant with the onset of acute GVHD of the skin and gut.

Discussion

Metastatic RCC is the solid tumor in which a GVT effect has been most expected. Childs *et al.*[3] and Childs and Otterud[24] have reported that of 50 patients with metastatic RCC who underwent allogeneic peripheral blood stem cell transplantation (PBSCT), 22 (44%) showed a disease response including four complete responses and 18 PRs, and five (10%) patients had a mixed response. However, the worldwide clinical experience of allogeneic SCT for metastatic RCC is limited, with approximately 200 cases reported in the literature.[3, 15,24] One of the major restrictions of this approach is the requirement that transplant candidates have an HLA-identical related donor. This requirement may limit the use of allogeneic stem cell transplantation to a minority of patients with metastatic RCC. Our patient achieved long-term survival following RI-UCBT, despite the lack of a suitable donor candidate among his family members.

Cord blood, which is collected from the umbilical cord and placenta of healthy newborns, is an alternative source of hematopoietic stem cells.[25] Compared to adult peripheral blood or bone marrow, cord blood contains a greater proportion of highly proliferative hematopoietic progenitor cells,[26] which may account for myeloid and lymphoid reconstitution after cord blood transplantation (CBT) despite the presence of fewer cells (by 1–2 logs) in cord blood than in bone marrow or mobilized peripheral blood.

It was originally thought in CBT that the immunological naivety of cord blood lymphocytes[26] might produce a

lowered GVT effect at the expense of a lower GVHD incidence. However, clinical studies revealed similar rates of disease relapse and lower rates of acute and chronic GVHD in adult patients with hematologic malignancies receiving CBT compared to those receiving allogeneic bone marrow transplantation or PBSCT.[17,19,21] Although it remains unclear whether such favorable effects also occur in patients with metastatic RCC who undergo CBT, several observations support the hypothesis that similar alloimmune effects mediated by donor T cells could work in these patients.[24,27 30] Although the target antigens in GVT effects after allogeneic transplantation against metastatic RCC have not been determined, clinical and laboratory observations suggested that minor histocompatibility antigens (mHAs) could be mainly involved as target antigens in GVT effects for metastatic RCC after PBSCT, and donor T cells responding to mHAs could be generated.[24,27,28] The fact that cord blood can generate cytotoxic T cells specific for the mHA in the same way as peripheral blood and bone marrow[29,30] might imply that mHA-specific donor T cells contributive to a GVT effect against metastatic RCC are inducible in a patient receiving a cord blood graft as well.

As allogeneic stem cell transplantation is associated with many and sometimes severe toxic effects, we used a reduced-intensity conditioning regimen as described in previous reports,[18,22] which included low-dose total body irradiation in combination with cyclophosphamide and fludarabine or with melphalan and fludarabine. This RI-UCBT regimen proved to be well tolerated and achieved durable donor engraftment with minimal GVHD. Although our patient required a second RI-UCBT because of graft refection after first RI-UCBT, the demonstrated feasibility of secondary transplantation may be of benefit in the treatment of older cancer patients with RI-UCBT. Of note, the observation in the patient that retroperitoneal and renal metastasis regressed, despite a mixed response, provides the first evidence of a GVT effect by a cord blood graft on RCC.

The advantages of CBT are the immediate availability of cells, the absence of risk to the donor and a lower need for HLA compatibility between the donor and the recipient.[16 22] Because of the establishment of many cord blood banks, nearly every patient can find a potential cord blood graft, suggesting that CBT could substantially expand the use of allogeneic transplantation in patients with metastatic RCC. Despite these potential advantages, there are several disadvantages such as susceptibility to graft rejection, prolonged recovery of hematopoiesis and unavailability of donor lymphocyte infusions. A clinical study focusing on minimizing toxicities and controlling infectious complications as well as enhancing GVT effects is needed to optimize the success of CBT for treatment of advanced RCC.

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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].

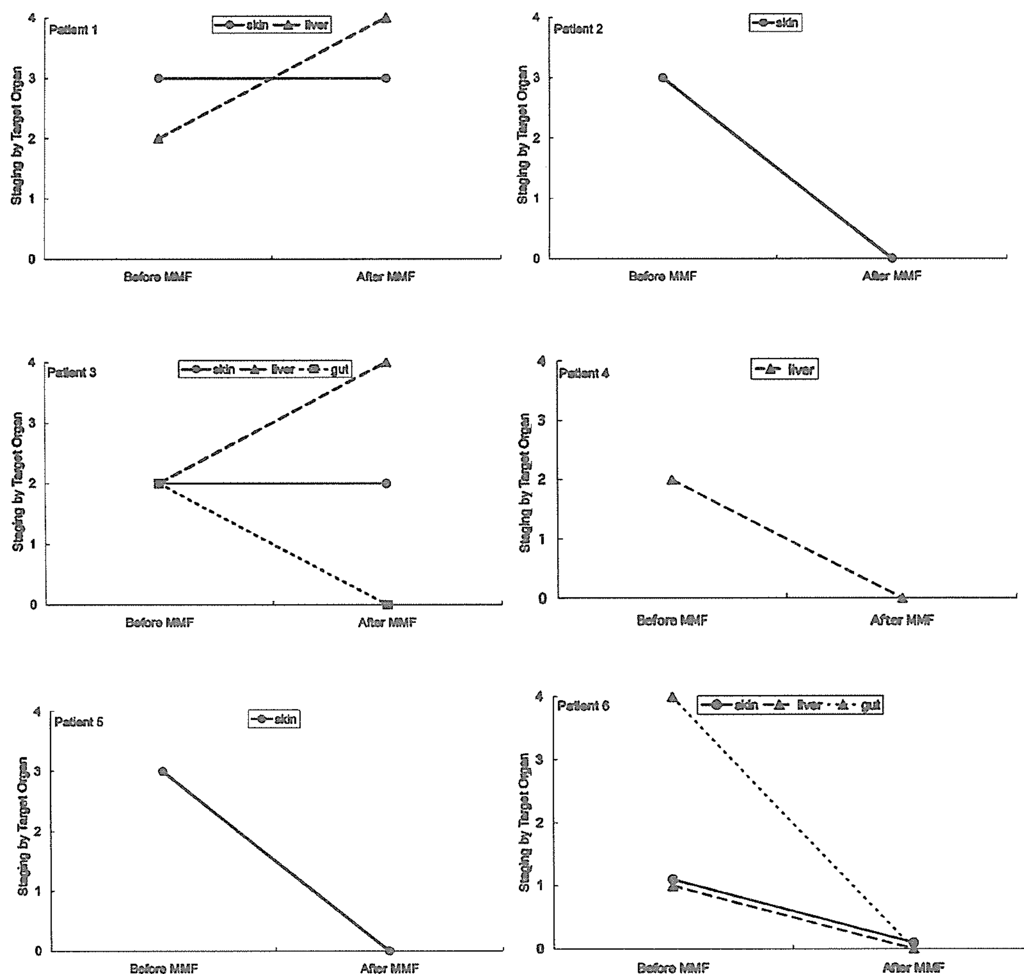

Figure 1. Response to mycophenolate mofetil (MMF) by target organ in 6 patients with acute graft-versus-host disease.

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

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We investigated the clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria (PNH)–type blood cells in patients with acquired aplastic anemia (AA). We quantified $CD55^-CD59^-$ granulocytes and red blood cells (RBCs) in peripheral blood from 122 patients with recently diagnosed AA and correlated numbers of PNH-type cells and responses to immunosuppressive therapy (IST). Flow cytometry detected 0.005% to 23.1% of $GPI\text{-}AP^-$ 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)

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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 ($GPI\text{-}AP^-$), 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 μg/m² or lenograstim, 5 μg/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

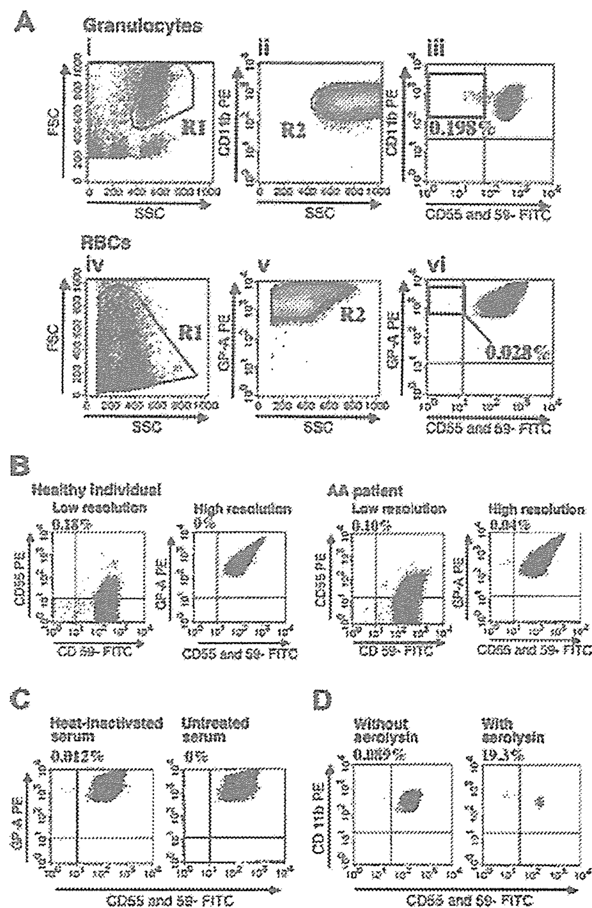

Figure 1. Validity of high-resolution flow cytometry. (A) An example of analysis on a patient with PNH^+ AA is shown. Gates were set up to exclude SSC^{dim} (i) and $CD11b^{dim}$ granulocytes and glycophorin A^{dim} RBCs (ii,v). Cells within rectangles showing horizontal distribution represent PNH-type 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

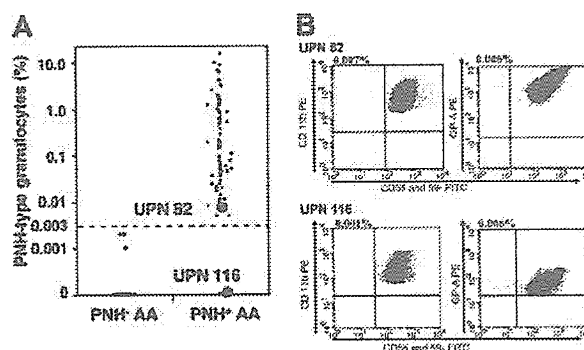

Figure 2. Proportions of PNH-type granulocytes. (A) Proportions of CD55$^-$CD59$^-$ granulocytes in each patient. (B) Histograms from one patient with PNH$^+$ (UPN 82) with minimal PNH-type cells and from another patient with increased PNH-type cells only in RBCs (UPN 116).

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, $\times 10^9$ (range)	2.1 (0.5-4.3)	1.9 (0.7-3.2)	.15
Median neutrophil count, $\times 10^9$/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, $\times 10^9$/L (range)	14.0 (2.0-60.0)	16.0 (1.0-87.0)	.65
Median reticulocyte count, $\times 10^9$/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.

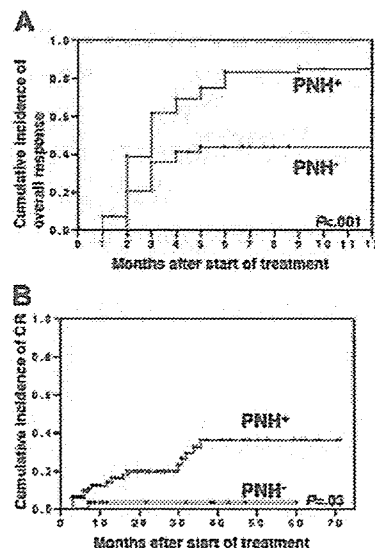

Figure 3. Response to immunosuppressive therapy. Incidence of overall (A) and complete (B) responses in patients with PNH+ and PNH−.

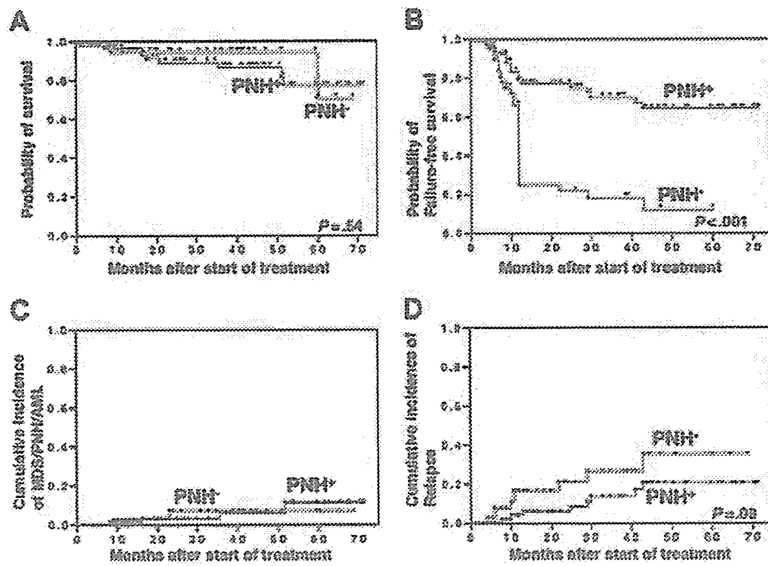

Figure 4. Prognosis after IST compared between patients with PNH⁺ and with PNH⁻. (A) Overall survival; (B) failure-free survival; (C) incidence of clonal hematologic disorders, including PNH, myelodysplastic syndrome, and acute myelogenous leukemia; and (D) incidence of relapse.

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

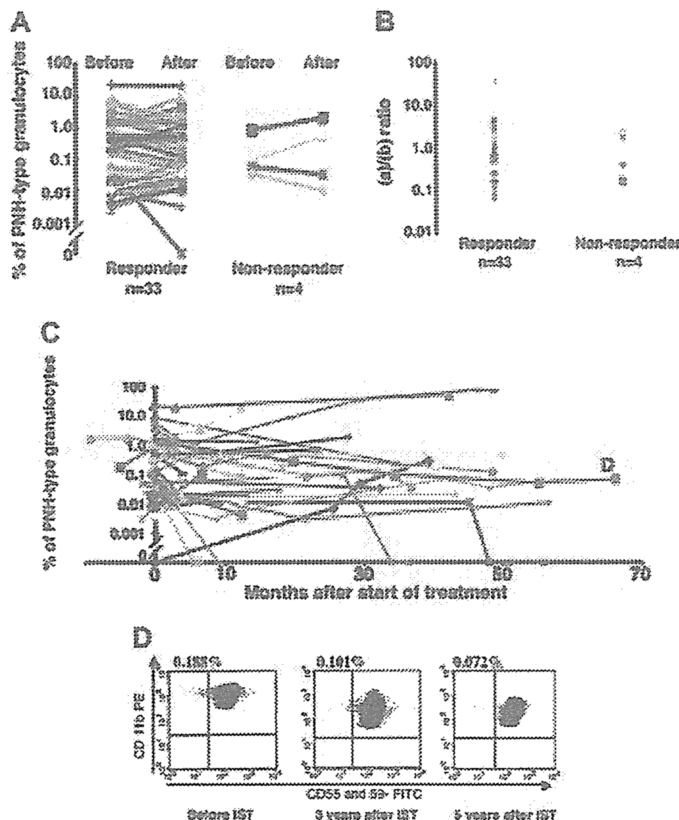

Figure 5. Changes in proportions of PNH-type granulocytes associated with responses to IST. (A) Change in responders and nonresponders. (B) Proportions of granulocyte counts after and before IST determined for PNH-type (a) and normal-type (b) granulocytes and ratios of PNH-type granulocytes (a) to normal-type 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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Diazepam-binding inhibitor-related protein 1: a candidate autoantigen in acquired aplastic anemia patients harboring a minor population of paroxysmal nocturnal hemoglobinuria–type cells

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To identify candidate antigens in aplastic anemia (AA), we screened proteins derived from a leukemia cell line with serum of an AA patient and identified diazepam-binding inhibitor-related protein 1 (DRS-1). Enzyme-linked immunosorbent assay (ELISA) revealed high titers of anti–DRS-1 antibodies (DRS-1 Abs) in 27 (38.0%) of 71 AA patients displaying increased paroxysmal nocturnal hemoglobinuria (PNH)–type cells (PNH+), 2 (6.3%) of 32 PNH− AA patients, 5 (38.5%) of 13 PNH+ myelodysplastic syndrome (MDS) patients, and none of 42 PNH− MDS patients. DRS-1 gene was abundantly expressed in myeloid leukemia cell lines and in CD34+ cells derived from healthy individuals. Stimulation of T cells from an AA patient displaying high DRS-1 Abs with a putative CD4+ T-cell epitope (amino acid residues [aa's] 191-204) presented by HLA-DR15, which overlapped with a hot spot (aa's 173-198) of DRS-1 Ab epitopes, gave rise to T cells cytotoxic for L cells (murine fibroblasts) that were transfected with DRB1*1501 and DRS-1. Enzyme-linked immunospot assay demonstrated increased frequency of T-cell precursors specific to the DRS-1 peptide in other HLA-DR15+ AA patients displaying high DRS-1 Ab titers. These findings indicate that DRS-1 may serve as an autoantigen eliciting immune attack against hematopoietic stem cells in a subset of AA patients characterized by increased PNH-type cells. (Blood. 2004;104:2425-2431)

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Introduction

Acquired aplastic anemia (AA), a bone marrow failure syndrome characterized by pancytopenia and bone marrow hypoplasia, has been the subject of study by hematologists for many years, as more than 70% of AA patients improve under immunosuppressive therapies such as antithymocyte globulin (ATG) and cyclosporine (CsA).[1-3] The dramatic effects of such T-cell suppressants on in vivo hematopoiesis suggest that immune system attack against hematopoietic stem cells plays an essential role in the development of AA.[4-6] However, despite extensive efforts to clarify the immune mechanisms of AA, the key antigens provoking immune response against hematopoietic stem cells remain unknown. This is largely due to a lack of animal models and the heterogeneity of pathogenesis in AA. Lack of good progenitor cell assays in humans has also hindered the elucidation of immune mechanisms in AA.

In organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM) and multiple sclerosis where autoreactive T cells play a primary role in pathogenesis, autoantibodies against target proteins of the pathogenic T cells are often detected.[7-10] Although such antibodies do not usually contribute to the pathogenesis of T-cell–mediated diseases, detection of the antibodies may prove useful in both identifying autoantigens and diagnosing immune mechanisms underlying the diseases.[11] We recently demonstrated that HLA-DRB1*1501 and increased paroxysmal nocturnal hemoglobinuria (PNH)–type cells represent prognostic markers for the immune mechanisms of AA.[12,13] Extensive investigation of antibodies in the sera of patients possessing HLA-DRB1*1501 and a minor population of PNH-type cells may be useful in identifying novel autoantigens in AA. Using immunofluorescent analysis, we previously found that antibodies to UT-7, a megakaryoblastic cell line, are frequently detectable in sera of AA patients who display increased PNH-type cells (PNH+ patients; unpublished observation, T.C. and S.N., May 2001). These antibodies may recognize antigens that elicit T-cell responses against hematopoietic stem cells, allowing expansion of PNH-type stem cells.[14,15]

To examine these hypotheses, we screened proteins derived from UT-7 cDNA library using serum from a PNH+ patient with HLA-DRB1*1501. Serologic identification of antigens by recombinant expression cloning (SEREX) analysis identified diazepam-binding inhibitor-related protein 1 (DRS-1) as an autoantigen that raises both antibody production and T-cell responses to antigen-presenting cells transfected with DRS-1 gene.

Patients, materials, and methods

Study subjects

Sera or plasma were obtained from 103 patients with AA (45 with severe AA and 58 with moderate AA); 55 patients with myelodysplastic syndrome

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(MDS), consisting of 46 with refractory anemia (RA) and 9 with refractory anemia with excess of blasts (RAEB); 5 patients with florid PNH; and 52 healthy individuals. Samples were cryopreserved at −80°C until use. All patients and controls provided informed consent according to the Declaration of Helsinki before supplying samples. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science.

AA and MDS were diagnosed in patients at Kanazawa University Hospital and other hospitals taking part in the bone marrow failure study group led by the Ministry of Health, Labor, and Welfare of Japan. MDS was diagnosed on the basis of cytopenia in peripheral blood, hypercellularity or normocellularity in the sternal or iliac bone marrow, and presence of dysplasia in at least 2 lineages of bone marrow cells. Cytogenetic abnormalities such as trisomy 8 and del(20)(q11) were noted in 14 of 46 RA patients and in 1 of 9 RAEB patients.

Detection of PNH-type cells

Percentages of $CD55^-CD59^-$ cells in $CD11b^+$ granulocytes and in glycophorin A^+ red blood cells (RBCs) were determined using 2-color flow cytometry as described previously.[12,16,17] Based on analytical results from 68 healthy individuals, presence of more than 0.003% $CD11b^+$ granulocytes and 0.005% glycophorin A^+ RBCs was considered abnormal.[12,17] Both thresholds exceeded the mean + 4 standard deviation (SD) for PNH-type granulocytes (0.0025%) and RBCs (0.0032%) determined on healthy individuals. Of the 103 AA patients, 71 (68.9%) displayed PNH-type cells ranged from 0.005% to 6.09%. The percentage of PNH-type cells was 0.005% to 0.01% in 7 (9.9%) patients, 0.01% to 0.1% in 22 (31.0%) patients, 0.1% to 1.0% in 32 (45.1%) patients, and 1.0% to 6.09% in 10 (14.1%) patients. Thirteen of the 46 (28.3%) RA patients displayed increased PNH-type cells, whereas none of the 9 RAEB patients did.

Preparation of cDNA library and SEREX

Poly(A) RNA was purified from UT-7 cells (kindly provided by Dr N. Komatsu of Jichi Medical School, Japan), and a cDNA expression library was constructed with a λZAPII expression vector using a cDNA library kit (Stratagene, La Jolla, CA). Screening for antigens recognized by autoantibodies in the patient's serum was performed as described previously.[18] Briefly, XL1-Blue *Escherichia coli* (Stratagene) was transformed with recombinant phages, plated on agar at 5×10^4 plaques per 150-mm Petri dish, and cultured at 37°C for 6 to 8 hours. Expression of recombinant proteins was induced by incubating bacterial lawns with an overlay of iso-propyl β-D-thiogalactoside (IPTG; Promega, Madison, WI) saturated nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Transfer of released proteins was allowed to proceed for an additional 4 hours at 37°C. Membranes were then washed with 25 mM tris(hydroxymethyl)aminomethane (Tris)–buffered solution (TBS) containing 150 mM NaCl, 2.5 mM KCl, and 0.05% Tween 20 (TBST; pH 7.5) to remove excess agar and blocked overnight with 5% nonfat dry milk in TBS at 4°C.

Serum was obtained from an untransfused 71-year-old AA patient (patient 1) who demonstrated CsA-dependent recovery of hematopoiesis and displayed an increase in PNH-type cells.[19] HLA-DRB1 alleles in this patient included 1501 and 0405. Serum was preabsorbed with bacterial lysates to minimize nonspecific antibody binding. Membranes were then incubated with the serum diluted at 1:200 in TBS containing 1% bovine serum albumin (BSA/TBS). Specific binding of immunoglobulin G (IgG) antibodies to recombinant proteins expressed on the lytic plaques was detected by incubating the membranes with alkaline phosphatase–conjugated antihuman IgG antibody (1:2000; Jackson ImmunoResearch, West Grove, PA). Antigen-antibody complexes were visualized by adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP; KPL, Guildford, United Kingdom) and nitroblue tetrezolium (NBT; KPL). cDNA inserts from reactive clones were subcloned to monoclonality, excised in vivo to the pBluescript SK(−) phagemid (Stratagene), and sequenced using an ABI PRISM3100 sequencer (PE Applied Biosystems, Foster, CA).

Purification of bacterially expressed fusion proteins and Western blotting

Full-length DRS-1 cDNA obtained from SEREX analysis was subcloned into the pET-44a (+) vector (Novagen, Madison, WI) for expression of a His-tag fusion protein. Synthesized proteins were purified using a His bind kit (Novagen) according to the manufacturer's instructions. A His-tag encoded by pET-44a (+) without the insert was also purified for use as a negative control. Native DRS-1 protein was released from His-tag DRS-1 protein using a thrombin cleavage kit (Novagen). Size of the recombinant proteins was confirmed by Western blotting using mouse anti-His monoclonal antibody (mAb; Amersham Pharmacia Biotech, Piscataway, NJ) as described previously.[20] To detect specific antibodies in serum, 1:200 diluted serum was incubated with blotted membranes.

Enzyme-linked immunosorbent assay (ELISA)

Each well of a 96-well Nunc-Immuno plate (Nalge-Nunc International, Roskilde, Denmark) was covered with 100 μL of coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) containing 1 μg/mL of purified recombinant DRS-1 protein and kept overnight at 4°C. Plates were washed and covered with phosphate-buffered saline (PBS) containing 10% fetal calf serum overnight at 4°C to block nonspecific binding of serum protein to DRS-1. Sera from patients were added to a final dilution of 1:1000 and incubated at room temperature for 1 hour. After washing, plates were incubated with peroxidase-conjugated goat antihuman IgG antibody (1:10 000; Jackson ImmunoResearch) at room temperature for 1 hour. Finally, plates were washed and incubated with 3,3′,5,5′-tetramethylbenzidine substrate (Pierce, Rockford, IL) at room temperature for 30 minutes, and the optic density (OD) absorbance at 450 nm was read using an SLT EAR 340AT ELISA reader (SLT-Labinstruments, Grödig, Austria). A positive reaction was defined as an absorbance value exceeding the mean + 2 SDs for the OD absorbance value of sera from the 52 controls.

Cell lines

A chronic myeloid leukemia cell line KH88 was kindly provided by Dr M. Yasukawa of Ehime University. K562, KU812, Daudi, U937, HEL, and Molt-4 were purchased from RIKEN BRC (Ibaraki, Japan). A murine fibroblast cell line, L cell, transfected with HLA-DRB1*1501 (1501-L cell) or with HLA-DRB1*0101 (0101-L cell) was provided by Dr Y. Nishimura of Kumamoto University. Lymphoblastoid cell lines (LCLs) were established from peripheral blood mononuclear cells (PBMCs) of 7 healthy individuals using B95-8 (ATCC, Manassas, VA).

Ribonuclease protection assay (RPA)

RNA probes were prepared for detecting gene expression of DRS-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by amplifying a cDNA fragment of DRS-1 and that of GAPDH using specific primer sets (sense primer 5′-CTATTCGATGCCGTGTATGC-3′, and antisense primer 5′-GCCTGGTCCAGACTTCTTTC-3′ for DRS-1; sense primer 5′-TGAACGGGAAGCTCACTGGC-3′, and antisense primer 5′-AGGTCCACCACCCTGTTGCT-3′ for GAPDH) followed by subcloning into a pGEM-T Easy vector (Promega). Linearized plasmid DNA containing DRS-1 or GAPDH by cutting with *Sal*I were used as templates to synthesize biotin-labeled RNA probes using MAXIscript T7 kit (Ambion, Austin, TX) with minor modifications. A total of 10 ng of the RNA probe was used for hybridization with 20 μg total RNA of each kind of cell line. RPA was performed using the Ribo-Quant RPA kit (PharMingen, San Diego, CA), and chemiluminescent signals were detected using the Non-Rad Detection kit (PharMingen) according to the manufacturer's instructions.

Isolation of $CD34^+$ cells and $CD4^+$ T cells

$CD34^+$ cells were isolated from the bone marrow of 3 healthy volunteers using a CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD4^+$ T cells were separated from cultured T cells using Dynabeads M-450 CD4 (Dynal Biotech ASA, Oslo, Norway).

Quantification of DRS-1–specific mRNA with real-time polymerase chain reaction (PCR)

Total RNA was extracted from PBMCs, CD34$^+$ cells of healthy volunteers, and leukemia cell lines using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription of 1 μg of RNA into cDNA was performed using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and stored at −20°C until use. Quantification of DRS-1 gene expression was performed using a LightCycler (Roche Diagnostics, Tokyo, Japan) with specific primers described under "Ribonuclease protection assay (RPA)." The LightCycler with a GAPDH primer kit (Search LC, Heidelberg, Germany) was used for quantification of mRNA for GAPDH, a housekeeping gene, in the same samples. The relative amount of DRS-1 mRNA to GAPDH mRNA (DRS-1/GAPDH) was used to represent expression level of the DRS-1 gene.

Epitope mapping

In order to detect hot spots of epitopes recognized by DRS-1 Abs of different patients, epitope mapping was performed using the NovaTope library construction and screening system (Novagen). Briefly, DRS-1 cDNA was randomly digested using DNase I into small fragments of 50 to 150 bp. After both ends were blunted and a single 3′-deoxyadenosine residue added, fragments were ligated into the pSCREEN T-Vector. NovaBlue (DE3)–competent cells were transformed using the constructed pSCREEN T-Vector for peptide expression. Colonies were transferred onto nitrocellulose filters and lysed in a chloroform vapor chamber. After denaturation and blocking, filters were incubated with sera from AA patients possessing DRS-1 Abs overnight at 4°C, then immunodetection was performed by incubating membranes with alkaline phosphatase–conjugated antihuman IgG antibodies (1:2000; Jackson ImmunoResearch). Antigen-antibody complexes were visualized by adding BCIP and NBT. Positive colonies were selected, and plasmid DNA was extracted from the colonies using a miniprep DNA purification system (Promega). Proteins derived from positive clones were purified using a His bind kit (Novagen) as described under "Purification of bacterially expressed fusion proteins and Western blotting."

Preparation of peptides as an epitope candidate within DRS-1

Peptide sequences within DRS-1 that can be presented by HLA-DR15 were deduced based on the TEPITOPE algorithm[21] with a prediction threshold (ie, percentage of best-scoring natural peptides) of 5%. Two positive peptides, amino acid residues (aa's) 191-204 (AVLLREFVGCFIDF, peptide 1) and aa's 351-364 (TNAVVNFLSRKSKL, peptide 2), as well as a negative peptide, aa's 95-108 (SSQVEPGTDRKSTG, peptide 3), which was predicted to display no binding to the HLA-DR15 molecule, were synthesized using a Rainin Symphony multiple-peptide synthesizer (Rainin, Woburn, MA). Synthetic peptides were lyophilized, reconstituted in dimethyl sulfoxide at 5 mg/mL, and diluted in RPMI 1640 (Gibco, Grand Island, NY) as needed.

Transduction of L cells with DRS-1 gene

The DRS-1 minigene was amplified by PCR using pBluescript SK(−) harboring full-length DRS-1 cDNA as a template. The primer set used for PCR included 5′-GGGCTCGAGCCCGCCGCCACCATGCTGACT-AACTTCACTGATATT-3′ and 5′-GCGGCCGCTCACAGTTTTGATTT-TCTGGATAA-3′ appended *Xho*I and *Not*I sites (underlined sequences) at the 5′ and 3′ ends of the DRS-1 minigene, respectively. PCR products were inserted into a pGEM-T Easy vector (Promega). After propagation, DRS-1 minigene was released by digestion with *Xho*I and *Not*I, then subcloned into pCAGIPuro vector (kindly provided by Dr H. Niwa, RIKEN). The pCAGIPuro vector harboring the DRS-1 minigene was used to transfect 1501- or 0101-L cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable transfectants were obtained by selection using RPMI 1640 medium containing puromycin (5 μg/mL; BD Biosciences Clontech, Palo Alto, CA).

Establishment of DRS-1–specific T cells and ^{3}H-thymidine incorporation assay

PBMCs obtained from an AA patient (patient 2) with HLA-DR15 displaying high titers of DRS-1 Abs were cultured for 14 days with irradiated 1501-L cells that were pulsed with DRS-1 peptides, and CD4$^+$ T cells were separated. Proliferative responses of cultured CD4$^+$ T cells to 1501-L cells or 0101-L cells transfected with the DRS-1 gene were measured using ^{3}H-thymidine incorporation assay. A total of 5×10^4 CD4$^+$ T cells were cultured in 96-well U-bottomed plates (IWAKI, Chiba, Japan) with the same number of 45 Gy-irradiated L-cell transfectants. After 3 days of incubation, 1 μCi (0.037 MBq) of ^{3}H-thymidine (6.7 Ci/mmol [2.48×10^{11} Bq/mmol]; Dupont NEN Products, Boston, MA) was added to each well. Cultured cells were harvested after 6 hours, and ^{3}H-thymidine incorporation was measured. Data were represented as relative proliferative index calculated as ^{3}H-thymidine incorporation by T cells cultured with L-cell transfectants relative to ^{3}H-thymidine incorporation by T cells cultured without L-cell transfectants.

^{51}Cr-release assay

L-cell transfectants were incubated with 100 μCi (3.7 MBq) of ^{51}Cr at 37°C for 1 hour after washing with PBS. Labeled cells were washed 3 times with PBS and suspended in complete medium containing 10% pooled human AB serum. Labeled cells (5×10^3) were incubated with various numbers of DRS-1–specific CD4$^+$ T cells for 4 hours. The release of ^{51}Cr into medium was measured using a γ-counter. Percentage of specific lysis (mean ± SD) obtained in the ^{51}Cr-release assay was determined from triplicate cultures as follows: 100 × (experimental release cpm − spontaneous release cpm)/(maximum release cpm − spontaneous release cpm), where cpm indicates counts per minute.

Determination of T-cell precursor frequencies specific to DRS-1 peptides

Approximately 2×10^6 PBMCs were cultured for 7 days in RPMI 1640 supplemented with heat-inactivated human serum (10%) and L-glutamine (2 mM) containing 20 μg/mL of a DRS-1–derived peptide. On day 7, 2×10^4 1501-L cells were pulsed with 20 μg/mL of the same peptide. After a 4-hour incubation, L cells were washed twice with RPMI1640, irradiated, and added to the cultured PBMCs. Interleukin 2 (IL-2) was added on day 8 at 100 U/mL. On day 14 of culture, CD4$^+$ T cells were separated and subjected to enzyme-linked immunospot assay (ELISPOT) using an interferon γ (IFN-γ) ELISPOT assay kit (BioSource International, Camarillo, CA). Briefly, 10^5 induced CD4$^+$ T cells were transferred into each well of the ELISPOT plate then cocultured with 2×10^4 peptide-pulsed L cells overnight. IFN-γ spots were then detected according to the manufacturer's instructions.

Statistics

Differences in prevalence of DRS-1 Ab titers in serum among different patient groups and in responses of DRS-1–specific T cells to different target cells were examined using Fisher exact test and Student *t* test, respectively. The logistic procedure was used to identify factors significantly associated with a good response to immunosuppressive therapy (IST).

Results

Identification of cDNA clones recognized by serum of an AA patient

SEREX analysis using the UT-7 cDNA library and diluted serum from an AA patient (patient 1) identified 6 independent clones including DRS-1, *Homo sapiens* KIAA0907 protein, α1HB subunit of voltage-dependent T-type calcium channel, U2 small nuclear ribonucleoprotein auxiliary factor 35-kDa subunit related-protein 2, hemoglobin γ-1 chain, and lens epithelium-derived growth factor. When sera from another 10 AA patients were screened for