Toxicity

The incidence of toxicity was comparable between G-CSF− and G-CSF+ groups. Acute allergic reaction during ATG therapy was observed in 81% of patients in the G-CSF− group and 92% of patients in G-CSF+ group ($P = .11$). A total of 2 patients in the G-CSF− group and 4 patients in the G-CSF+ group had serum sickness. Toxicity greater than grade III was noted in 2 patients (anaphylactic reaction to ATG and delirium associated with CyA administration) in the G-CSF− group, and in 1 patient (liver dysfunction related to ATG administration) in the G-CSF+ group.

Discussion

The results of this prospective multicenter study showed that the addition of G-CSF to IST plays some role in the treatment of SAA. The hematologic response rate at 6 months in the G-CSF+ group was significantly higher compared with the G-CSF− group (77% in the G-CSF+ group, 57% in the G-CSF− group), but at 1 year was comparable (79% in the G-CSF+ group, 76% in the G-CSF− group). This indicates that G-CSF can accelerate the recovery of hematopoiesis in patients with AA when used in combination with IST. Our result is inconsistent with a similar study conducted in Japanese children with SAA, which showed no difference between the G-CSF+ and the G-CSF− group in terms of hematologic response.[14] A European group also reported the result of a similar study in patients with SAA (age, 1-82 years), which showed that G-CSF only enhanced the recovery of neutrophils.[15] The reasons why different results were obtained in these studies are uncertain. However, it is possible that patient's age might influence the results, because the distribution of patient's age is apparently different among the studies.

Our study showed that the accumulated relapse rate was significantly lower in the G-CSF + group (42% in the G-CSF+ group and 15% in the G-CSF− group at 4 years; $P = .01$). This indicates that G-CSF can reduce the relapse rate in patients who have responded to IST. The reason why G-CSF has an impact on the occurrence of relapse is uncertain. However, as G-CSF has a stimulatory effect on the growth of hematopoietic stem and progenitor cells in AA,[22] it is possible that hematopoietic stem and progenitor cells, which can escape from the immune attack, might expand in patients who are successfully treated with IST in combination with G-CSF. If so, it is possible that relapse rate is low in the G-CSF+ group because of the high expansion of immune attack-resistant hematopoietic cells. A Japan childhood AA study group showed that there was a trend of low relapse rate in the G-CSF+ group (the risk for relapse at 4 years was 29% in the G-CSF+ group and 64% in the G-CSF− group), although the difference between the 2 groups was not statistically significant ($P = .10$), which might be due to a low number of patients enrolled.[14] A European group reported that the actuarial risk for relapse after IST was 35% at 10 years, and the relapse occurred at any time from a few months to 10 years after IST without a particular period of higher occurrence.[23] Because the median follow-up period of our study was not so long, the possibility that G-CSF only delays the time of relapse cannot be excluded. To elucidate whether G-CSF actually prevents relapse or only delays the time of relapse, further follow-up is required

To date, there was no significant difference between the G-CSF+ and the G-CSF− group in terms of overall survival (94% to 88% at 4 years). This finding is in keeping with that of previous reports.[14,15] It has been reported that the overall survival in patients who do not relapse is better than that of patients who relapse.[23] Therefore, better survival in the G-CSF+ group will be expected because of the low incidence of relapse in the G-CSF+ group. Further follow-up is necessary to conclude whether a difference in overall survival exists between the G-CSF+ and the G-CSF− group.

There was no difference in the incidence of documented infections and febrile episodes during the first 12 weeks between the G-CSF+ and G-CSF− groups, although the addition of G-CSF to IST resulted in an increase in the neutrophil counts. This result is in agreement with those of previous studies[14,15] suggesting that G-CSF has no preventive effect on infections during the IST. In our study, the proportion of patients who contracted infection was relatively higher in the G-CSF+ group compared with the G-CSF− group, although the difference between the 2 groups was not statistically significant (58% vs 40%; $P = .07$). This might be due to the relatively high proportion of patients with vSAA (neutrophil count, $< 0.200 \times 10^9$/L [200/μL]) in the G-CSF+ group compared with the G-CSF− group (40% in the G-CSF+ group and 23% in the G-CSF− group) who were more susceptible to infections.

Over the long term, patients with AA who have been treated with IST have an increased risk (10%-47%) of developing MDS or AML.[24] However, it is uncertain whether evolution to MDS/AML is a reflection of the natural history of AA or secondary disease related to IST. In addition, it has been reported that administration of G-CSF was associated with the development of MDS/AML,[25-27] although conflicting results have been reported.[28] In a retrospective study from Japan, it was demonstrated that 4 (22%) of 18 adult patients with AA patients treated with both IST and G-CSF developed MDS, and a high cumulative dose of G-CSF and the use of G-CSF for more than 1 year were the significant risk factors for developing MDS.[25] Another study showed that the cumulative incidence of developing MDS/AML was 13.7% in children with AA who received IST and danazol with or without G-CSF, and long-term use of G-CSF and no response to therapy at 6 months were significant risk factors for developing MDS.[26] In contrast, the Italian group showed that the risk for developing secondary malignancies at 60 months in patients treated with IST with or without G-CSF treatment was 9% and 7%, respectively ($P = .99$).[28] This study concluded that large doses of G-CSF (36 000 μg/patient) administered over a long period of time (6 months) in conjunction with IST do not increase the actuarial risk of developing MDS/AML. In our study, the risk for developing MDS/AML at 4 years is 3% for the G-CSF− group and 5% for the G-CSF+ group ($P = .63$). Our finding suggests that use of G-CSF with IST in a relatively short period of time (12 weeks) does not increase the risk for developing MDS/AML, at least during the several years after IST. Longer follow-up (at least 10 years) is necessary to determine the role of G-CSF in the development of MDS/AML. Until then, routine use of G-CSF is not recommended unless part of a clinical trial.

It is well known that chromosomal abnormalities develop in some patients after IST.[29] In addition, Japanese studies suggest a close relationship between the use of G-CSF and development of monosomy 7.[25,26] In our study, 8 (11%) of 74 evaluable patients who received IST developed chromosomal abnormalities, and there was no significant difference in the incidence of development of chromosomal abnormalities between the G-CSF+ and G-CSF− groups. This result is consistent with data from an Italian group.[28] In our study, however, it should be noted that monosomy 7 was only developed in the G-CSF+ group (2 patients), and 1 patient

with monosomy 7 developed MDS. It has recently been shown that G-CSF preferentially stimulates the proliferation of monosomy 7 cells expressing the class IV G-CSF receptor, which is defective in signaling cell maturation.[29]

A transient appearance of chromosomal abnormalities in patients with AA after IST is also a well-documented phenomenon.[28,30,31] An Italian group showed 9 patients (4 in the G-CSF+ group, 5 in the G-CSF− group) with transient chromosomal abnormalities.[28] In the present study, transient chromosomal abnormalities were observed in 4 patients (3 in the G-CSF+ group, 1 in the G-CSF− group). Therefore, the appearance of abnormal cytogenetic clones after IST does not necessarily mean the subsequent expansion of those clones, and appeared to be unrelated to the combined use of G-CSF.

The present study suggests that combined use of immunosuppressive agents and G-CSF has some benefits in terms of the promotion of hematopoietic recovery and suppression of relapse rate, which result in reducing the need for subsequent treatments such as blood transfusion and second IST. G-CSF support of IST might be feasible for the treatment of adult SAA; however, further follow-up is required to elucidate whether G-CSF increases the risk for MDS/AML. In addition, it is important to discuss whether G-CSF support of IST is appropriate in terms of cost-effectiveness. To address this issue, long-term follow-up is necessary.

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Authorship

Author contributions: M.T., S.N., A.U., M.O., and H.M. designed research; M.T analyzed data and drafted the paper; A.K., S.I, and Y.Y. contributed to the enrollment of patients.

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Specific antibodies to moesin, a membrane-cytoskeleton linker protein, are frequently detected in patients with acquired aplastic anemia

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To identify novel autoantibodies in acquired aplastic anemia (AA), we screened the sera of patients with AA possessing small populations of paroxysmal nocturnal hemoglobinuria (PNH)–type cells for the presence of antibodies (Abs) which recognize proteins derived from a leukemia cell line, UT-7. Immunoblotting using proteins derived from lysates or culture supernatants of UT-7 cells revealed the presence of IgG Abs specific to an 80-kDa protein. Peptide mass fingerprinting identified this 80-kDa protein as moesin. Enzyme-linked immunosorbent assay (ELISA) using recombinant moesin showed high titers of antimoesin Abs in 25 (37%) of 67 patients with AA. Moesin was secreted from several myeloid leukemia cell lines other than UT-7, such as OUN-1 and K562, as an exosomal protein. The presence of antimoesin Abs was significantly correlated with the presence of PNH-type cells and antidiazepam-binding inhibitor-related protein-1 (DRS-1) Abs. Patients with AA that did not show any of these 3 markers tended to respond poorly to immunosuppressive therapy. These findings suggest that a B-cell response to moesin, possibly derived from hematopoietic cells, frequently occurs in patients with AA and that detection of antimoesin Abs in combination with other markers may be useful in diagnosing immune pathophysiology in patients with AA. (Blood. 2007;109:2514-2520)

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Introduction

Acquired aplastic anemia (AA) is a syndrome characterized by pancytopenia and bone marrow hypoplasia. Immune-mediated suppression of hematopoiesis is considered to be the most important mechanism responsible for the development of this syndrome,[1-3] but this mechanism does not underlie all patients with AA. Approximately 30% of patients with AA do not respond to immunosuppressive therapy (IST) with antithymocyte globulin (ATG) plus cyclosporine (CsA),[4,5] and some of the patients treated with IST undergo progression to myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML). For patients with nonimmune mediated AA, IST might even be harmful because of an increased risk of opportunistic infections. Despite the understanding that not all patients with AA have an immune pathophysiology, patients with AA have been unconditionally placed on IST because there is no good marker to differentiate nonimmune-mediated AA from immune-mediated AA.

We recently reported that the presence of a small population of $CD55^-CD59^-$ blood cells serves as a marker for immune pathophysiology of AA.[6-8] However, the highly sensitive flow cytometric assay which determines whether there is an increase in the proportion of such paroxysmal nocturnal hemoglobinuria (PNH)–type cells requires fresh blood. The assay to detect PNH-type granulocytes is difficult to use on patients with AA whose neutrophil counts are less than $0.1 \times 10^9/L$. Moreover, even in the absence of an increased proportion of PNH-type cells, greater than 40% of patients with AA respond to IST.[8] Accordingly, laboratory markers other than small populations of PNH-type cells are necessary to diagnose immune pathophysiology prior to IST in patients with AA.

In autoimmune diseases in which T cells play a primary role in the pathogenesis, antibodies (Abs) specific to antigens derived from T-cell target proteins, such as myelin basic protein in multiple sclerosis (MS) and glutamate decarboxylase in insulin-dependent diabetes mellitus (IDDM), are often detected, and they have served as a marker of immune pathophysiology of the diseases.[9-12] If Abs specific to the antigens abundantly expressed by hematopoietic progenitor cells were detectable in the serum of patients with AA, they might reflect the immune pathophysiology of their patients. We previously identified Abs specific to diazepam-biding inhibitor-related protein-1 (DRS-1) in the serum of 38% of patients with AA showing small populations of PNH-type cells (patients with PNH^+ AA).[13] The serum of patients with PNH^+ AA may also contain other autoantibodies (auto-Abs) specific to self-antigens because of a breakdown of tolerance that is thought to occur in organ-specific autoimmune diseases such as MS,[14] IDDM,[15] and Basedow disease.[16] The detection of such Abs may complement the diagnosis of immune pathophysiology in patients with AA.

To identify novel auto-Abs in AA, we screened sera from patients with PNH^+ AA for the presence of Abs recognizing antigens derived from a megakaryocytic leukemia cell line, UT-7, a cDNA library of which was used for the identification of anti–DRS-1 Abs.[13] A new method taking advantage of culture supernatant of the cells for Western blotting followed by mass spectrometry identified an Ab specific to a membrane-cytoskeleton linking protein, moesin.

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Patients, materials, and methods

Study subjects

Sera or plasma were obtained from 67 patients with AA (23 with severe AA and 44 with moderate AA), 21 patients with refractory anemia (RA) of MDS, 49 patients with rheumatoid arthritis, and 48 healthy individuals. The severity of AA was classified according to the criteria proposed by Camitta et al.[17] Samples were cryopreserved at −80°C until use. All patients and healthy volunteers were provided informed consent in accordance with the Declaration of Helsinki, before collecting samples. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science. AA was diagnosed in patients at Kanazawa University Hospital and other hospitals taking part in the bone marrow failure study led by the Ministry of Health, Labor, and Welfare of Japan.

Detection of PNH-type cells

Proportions of $CD55^-CD59^-$ cells in $CD11b^+$ granulocytes and in glycophorin A^+ red blood cells were determined using 2-color flow cytometry as described previously.[8]

Cell lines

The following leukemia cell lines and synovial cells were kindly provided by each respective researcher. A megakaryoblastic leukemia cell line UT-7 by Dr N. Komatsu of Jichi Medical School; myeloid leukemia cell lines KH88, OUN-1, SAS413, NB4, and KG-1 by Dr M. Yasukawa of Ehime University; a myelodysplastic syndrome cell line TF-1 by Dr S. Ogawa of the University of Tokyo; synovial cells of a patient with rheumatoid arthritis by Dr M. Kawano of Kanazawa University. K562 was purchased from the Health Science Research Resources Bank (Osaka, Japan).

Isolation of $CD34^+$ cells, $CD3^+$ T cells, and $CD19^+$ B cells

Peripheral blood buffy coat cells were collected from a healthy volunteer donor after G-CSF administration. $CD34^+$ cells were isolated from the buffy coat cells using a CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD3^+$ T cells and $CD19^+$ B cells were isolated from the same cells using CD3 and CD19 microbeads (Miltenyi Biotec), respectively. The purity of isolated $CD34^+$, $CD3^+$, and $CD19^+$ cells was greater than 95% as demonstrated by flow cytometry.

Immunofluorescence analysis of UT-7 cells

UT-7 cells were fixed with 4% formaldehyde for 5 minutes and treated with 0.1% Tween 20 for 10 minutes for permeabilization. The cells were incubated in PBS containing 0.5% sera from a patient with AA for 30 minutes. After washing, the cells were incubated in PBS containing 0.1% FITC-labeled goat anti–human IgG and viewed with an immunofluorescent microscope (Axioplan2 imaging; Carl Zeiss, Jena, Germany) equipped with a 100×/0.4 NA oil objective lens and M1 digital camera (Carl Zeiss); Isis software version 5.0 (Carl Zeiss) was used to acquire digital images.

Purification of bacterially expressed moesin proteins

Full-length human moesin cDNA and the cDNA fragments of human moesin were synthesized by the reverse transcription–polymerase chain reaction (RT-PCR) amplification method. Briefly, cDNA was reverse transcribed from the mRNA of UT-7 cells by using SuperScript First-Strand Synthesis (Invitrogen, Carlsbad, CA). Thirty cycles of PCR were performed using each set of primers; for the full-length moesin termed M0 (S0, 5′-CGGAATTCGCCTTTGCCGCCACCATGCCC-3′; A0, 5′-CGGTCGACTCCCTAGAGGCTGGGTGCCCA-3′), for the carboxyl (C)–terminal portion of moesin (moesin 399-500) termed M1 (S1, 5′-CGCGGATCCGCCAAGGAGGCCTTGCTGCAG-3′; A1, 5′-CGGAATTCGGCCATAGCATCAGCCCGTAG-3′), and for the C-terminal portion of moesin (moesin 494-577) termed M2 (S2, 5′-CGCGGATCCCTACGGGCTGATGCTATGGCC-3′; A2, 5′-CGGAATTCTCCCTAGAGGCTGGGTGCCCA3-′). Each cycle consisted of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. PCR products purified from an agarose gel were inserted into the pGEX-6P-1 vector (GE Healthcare, Fairfield, CT) between the *Eco*RI and *Sal*I sites for expression of a GST-tag fusion protein M0, and between the *EcoRI* and *Bam*HI sites for the GST-tag fusion proteins M1 and M2 using BL21 competent cells (Novagen, Madison, WI). Synthesized proteins were purified using glutathione Sepharose 4B (Amersham Biosciences, Piscataway, NJ). Native moesin protein and C-terminal moesin protein fragments were released from GST-tag moesin proteins using PreScission Protease (Amersham Biosciences) according to the manufacturer's instructions. The proper size of the recombinant proteins was confirmed by Western blotting with mouse antimoesin mAb (Clone 38; BD Biosciences, San Jose, CA). To detect a specific Ab in serum, blotted membranes were incubated in 3% BSA-PBS containing serum diluted 1:200.

Western blotting

UT-7 cells were suspended in Laemmli sample buffer and sonicated for the preparation of cell lysates. Approximately 10 μg UT-7 lysate, 5 μg Grp78, and 5 μg recombinant native moesin protein per lane were electrophoresed in a 12% polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated in 3% BSA-PBS containing serum diluted 1:200 from patients with AA or healthy persons.

Peptide mass fingerprinting

UT-7 cells (1×10^7) were suspended in 1 mL RPMI 1640 medium containing 10% fetal calf serum (FCS) and incubated for 1 hour at 37°C in the CO_2 incubator. Culture supernatants were collected by centrifugation at 500*g* for 5 minutes. Mass spectrometric identification of an 80-kDa protein derived from the UT-7 cell supernatants was performed as previously described.[18] Briefly, proteins fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were visualized by Coomassie Brilliant Blue (CBB) staining, and the 80-kDa band was excised from gels, followed by in-gel digestions with trypsin (Promega, Madison, WI) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C. Molecular mass analyses of the triptic peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using an ultraflex TOF/TOF (Bruker Daltonics, Billerica, MA). Proteins were identified by comparison between the molecular weights determined by MALDI-TOF/MS and the theoretical peptide masses of proteins registered in NCBInr.

Enzyme-linked immunosorbent assay (ELISA)

Each well of a 96-well Nunc-Immuno plate (Nalge-Nunc, Roskilde, Denmark) was filled with 50 μL coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) containing 5 μg/mL purified recombinant moesin protein and was incubated overnight at 4°C. The plates were washed and filled with PBS containing 10% FCS for 2 hours at 37°C to block nonspecific binding of proteins to moesin. Sera from the patients were added to the wells at a final dilution of 1:200, and the plates were incubated at 37°C for 1 hour. After washing, peroxidase-conjugated goat anti–human IgG antibodies (1:80 000; Jackson ImmunoResearch, Baltimore, PA) were added to the wells, and the plates were incubated at 37°C for 1 hour. Finally, the plates were washed and incubated with 3,3,5,5-tetramethylbenzidine substrate (Pierce, Rockford, IL) at room temperature for 30 minutes, and the optical density (OD) absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments, Grödig, Austria). A positive reaction was defined as an absorbance value exceeding the mean +2 SDs of the OD absorbance values from the sera of the 20 or 48 controls.

Enrichment of exosomes from culture supernatants of leukemia cell lines and Western blotting

Leukemia cell lines (K562, OUN-1, TF-1, and UT-7) cells, $CD34^+$ cells, $CD3^+$ T cells, $CD19^+$ B cells, and synovial cells were cultured at 2×10^9/L for 48 hours, and the supernatants were collected. The culture supernatants

of leukemia cell lines and synovial cells were subjected to 3 successive centrifugations to remove cells and cell debris at 300*g* for 10 minutes, 2000*g* for 20 minutes, and finally at 10 000*g* for 30 minutes, all at 4°C. Exosomes were then pelleted at 64 000*g* for 100 minutes. Pellets were resuspended and washed in PBS and centrifuged at 100 000*g* for 1 hour using a SW28 rotor (Beckman Coulter Instruments, Fullerton, CA).[19] The exosomes were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer of pH 7.4 for 1 hour and then were postfixed with 0.5% OsO_4 for 20 minutes at 4°C. Next, they were stained with 0.5% uranyl acetate for 20 minutes, dehydrated with a graded ethanol series, and were embedded in an epoxy resin (Selva Feinbiochemica GmbH, Heidelberg, Germany). Ultrathin sections were prepared and examined using an electron microscope (JEM-1210; JEOL, Tokyo, Japan) after brief staining with uranyl acetate and lead citrate. Exosomes were resuspended in PBS, divided into aliquots, and stored at −80°C. Approximately 1 μg exosomal protein per lane was electrophoresed in a 12% polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated in PBS containing a mouse antimoesin mAb (Clone 38/87; NeoMarkers, Fremont, CA) at 0.2 μg/mL or a rabbit antimoesin polyclonal Ab (TK89) which was kindly provided by Dr S. Tsukita of Kyoto University.

Immunosuppressive therapy and response criteria

ATG (Lymphoglobuline; Aventis Behring, King of Prussia, PA; 15 mg/[kg/day], 5 days) plus cyclosporine (CsA; Novartis, Basel, Switzerland; 3-6 mg/[kg/day]), or CsA alone was administered following the standard protocol. 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. 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.[20]

Statistics

Differences in the prevalence of antimoesin Ab titers in serum among different patient groups were examined using Fisher exact test.

Results

Detection of Abs specific to proteins derived from UT-7 cells in the sera of patients with AA

We first screened sera of patients with AA showing increased PNH-type cells for the presence of Abs reactive to UT-7 cell proteins using an immunofluorescence analysis. Among the sera from 9 patients with AA, 6 patients' sera stained the cytoplasm of UT-7 cells as shown in Figure 1A, indicating the presence of IgG antibodies specific to UT-7 proteins. To identify these proteins, UT-7 lysate was subjected to Western blotting using sera from patients with AA. Sera from several patients with PNH^+ AA produced a clear band with a size of 80 kDa (Figure 1B). The Ab specific to the 80-kDa protein was detected in the sera of 9 (43%) of 21 patients with PNH^+ AA ,whereas it was undetectable in any of the 7 patients not showing increased PNH-type cells (PNH^- patients) and 11 healthy persons.

Identification of the 80-kDa protein

To identify the 80-kDa protein, we cut out the clear band from a CBB-stained gel and tried to determine the amino acid sequence of the protein using peptide mass fingerprinting. However, this attempt failed to identify a single protein because of the presence of various other proteins in the total amount eluted from the 80-kDa band. We then examined culture supernatants of UT-7 cells for the presence of the 80-kDa protein because some intracellular proteins are reportedly secreted from cell lines into culture medium.[19,21,22] Figure 2 shows the results of Western blotting. Incubation of the membrane blotted with the culture supernatant proteins in PBS containing sera from patients with AA revealed a similar but more distinct 80-kDa band. When the corresponding band was cut out from the original CBB-stained gel (Figure 2, lane 5) and subjected to peptide mass fingerprinting, this protein was identified as either moesin or Grp 78. Serum of patients with AA failed to reveal an 80-kDa band when a Grp 78 protein blotted membrane was used. The approximately 70-kDa bands in lanes 3 and 6 of Figure 2 were thought to be nonspecific bands because of a low purity of the recombinant Grp 78 protein.

Prevalence of antimoesin Abs in patients with AA or MDS

To confirm the presence of Abs specific to moesin in the sera of patients with AA, a recombinant human native moesin protein was prepared. Figure 3A shows the results of Western blotting using the native moesin. Clear bands corresponding to the moesin were produced by sera of 3 patients with PNH^+ AA but not by sera from 2 healthy persons. When we used the patient serum at different dilutions of more than 1:200, including 1:400, 1:800, 1:1600, 1:6400, and 1:25 600, the serum diluted up to 1:6400 could detect moesin by Western blotting. To measure the titers of moesin Abs in the serum, we established an ELISA using recombinant moesin protein. Higher antimoesin Ab titers than the cutoff value were detected in 25 (37%) of 67 patients. The prevalence was 44% in 41 patients whose duration of illness was shorter than 1 year. Figure 3B shows titers of antimoesin Ab in the sera of 67 patients with AA consisting of 43 patients with

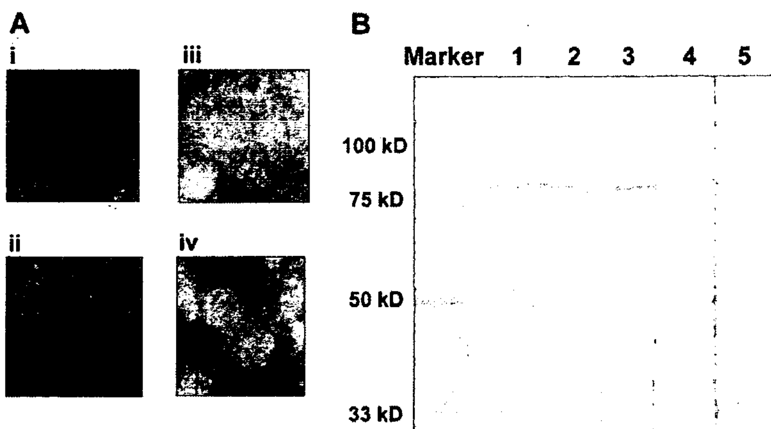

Figure 1. Detection of antibodies (Abs) specific to proteins derived from UT-7 cells in the sera of patients with AA. (A) Immunofluorescence analysis of UT-7 cells using 1:200 diluted sera and FITC-labeled anti–human IgG. (i-ii) Healthy persons; (iii-iv) patients with AA. (B) Western blotting for UT-7 lysates with sera from 3 patients with PNH^+ AA (lanes 1-3) and 2 healthy persons (lanes 4-5). Images were obtained using 1000× magnification.

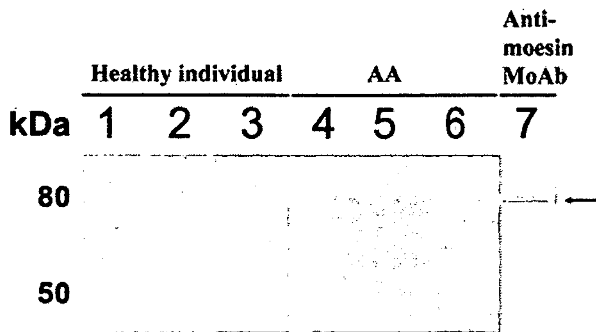

Figure 2. Identification of the 80-kDa protein recognized by IgG Abs of patients with AA. UT-7 lysates (lanes 1, 4), culture supernatants of UT-7 cells (lanes 2, 5, 7), and recombinant Grp 78 protein (lanes 3, 6) were subjected to Western blotting using sera from a healthy person (lanes 1-3), a patients with PNH^+ AA (lanes 4-6), and antimoesin monoclonal Ab (lane 7). The arrow indicates 80-kDa protein bands.

PNH^+ and 24 patients with PNH^-, 21 patients with MDS-RA consisting of 14 patients with PNH^+ and 7 patients with PNH^-, and 48 healthy persons. Twenty (47%) of the patients with PNH^+ AA showed Ab titers greater than the cutoff value, whereas 5 (21%) of the patients with PNH^- AA showed increased titers. There was a significant difference in the prevalence of higher antimoesin Ab titers between patients with PNH^+ AA and patients with PNH^- AA ($P = .03$). Nine (64%) of the patients with PNH^+ MDS-RA showed Ab titers greater than the cutoff value, whereas 3 (43%) of the patients with PNH^- MDS-RA showed increased titers. No significant difference was observed in the prevalence of higher antimoesin Ab titers between patients with PNH^+ MDS-RA and patients with PNH^- MDS-RA ($P = .32$). Among patients with recently diagnosed AA examined before therapy, the prevalence of patients showing higher antimoesin Ab titers than the cutoff value in 31 patients with PNH^+ (52%) was also higher than that in 10 patients with PNH^- (20%), although the difference in the prevalence between the 2 groups was not statistically significant ($P = .08$; Figure 3C). None of the sera from 6 patients undergoing chemotherapy (3 with acute myelogenous leukemia and 3 with non-Hodgkin lymphoma) were positive for antimoesin Abs.

Epitope analysis of antimoesin Abs in patients with AA and those with rheumatoid arthritis

A previous study demonstrated that antimoesin Abs were detectable in 14% of patients with rheumatoid arthritis.[23] Our ELISA revealed high titers of antimoesin Abs in 34% of patients with rheumatoid arthritis who did not show any cytopenias (data not shown). To determine whether a difference exists in the specificity of antimoesin Abs between patients with AA and rheumatoid arthritis, 2 different C-terminal fragments of moesin (M1 and M2) were prepared and used for the ELISA. When we screened the sera from 3 patients showing high titers of antimoesin Abs against the full-length moesin for the presence of Abs specific to N-terminal proteins (amino acid residues 1-398) using Western blotting, none of the sera from these patients revealed a band of the N-terminal protein (data not shown). We therefore focused on the C-terminal protein to detect specific Abs with ELISA. As shown in Figure 3D-E, 12 (19%) patients with AA and 10 patients (20%) with rheumatoid arthritis showed anti-M1 Ab titers, whereas 11 (18%) patients with AA and 5 patients (10%) with rheumatoid arthritis showed anti-M2 Ab titers greater than the cutoff value. Therefore, the 2 groups of patients showed a similar pattern of Ab titers to each fragment.

Secretion of moesin from leukemia cell lines, $CD34^+$ cells, $CD3^+$ T cells, $CD19^+$ B cells, and synovial cells

The secretion of moesin may be common to immature myeloid leukemia cells. We therefore examined culture supernatants of myeloid leukemia cell lines other than UT-7 cells as well as of $CD34^+$ cells, $CD3^+$ T cells, and $CD19^+$ B cells from a healthy volunteer donor. Moesin was detectable in culture supernatant of 4 (K562, OUN-1, NB4 and UT-7) of 7 myeloid leukemia cell lines, $CD34^+$ cells, and $CD19^+$ B cells (Figure 4A). Because B lymphocytes reportedly secrete moesin as a form of exosome,[24] we examined the exosomal fraction of culture supernatant derived from leukemia cell lines and synovial cells of a patient with rheumatoid arthritis for the presence of moesin. As shown in Figure 4B, moesin was detectable in the exosome fraction of culture supernatants from all 4 leukemia cell lines and synovial cells.

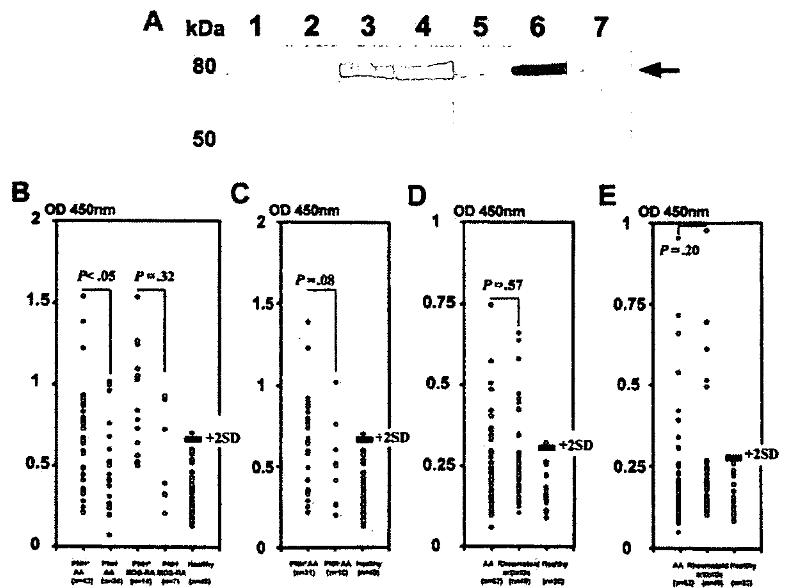

Figure 3. Detection of antimoesin Abs in the sera of patients with AA, MDS-RA, and rheumatoid arthritis. (A) Detection of specific Abs to recombinant moesin in the sera of patients with AA. Purified recombinant native moesin protein was used to detect Abs specific to moesin in sera from 2 healthy persons (lanes 1-2) and 3 patients with PNH^+ AA (lanes 3-5). Antimoesin monoclonal Ab was used as a positive control (lane 6). The serum of a patient with PNH^- AA diluted to 1:6400 was also used (lane 7). The arrow indicates recombinant moesin bands. (B-C) Titration of moesin Abs in sera of patients with AA and MDS-RA using ELISA. Antimoesin Ab titers were determined for the sera of 43 patients with PNH^+ AA, 24 patients with PNH^- AA, 14 patients with PNH^+ MDS-RA, 7 patients with PNH^- MDS-RA, and 48 healthy persons. (B) All patients. (C) Patients with recently diagnosed AA examined before therapy. (D-E) Titration of Abs specific to C-terminal moesin fragment in the sera of patients with AA and rheumatoid arthritis. Titers of anti–C-terminal moesin fragment Abs were determined in the sera of 62 patients with AA, 49 patients with rheumatoid arthritis, and 20 healthy persons. (D) Antimoesin C-terminal fragment (399-500) M1 Abs. (E) Antimoesin C-terminal fragment (494-577) M2 Abs. The solid line denotes a cutoff value defined as the mean + 2 SDs of the absorbance in healthy persons. P values indicate the differences in the prevalence of patients showing higher antimoesin or moesin-fragment Ab titers between 2 different groups.

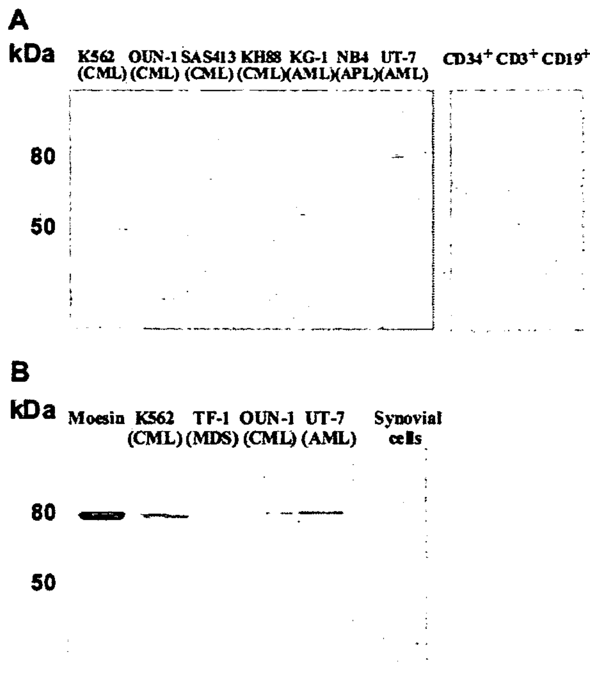

Figure 4. Detection of moesin in the culture supernatant of myeloid leukemia cell lines, CD34⁺ cells, CD3⁺ T cells, CD19⁺ B cells, and synovial cells. (A) Culture supernatants of various myeloid leukemia cell lines, CD34⁺ cells, CD3⁺ T cells, and CD19⁺ B cells were subjected to Western blotting using antimoesin Abs. (B) Exosomal fractions prepared from the culture supernatants of the 4 positive cell lines and synovial cells were also examined by Western blotting.

When the purified exosome fraction was examined by electron microscopy, the presence of small particles which were compatible in size with that of the exosomes was observed (data not shown).

Relationship of antimoesin Abs with either other markers or the response to IST

We previously reported that Abs specific to DRS-1 are detectable in 38% of patients with PNH^+ AA.[13] We then studied the relationship between the presence of antimoesin Abs with the presence of anti–DRS-1 Abs in 45 patients with AA. Antimoesin Abs were detectable in 68% of patients with anti–DRS-1 Ab^+, whereas they were detectable in only 27% of patients with anti–DRS-1 Ab^- ($P = .007$). Twenty-eight patients recently diagnosed underwent ATG + CsA or CsA therapy after examination of their blood for the presence of antimoesin Abs, anti–DRS-1 Abs, and small populations of PNH-type cells (Table 1). There were no significant differences in the rate of response to ATG + CsA or CsA between patients showing antimoesin Abs and those not showing antimoesin Abs (75% versus 67% for ATG + CsA; 100% versus 75% for CsA). When all 3 markers were assessed, 13 (76%) of 17 and 9 (100%) of 9 patients showing at least 1 of the 3 makers improved with ATG + CsA and CsA alone, respectively, whereas none of 2 patients not showing any of these markers responded.

Discussion

The present study revealed that Abs specific to moesin are detectable in the serum of approximately 37% of patients with AA.

Table 1. Relationships between the presence of PNH-type cells and auto-Abs in patients recently diagnosed with AA and clinical significance of antimoesin Abs

Patient	Age, y	Sex	Severity	PNH	Anti-DRS-1 Ab	Antimoesin Ab	Response to IST	IST
1	25	Male	M	+	+	+	+	ATG + CsA
2	29	Female	M	+	+	+	+	ATG + CsA
3	60	Male	M	+	+	+	+	ATG + CsA
4	64	Female	S	+	+	+	+	ATG + CsA
5	66	Female	M	+	+	+	+	ATG + CsA
6	73	Female	M	+	+	+	+	ATG + CsA
7	49	Male	S	+	+	+	−	ATG + CsA
8	66	Male	M	+	+	−	+	ATG + CsA
9	17	Male	M	+	−	+	+	ATG + CsA
10	73	Male	S	+	−	+	+	ATG + CsA
11	72	Male	M	+	−	+	−	ATG + CsA
12	22	Male	S	+	−	−	+	ATG + CsA
13	62	Male	S	+	−	−	+	ATG + CsA
14	83	Female	S	+	−	−	+	ATG + CsA
15	23	Male	S	−	+	−	−	ATG + CsA
16	63	Male	M	−	−	+	+	ATG + CsA
17	77	Female	S	−	−	+	−	ATG + CsA
18	28	Male	M	−	−	−	−	ATG + CsA
19	34	Female	M	+	+	+	+	CsA
20	42	Male	M	+	+	+	+	CsA
21	74	Female	S	+	+	+	+	CsA
22	78	Female	M	+	+	+	+	CsA
23	70	Female	M	+	+	−	+	CsA
24	75	Female	M	+	−	+	+	CsA
25	47	Female	M	+	−	−	+	CsA
26	79	Male	M	+	−	−	+	CsA
27	43	Female	M	−	−	+	+	CsA
28	39	Female	M	−	−	−	−	CsA

M indicates moderate; S, severe; +, positive; and −, negative.

Moesin is an intracellular protein that links the cell membrane and cytoskeleton and mediates the formation of microtubules and cell-adhesion sites as well as ruffling of the cell membrane.[25] This membrane-linking protein is expressed by various blood cells, including megakaryocytes and granulocytes, but its expression is localized inside the cell membrane and not on the cell surface. It is therefore unlikely for antimoesin Abs to affect the function and viability of hematopoietic cells. It is also unlikely that T cells specific for moesin play a role in the inhibition of hematopoietic stem cells in patients with AA because moesin is expressed by various kinds of cells other than blood cells.[26,27] Nevertheless, the presence of antimoesin Abs appears to reflect the immune pathophysiology of bone marrow failure because it correlates with the presence of small population of PNH-type cells which are well associated with the immune pathophysiology of AA.[8]

A previous study demonstrated the presence of antimoesin Abs in 14% of patients with rheumatoid arthritis.[23] None of the patients with antimoesin Abs$^+$ with rheumatoid arthritis in the previous report and of the 11 patients in our study showed cytopenia. In contrast, none of our patients with AA with antimoesin Abs showed symptoms characteristic of rheumatoid arthritis or laboratory findings such as positive rheumatoid factors. There was no difference in the specificity pattern of antimoesin Abs in patients with AA and rheumatoid arthritis. However, a case-control study on AA conducted by IAAS revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA,[28] and moesin was detectable in the exosomes derived from leukemia cell lines as well as from synovial cells of a patients with rheumatoid arthritis. It is therefore possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin.

In T-cell diseases such as AA, the presence of auto-Abs has not attracted much attention from hematologists for a long time. Recently, 3 different auto-Abs specific to kinectin,[29] DRS-1,[13] and anti-postmeiotic segregation increased 1[30] were detected in the serum of patients with AA. All of these Abs were identified through the immunoscreening of serum with a cDNA library derived from fetal liver cells or a leukemia cell line. The Abs to moesin are unique in that they were identified through their direct binding to protein in the denatured lysate of UT-7 cells. Moreover, moesin is the first autoantigen that was successfully identified in autoimmune diseases by using auto-Abs in patient's serum and peptide mass fingerprinting.

Although the serologic identification of antigens by recombinant expression cloning (SEREX) is a useful assay for identifying novel antigens recognized by a small amount of auto-Abs in serum,[13,29] insignificant antigens are often identified because of the low specificity of the assay. Antigens detected by immunoblotting with serum may be significant when the assay revealed distinct bands. However, it is often difficult to identify the amino acid sequence of target antigens in cell lysates with peptide mass fingerprinting because of the presence of many proteins other than the target antigens which are eluted from the cut band. Moesin was successfully identified using the culture supernatant of UT-7 cells as a template because it was secreted from the cell line as a protein in the exosome and formed a single band in the polyacrylamide gel. Immunoblotting of exosomal fractions from culture supernatants followed by peptide mass fingerprinting thus appears to be a powerful method for identifying novel antigens which potentially elicit antibody production.

Our study showed that moesin was secreted into culture medium from various leukemia cell lines and synovial cells of a rheumatoid arthritis patient as an exosomal protein. Previous studies have shown that exosomes secreted from B lymphocytes and mesothelioma cells contain various intracellular proteins, including moesin,[19,24] and that some leukemia/lymphoma cell lines such as K562[21] and Daudi[22] secrete exosome-containing cytosolic proteins. However, moesin has not yet been detected in the exosomal fractions derived from cells from leukemia cell lines and synovial cells. Immunoblotting in the present study has shown the exosome fraction derived from the culture supernatants of the leukemia cell lines to contain moesin. Exosomes are known to be capable of presenting antigen by itself directly or indirectly through being ingested by antigen-presenting cells to helper T cells and eliciting specific Ab production.[31,32] It is conceivable that immature hematopoietic cells and B lymphocytes of patients with AA may also secrete a low amount of moesin as an exosome, leading to the induction of antimoesin Abs. The presence of moesin in the culture supernatant of CD34$^+$ cells and B cells from a healthy person supports this hypothesis.

The prevalence of high antimoesin Abs was significantly higher in patients with PNH$^+$ than in patients with PNH$^-$. A similar association was observed between anti–DRS-1 Ab and PNH-type cells in our previous study. In immune-mediated bone marrow failure such as PNH$^+$ AA, the immune system attack, particularly by CD4$^+$ T cells, is thought to damage hematopoietic stem cells. Various cytokines produced by CD4$^+$ T cells in the process of the immune attack may stimulate hematopoietic cells and B cells to secrete moesin, leading to a breakdown of immune tolerance toward moesin. Although the presence of antimoesin Abs was not associated with a good response to IST in our small series of patients, the detection of antimoesin Abs in combination with anti–DRS-1 Abs and PNH-type cells may help predict a good response to IST. Indeed, the rate of response to IST in 28 patients with AA showing at least 1 of the 3 makers was 85%, whereas none of the 2 patients with AA not showing any of the markers responded. The presence of such auto-Abs may particularly serve as a good marker for immune pathophysiology when sufficient numbers of blood cells for flow cytometry to detect PNH-type cells are not available because of severe bone marrow failure. The predictive value of these autoantibodies needs to be tested in a prospective clinical study.

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Authorship

Contribution: H.T., S.N., and T.C. designed the research, analyzed data, and wrote the paper; M.Y. and S.I. performed electron microscopic examination; X.F. and X.L. performed research; K.O. performed peptide mass fingerprinting; C.S. analyzed data.

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Clinical Significance of a Small Population of Paroxysmal Nocturnal Hemoglobinuria–Type Cells in the Management of Bone Marrow Failure

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Abstract

Although increased blood cell deficiency of glycosyl phosphatidylinositol–anchored membrane proteins has often been detected in patients with aplastic anemia (AA) and myelodysplastic syndrome (MDS), the clinical significance of such paroxysmal nocturnal hemoglobinuria (PNH)-type cells remains to be elucidated. We established a sensitive flow cytometric assay capable of detecting less than 0.01% of CD59$^-$CD55$^-$ blood cells in a sample and used the assay to examine a large number of patients with bone marrow failure. An increase in the proportion of PNH-type cells was detectable in approximately 60% of all AA patients and in 20% of all refractory anemia (RA)-MDS patients. The increase was undetectable in patients with RA with an excessive number of blasts, acute myelogenous leukemia, multiple myeloma, or systemic lupus erythematosus. Our study showed that the presence of an increased number of PNH-type cells was predictive of a good response to immunosuppressive therapy and a favorable prognosis among patients with recently diagnosed AA and RA. A sensitive flow cytometric analysis for detection of a small population of PNH-type cells in peripheral blood cells is one of the most important examinations in the management of bone marrow failure.

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Key words: Paroxysmal nocturnal hemoglobinuria–type cells; Bone marrow failure

1. Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia characterized by an increased number of glycosyl phosphatidylinositol (GPI)-anchored membrane protein–deficient cells derived from hematopoietic progenitor cells with a *PIG-A* gene mutation [1]. A small population of GPI-deficient blood cells are often detected in patients with bone marrow failure who do not have clinical or laboratory signs of hemolysis [2,3]. This type of marrow failure has been defined as subclinical PNH (PNH-sc) by the International PNH Interest Group [4]. In several studies investigators have attempted to clarify the clinical significance of such PNH-type cells in bone marrow failure patients. Some studies have revealed that the presence of PNH-type cells in patients with myelodysplastic syndrome (MDS) [5] and aplastic anemia (AA) [6] is predictive of the response to immunosuppressive therapy (IST). Other studies, however, have not confirmed the predictive value of PNH-type cells [7,8]. This discrepancy may be due to differences in the sensitivity and specificity of the flow cytometric assays used in previous studies to detect PNH-type cells.

We have been using a high-sensitivity flow cytometric assay to detect PNH-type cells in the peripheral blood of patients with various types of bone marrow failure and thus postulated the significance of an increase in the proportion of PNH-type cells as a marker of the immune pathophysiology of AA and refractory anemia (RA)-MDS [9-11]. In this review, we summarize our experience over the past 6 years in the detection of PNH-type cells in patients with various hematologic diseases. We emphasize the importance of using a high-sensitivity flow cytometric assay to detect small populations of PNH-type cells in the management of bone marrow failure.

2. Detection of a Small Population of PNH-Type Cells

A small number of CD55$^-$CD59$^-$ cells can be detected in healthy individuals [12,13]. When we used a 2-color flow cytometric assay to examine 1,000,000 peripheral blood granulocytes from 5 healthy individuals, 12 to 28 CD55$^-$CD59$^-$ cells

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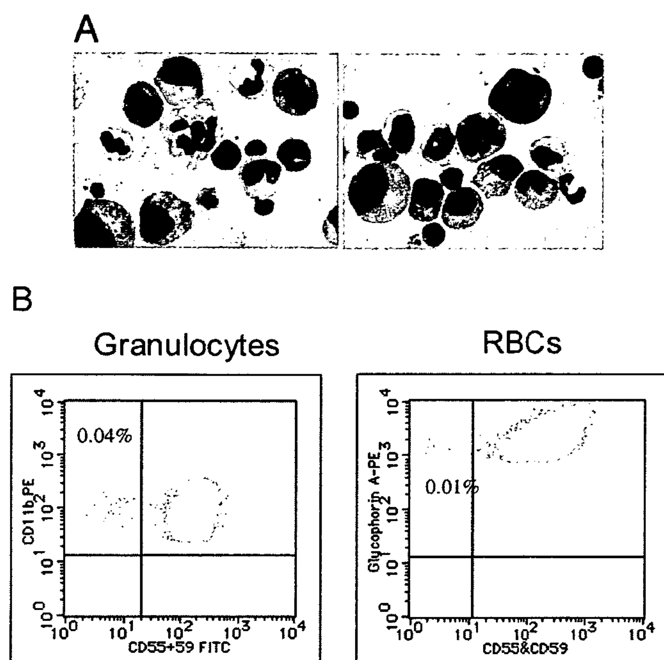

Figure 1. Bone marrow morphology and flow cytometric examination of a typical patient with subclinical paroxysmal nocturnal hemoglobinuria. Bone marrow photomicrograph (A) and results of high-sensitivity flow cytometric analysis (B) of a 28-year-old woman whose condition was diagnosed as myelodysplastic syndrome refractory cytopenia with multilineage dysplasia. RBCs indicates red blood cells; FITC, fluorescein isothiocyanate.

were detected [9]. When we examined 100,000 granulocytes, an appropriate number for routine examination of patient samples, from 68 healthy individuals, 26.5% of the cells exhibited either 1 or 2 $CD55^-CD59^-$ granulocytes, and 50.0% exhibited 1 or 2 $CD55^-CD59^-$ red blood cells (RBCs) [10]. No healthy individual had 3 or more $CD55^-CD59^-$ cells per 100,000 granulocytes and 100,000 RBCs. On the basis of these results, we defined the presence of more than 0.003% $CD55^-CD59^-$ cells among both granulocytes and RBCs as a definite increase in the number of PNH-type cells.

2.1. Importance of Detecting a Minor Population of PNH-Type Cells

The best example for demonstrating the significance of PNH-type cells is moderate AA mimicking RA-MDS. A slight increase in the proportion of PNH-type cells is often detected in patients with this type of bone marrow failure [10]. Because of the presence of moderate signs of dysplasia and normocellularity in iliac bone marrow, the condition of these PNH-sc patients usually is diagnosed as either RA or refractory cytopenia with multilineage dysplasia (RCMD) rather than moderate AA.

Figure 1A shows the marrow characteristics of a typical PNH-sc patient. Because of the presence of erythroid and myeloid dysplasia and of normocellularity in the bone marrow, the condition of this 28-year-old woman was diagnosed as RCMD during pregnancy. The physician in charge planned to treat the patient with allogeneic transplantation from an unrelated donor after delivery of the infant. However, our flow cytometric analysis revealed a small population of $CD55^-CD59^-$ cells in both the granulocytes and the RBCs (Figure 1B). We therefore treated the patient with antithymocyte globulin plus cyclosporine after delivery. Her blood cell counts completely normalized within 1 year after IST. The patient was in complete remission more than 5 years after treatment. Recent bone marrow aspiration revealed no signs of dysplasia.

This patient was about to be treated improperly. PNH-sc patients are likely to be treated with cytotoxic drugs or stem cell transplantation from unrelated donors or to be observed with supportive care unless a high-sensitivity flow cytometric assay is used to examine their blood for the presence of a small population of PNH-type cells.

2.2. Reasons for High Sensitivity of the Flow Cytometric Assay

Conventional flow cytometric assays have defined the presence of 1% or more $CD55^-CD59^-$ cells as indicating a significant increase in the proportion of PNH-type cells, because $CD55^-CD59^-$ cells up to 1% of the total granulocyte and RBC counts are frequently detected in healthy individuals [14]. We used a highly sensitive flow cytometric assay to examine the peripheral blood of more than 600 patients with bone marrow failure for the presence of PNH-type cells. As shown in Figure 2, 56.4% of AA and 19.4% of RA patients were judged to have PNH-sc. Notably, more than 80% of the PNH-sc patients had less than 1% PNH-type cells. Therefore these patients, who are potentially responsive to IST, are overlooked on the basis of findings of conventional flow cytometry.

2.3. Accurate Detection of Small Populations of PNH-Type Cells

In flow cytometry, damaged cells and dead cells can be often be mistakenly identified as $CD55^-CD59^-$ cells owing to

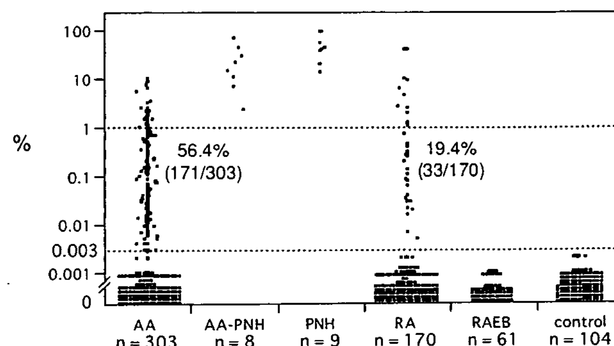

Figure 2. Proportions of PNH-type cells in $CD11b^+$ granulocytes of various patients. Squares indicate proportion of $CD55^-CD59^-CD11b^+$ granulocytes determined with high-sensitivity flow cytometric analysis; AA, aplastic anemia; PNH, paroxysmal nocturnal hemoglobinuria; RA, refractory anemia; RAEB, RA with excess of blasts.

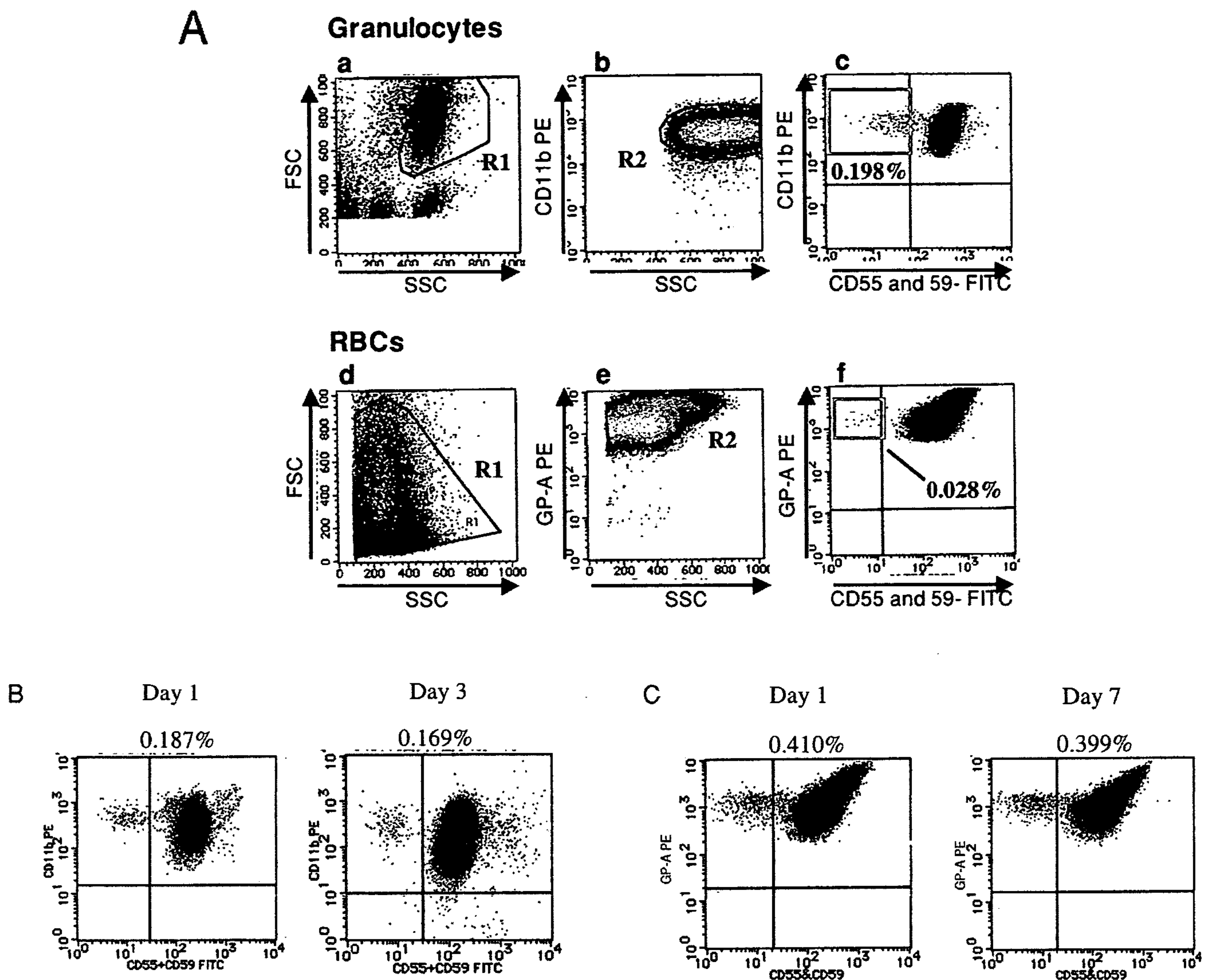

Figure 3. High-sensitivity flow cytometric analysis. A, Example of analysis on granulocytes (A, B, C) and red blood cells (RBCs) (D, E, F) of a patient with aplastic anemia and an increased proportion of paroxysmal nocturnal hemoglobinuria–type cells. Gates were set up to exclude SSC^{dim} (A) and CD11b^{dim} (B) granulocytes and glycophorin A^{dim} (E) RBCs. FSC indicates forward scatter; SSC, side scatter; GP-A PE, glycoprotein A phycoerythrin; FITC, fluorescein isothiocyanate. B, Influence of time from blood collection to treatment of granulocytes with monoclonal antibodies. C, Influence of time from blood collection to treatment of RBCs with monoclonal antibodies.

poor binding of antibodies to these GPI-anchored proteins. It is necessary to eliminate the damaged and dead cells from analysis to reduce the cutoff value to less than 0.01%. By careful gating, as shown in Figure 3A, our sensitive flow cytometric analysis excludes SSC^{dim} and CD11b^{dim} granulocytes and glycophorin A^{dim} RBCs on the histograms [15].

The time from blood collection to treatment of blood cells with monoclonal antibodies is an important factor influencing the results of flow cytometry. Granulocytes have to be treated within 24 hours after blood collection, because CD11b expression by granulocytes diminishes over time (Figure 3B). On the other hand, the time period from 7 to 14 days after blood collection does not affect the results of RBC analysis (Figure 3C). Only 10% of patients have an increase in the proportion of PNH-type cells in granulocytes but not in RBCs [15]. Accordingly, 90% of cases of PNH-sc can be precisely diagnosed with examination of RBCs alone.

3. Clinical Significance of PNH-Type Cells in Patients with Recently Diagnosed AA

We recently compared the IST responses of 83 AA patients who had an increased proportion of PNH-type cells (PNH$^+$ patients) with that in 39 AA patients who did not have such an increase (PNH$^-$ patients) [15]. Figure 4A shows the cumulative incidence of achieving a partial response to IST as defined by Camitta [16]. PNH$^+$ patients had a significantly better and faster response than PNH$^-$ patients. Although the

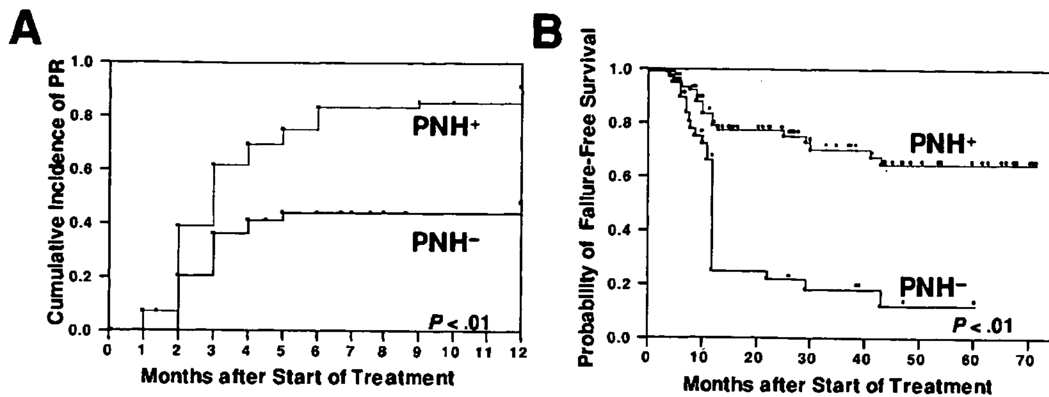

Figure 4. Response to immunosuppressive therapy and prognosis in patients with aplastic anemia. Cumulative incidence of overall responses (A) and failure-free survival (B) in patients with an increased proportion of paroxysmal nocturnal hemoglobinuria–type cells (PNH^+) and patients without such an increase (PNH^-). PR indicates partial response.

overall 5-year survival rate was similar for PNH^+ (77.0%) and PNH^- (70.8%) patients, the probability of surviving failure free for 5 years was markedly higher among PNH^+ patients (64.2%) than PNH^- patients (12.3%) (Figure 4B). These results indicate that a minor population of PNH-type cells is a reliable marker of a positive IST response and a favorable prognosis among patients with AA. The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in AA thus needs to be evaluated with a prospective study. A cooperative study led by the Intractable Disease Study Group of Japan and other referring institutions is underway in Japan.

4. Increase in Proportion of PNH-Type Cells in Diseases Other than AA and RA

Studies have revealed an increased number of PNH-type cells in patients with systemic lupus erythematosus (SLE) [17] and multiple myeloma (MM) [18]. Using high-sensitivity flow cytometry, we analyzed the cases of 43 SLE patients and 14 MM patients and found no small populations of PNH-type cells in any of these patients. As a result, an increase in the proportion of PNH-type cells is thus considered a characteristic of bone marrow failure.

Among bone marrow failure patients, an increased number of PNH-type cells was undetectable in any of 61 patients with RA with excess of blasts or 11 patients with acute myelogenous leukemia (Figure 2). These findings are in line with our observation that clonal hematopoiesis arose more frequently in PNH^- than in PNH^+ AA or RA patients [11]. Accordingly, the presence of PNH-type cells in bone marrow failure thus represents a benign feature of bone marrow failure.

5. Bone Marrow Cellularity in PNH-sc

One of the main reasons for the misdiagnosis of PNH-sc as MDS is that bone marrow aspirate and biopsy specimens from PNH-sc patients often show either normocellularity or hypercellularity. Although most areas of bone marrow are replaced with fat tissue, PNH-sc patients usually have residual hematopoietic nests that produce confusing results during pathological diagnosis. Figure 5A shows a sagittal magnetic resonance image (MRI) of the pelvis from a PNH-sc patient. In this T1-weighted image, dark (cellular) spots are present on the white (fatty) background. Biopsy specimens acquired by chance from the cellular spot are judged normocelluar. A T1-weighted MRI of the thoracic and lumbar vertebrae of the same patient shows fatty marrow with a few cellular spots (Figure 5B), indicating marrow cellularity to be low on the whole. As a result, the bone marrow cellularity of marrow failure patients cannot be determined with bone marrow biopsy alone. MRIs of the thoracic and lumbar vertebrae also must be obtained, particularly when the bone marrow biopsy findings from patients with pancytopenia show either normocellularity or hypercellularity. Inhomogeneous MRI patterns are commonly seen not only in MDS patients [19] but also in PNH-sc patients. A similar MRI pattern of a PNH-sc patient has been reported by Kouba et al [20].

6. Conclusions

Detection of a small population of PNH-type cells is the most fundamental examination for assessing the pathophys-

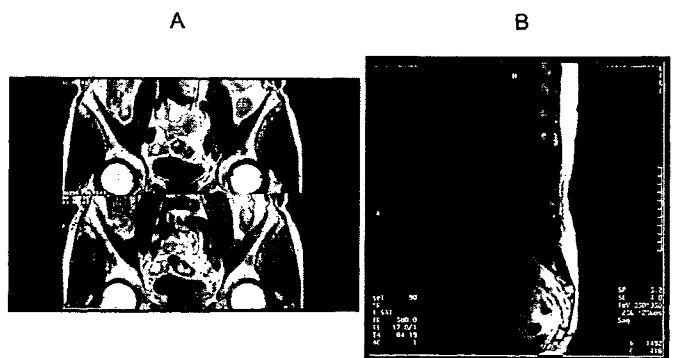

Figure 5. Magnetic resonance images of a patient with subclinical paroxysmal nocturnal hemoglobinuria. A, T1-weighted image of the pelvis. B, T1-weighted image of the thoracic and lumbar vertebrae.

iology of bone marrow failure. High-sensitivity flow cytometry is easily performed. A PDF file that describes use of flow cytometry detection of small populations of PNH-type cells in the peripheral blood can be downloaded from http://web.kanazawa-u.ac.jp/~med18/.

The predictive value of an increased proportion of PNH-type cells for a favorable prognosis in bone marrow failure warrants a worldwide prospective study with non-Japanese patients with AA.

Acknowledgments

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Roles of DRB1*1501 and DRB1*1502 in the pathogenesis of aplastic anemia

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Objective. **Although a number of reports have documented a significantly increased incidence of HLA-DR15 in aplastic anemia (AA), the exact role of HLA-DR15 in the immune mechanisms of AA remains unclear. We herein clarify the difference between DRB1*1501 and DRB1*1502, the two DRB1 alleles that determine the presentation of HLA-DR15, in the pathophysiology of AA.**

Materials and Methods. **We investigated the relationships of the patients* HLA-DRB1 allele with both the presence of a small population of $CD55^-CD59^-$ (PNH-type) blood cells and the response to antithymocyte globulin (ATG) plus cyclosporin (CsA) therapy in 140 Japanese AA patients.**

Results. **Of the 30 different DRB1 alleles, only DRB1*1501 (33.6% vs 12.8%, $p_c < 0.01$) and DRB1*1502 (43.6% vs 24.4%, $p_c < 0.01$) displayed significantly higher frequencies among the AA patients than among a control. AA patients possessing HLA-DR15 tended to be old, and especially, the frequency of DRB1*1502 in patients 40 years of age and older (52.4%) was markedly higher than that in those younger than 40 years old (16.2%, $p_c < 0.01$). Only DRB1*1501 was significantly associated with the presence of a small population of PNH-type cells and it also showed a good response to ATG plus CsA therapy in a univariate analysis. A multivariate analysis showed only the presence of a small population of PNH-type cells to be a significant factor associated with a good response to the immunosuppressive therapy ($p < 0.01$).**

Conclusions. **Although both DRB1*1501 and DRB1*1502 contribute to the development of AA, the methods of contribution differ between the two alleles.** **© 2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

Aplastic anemia (AA) is a syndrome characterized by pancytopenia and bone marrow hypoplasia. Although the etiology remains unclear, the immune destruction of hematopoietic stem cells has been considered the most important mechanism of bone marrow failure in AA [1]. One important finding supporting the role of such autoimmune mechanisms in AA is the high incidence of a certain HLA allele in AA patients. A number of reports have documented a significantly increased incidence of HLA-DR2 or the split antigen HLA-DR15 in AA [2–5]. We previously demonstrated a strong association between DRB1*1501 and a susceptibility to AA, in which the hematopoietic function improves with administration of cyclosporin A (CsA) [6]. Some reports have also demonstrated that HLA-DR15 or DRB1*1501 can predict the response to immunosuppressive therapy (IST) in patients with AA and myelodysplastic syndrome (MDS) [7–9], while others have failed to identify HLA-DR15 as a predictor for the response to antithymocyte globulin (ATG) therapy [3,10,11].

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In our previous study, AA patients carrying DRB1*1502, another major allele corresponding to HLA-DR15 in Japanese, did not show a better response to CsA than those without HLA-DR15 [6]. The exact role of HLA-DR15 in the immune mechanisms of AA thus remains unclear, probably because of the low number of patients that have been studied for DRB1 alleles and the general heterogeneity in the pathogenesis of AA.

Another interesting aspect of HLA-DR15 is the association with the expansion of paroxysmal nocturnal hemoglobinuria (PNH) clones. Several studies have revealed the frequency of HLA-DR15 to be significantly higher in patients with AA and MDS possessing PNH-type blood cells and in florid PNH than in normal controls [10,12], however, the relationship between DRB1 alleles corresponding to DR15 and increased PNH-type cells in AA has not yet been studied in detail. The close relationship between HLA-DR15 and the expansion of PNH clones suggests that the T-cell responses against certain antigen presented by HLA-DR15 or other HLA-class II alleles in linkage disequilibrium with DR15 in hematopoietic stem cells may cause bone marrow failure, thus allowing PNH-type stem cells to survive.

We previous demonstrated the frequency of HLA-DR15 to markedly increase in patients with MDS-refractory anemia (RA) and a small population of PNH-type cells (>0.003% for granulocyte, >0.005% for red blood cells [RBCs]), as demonstrated by sensitive flow cytometry [13]. In that study, RA patients possessing a small population of PNH-type cells displayed favorable responses to CsA. An investigation of a large number of AA patients treated with IST using the same methods to detect small populations of PNH-type cells would thus clarify the role of DRB1 alleles corresponding to HLA-DR15 and PNH-type cells in the immune mechanisms of AA and their mutual relationships. To test this hypothesis, we investigated the relationship between the DRB1 allele in such patients and both the presence of a small population of PNH-type cells and the response to ATG plus CsA therapy in 140 Japanese AA patients.

Materials and methods

Patients

Table 1 summarizes the patient characteristics. The 140 Japanese AA patients were diagnosed at Kanazawa University Hospital, hospitals that participate in a cooperative study led by the Intractable Disease Study Group of Japan, and other referring institutions from April 1999 through November 2005. The study subject included 77 patients who were tested for any correlation between the presence of a minor population in PNH-type cells and the response to IST in our previous study [14]. The severity of AA was classified according to the criteria proposed by Camitta [15] and Marsh et al. [16]. All participants provided written, informed consent to all procedures associated with the study, which was approved by the Ethical Committee at our institution (study number 46). This study also conforms to the recently revised tenets of the Helsinki protocol.

Table 1. Patient characteristics

Characteristics	n	Range
Total (n)	140	NA
Age at diagnosis (y)	60	12–92
Gender: Male/female	65/75	NA
Severity: Severe/moderate	65/75	NA
Neutrophil count ($\times 10^9$/L)	720	0–2226
Platelet count ($\times 10^9$/L)	20	2–118
Reticulocyte count ($\times 10^9$/L)	28	2–106
No. of patients with clonal abnormality (n)	11	NA

NA = not applicable.

Detection of PNH-type cells

We performed two-color flow cytometry of the granulocytes and RBCs according to our previously described method [14,17,18]. First, 3–5 mL heparinized blood was drawn from each patient. To detect the PNH-type granulocytes, phycoerythrin (PE)-labeled anti-CD11b monoclonal antibodies (mAbs; Becton Dickinson, Mountain View, CA, USA), fluorescein-isothiocyanate (FITC)-labeled anti-CD55 mAbs (clone IA10, mouse IgG2a; Pharmingen, San Diego, CA, USA), and FITC-labeled anti-CD59 mAbs (clone p282, mouse IgG2a; Pharmingen) were used in combination with isotype-matched control mAbs, as described previously. To detect PNH-type RBCs, PE-labeled anti-glycophorin A mAbs (clone JC159, DAKO, Glostrup, Denmark) were used instead of anti-CD11b mAbs . Fresh blood was diluted to 3% using phosphate-buffered saline, and 50 mL diluted blood was incubated with 4 mL PE-labeled anti-glycophorin A mAbs , FITC-labeled anti-CD55 and anti-CD59 mAbs on ice for 25 minutes. A total of at least 1 × 10^5 $CD11b^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analyzed using FACScan flow cytometry (Becton Dickinson). In order to avoid any false-positive results, we excluded $CD11b^{dim}$ and glycophorin A^{dim} cells from the analyses using careful gating because these cells include damaged cells those are often mistakenly judged to be PNH-type cells because of their poor binding to anti-CD55 and anti-CD59 mAbs. This flow cytometry method failed to detect 0.003% or more $CD55^-CD59^-CD11b^+$ granulocytes or 0.005% or more $CD55^-CD59^-$glycophorin-A^+ RBCs in any of 183 healthy individuals. We, therefore, defined the presence of >0.003% $CD55^-CD59^-CD11b^+$ granulocytes $CD55^-CD59^-$glycophorin-A^+ RBCs to be abnormal [14,18].

Determination of DRB1 alleles

DRB1 alleles of 140 AA patients and 491 healthy Japanese randomly selected from general population [19] were determined using polymerase chain reactions with sequence-specific primers (PCR-SSP) (Micro SSP HLA DNA typing trays; One Lambda, Canoga Park, CA, USA). Genomic DNA was prepared from blood samples using a DNA extraction kit (Generation capture column kit; Gentra, Minneapolis, MN, USA).

ATG plus CsA therapy and response criteria
Seventy-seven of 140 patients (55.0%) were treated with ATG (15 mg/kg/day, 5 days; Lymphoglobuline, Aventis Behring, King of Prussia, PA, USA) and CsA (Novartis, Basel, Switzerland, 6 mg/kg/day) within 1 year of diagnosis. The dose of CsA was adjusted to maintain trough levels at between 150 and 250 ng/mL and the appropriate dose was administered for at least 6 months. Granulocyte colony-stimulating factor (filgrastim, 300 μg/m^2 or lenograstim, 5 μg/kg) was administered to some patients. The response to ATG plus CsA therapy was evaluated according to the response criteria described by Camitta [20]. A complete response was defined as hemoglobin normal for age, neutrophil count $>1.5 \times 10^9$/L, and platelet count more than 150×10^9/L. A partial response 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 of the normalization of at least one cell line or an increase in the baseline hemoglobin of more than 30 g/L (if initially <60 g/L), a neutrophil count of $>0.5 \times 10^9$/L (if initially $<0.5 \times 10^9$/L), and a platelet count of more than 10×10^9/L (if initially $<20 \times 10^9$/L) in patients with moderate AA.

Statistical analysis
The allele frequency defined as the proportion of patients with at least one copy of a specific gene was determined by direct counting. The χ^2 test compared the allele frequencies of HLA-DRB1 between the patient groups and a Japanese control population, composed of 491 healthy unrelated individuals selected at random from the general population [19]. The corrected value of p (p_c) was calculated by multiplying p with the number of alleles tested (n = 30). The χ^2 test, Fisher's exact test, and logistic procedures [21] analyzed associations between prevalence of increased PNH-type cells and genetic factors, and between individual pretreatment variables and the response to ATG plus CsA therapy. The Kaplan-Meier methods graphically compared the cumulative incidence of the response to ATG and CsA therapy and the time to event, while the log-rank test analyzed differences between the patients who possess HLA-DRB1*1501, DRB1*1502 and DRB1 alleles other than these two alleles. All statistical analyses were performed using the JMP version 5.0.1J software program (SAS Institute, Cary, NC, USA).

Table 2. Frequencies of HLA-DRB1 alleles in Japanese AA patients and controls

	AA patients (n = 140)		Controls (n = 491)		
HLA-DRB1 allele	n	%*	n	%*	p_c value**
0101	10	7.1	64	13.0	NS
0301	0	0.0	4	0.8	NS
0401	2	1.4	17	3.5	NS
0403	4	2.9	18	3.7	NS
0404	0	0.0	2	0.4	NS
0405	35	25.0	129	26.3	NS
0406	5	3.6	32	6.5	NS
0407	2	1.4	2	0.4	NS
0409	0	0.0	1	0.2	NS
0410	1	0.7	17	3.5	NS
0701	0	0.0	2	0.4	NS
0801	0	0.0	0	0.0	NS
0802	6	4.3	36	7.3	NS
0803	8	5.7	84	17.1	NS
0901	36	25.7	148	30.1	NS
1001	2	1.4	2	0.4	NS
1101	7	5.0	22	4.5	NS
1201	7	5.0	34	6.9	NS
1202	2	1.4	12	2.4	NS
1301	0	0.0	4	0.8	NS
1302	11	7.9	61	12.4	NS
1401	2	1.4	21	4.3	NS
1402	0	0.0	2	0.4	NS
1403	4	2.9	13	2.6	NS
1405	4	2.9	18	3.7	NS
1406	2	1.4	10	2.0	NS
1407	0	0.0	1	0.2	NS
1501	47	33.6	63	12.8	<0.01
1502	61	43.6	120	24.4	<0.01
1602	2	1.4	4	0.8	NS

AA = aplastic anemia; NS = not significant.
*Allele frequencies were determined by dividing the number of patients carrying one or two specific alleles by the total number of individuals.
**Corrected p value (p_c) was calculated by multiplying the p value with the number of alleles (n = 30) tested.

Results

Frequencies of DRB1 alleles in AA patients
Table 2 summarizes the frequencies for the 30 different DRB1 alleles identified in the 140 AA patients and 491 controls. Only the frequencies of DRB1*1501 (33.6% vs 12.8%, $p_c < 0.01$, odds ratio = 3.43) and DRB1*1502 (43.6% vs 24.4%, $p_c < 0.01$, odds ratio = 2.39) were significantly higher among the AA patients than among controls. Figure 1 illustrates the numbers of patients with DRB1*1501 and/or DRB1*1502 and the patients without either of the two alleles in the different age groups. Two peaks in the age distribution of the patients were noted, namely, at 20 to 29 years old and at 60 to 79 years old. After dividing the patients into young (younger than 40 years old, n = 37) and old (40 years or older, n = 103) groups, 82.5% of patients in the older group carried at least one of DRB1*1501 or DRB1*1502. Frequency of DRB1*1502 in the older group (54 of 103 patients, 52.4%) was significantly higher (p_c = 0.03) than that in the younger group (6 of 37 patients, 16.2%). No significant difference in the frequency of DRB1*1501 was identified between the two groups (36 of 103 patients, 35.0% vs 11 of 37 patients, 29.7%, p = 0.56).

Prevalence of patients possessing PNH-type cells
A wide range of PNH-type granulocytes (0.005–23.0%; median, 0.153%) and PNH-type RBCs (0.007–6.57%; median, 0.094%) were detected in 92 of 140 (65.7%) AA patients. When patients were divided into four groups according to presence of DRB1*1501 and DRB1*1502, the proportions of PNH$^+$ patients were 66.7% (4 of 6 patients) in the

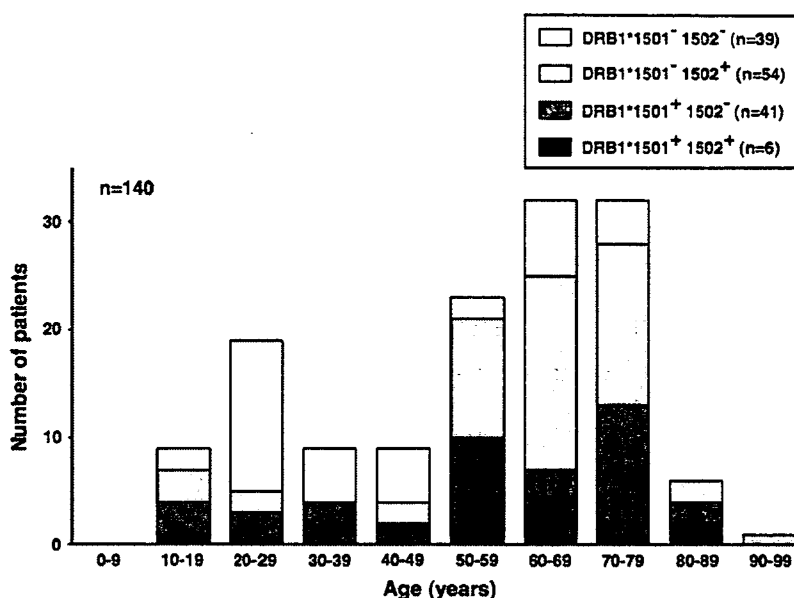

Figure 1. Age distribution of aplastic anemia (AA) patients with or without HLA-DR15. The number of AA patients with or without HLA-DR15 in different age groups is shown. DRB1*1501$^+$1502$^+$, patients with both DRB1*1501 and DRB1*1502; DRB1*1501$^+$1502$^-$, patients with DRB1*1501 but not DRB1*1502; DRB1*1501$^-$1502$^+$, patients with DRB1*1502 but not DRB1*1501; DRB1*1501$^-$1502$^-$, patients with neither DRB1*1501 nor DRB1*1502.

DRB1*1501$^+$1502$^+$ patients, 85.3% (35 of 41 patients) in DRB1*1501$^+$1502$^-$, 59.3% (32 of 54 patients) in DRB1*1501$^-$1502$^+$ and 53.8% (21 of 39 patients) in DRB1*1501$^-$1502$^-$.

Allele frequencies in the PNH$^+$ and PNH$^-$ AA patients
We next divided the 140 AA patients for whom both DRB1 alleles were determined into PNH$^+$ patients (n = 92) and patients without a small population of PNH-type cells (PNH$^-$ patients, n = 48), and then compared the frequency of each DRB1 allele among the three different groups including the PNH$^+$ patients, PNH$^-$ patients, and controls (Fig. 2). The frequency of DRB1*1501 compared to the controls was significantly higher in only the PNH$^+$ patients (39 of 92 patients, 42.4%, $p_c < 0.01$), not in PNH$^-$ patients (8 of 48 patients, 16.7%). On the other hand, the frequency of DRB1*1502 in comparison to the controls was higher in both the PNH$^+$ patients (37 of 92 patients, 40.2%, $p_c = 0.05$) and PNH$^-$ patients (24 of 48 patients, 50.0%). Frequencies of other DRB1 alleles, including DRB1*0405, were similar among PNH$^+$ patients, PNH$^-$ patients, and controls.

Correlation of HLA-DR15 alleles with the prevalence of increased PNH-type cells in AA patients
We analyzed the associations between the prevalence of PNH-type cells and genetic factors, such as age, sex, severity, chromosomal abnormality, and HLA-DRB1 allele to determine which factors might contribute to a slight increase in PNH-type cells in our AA patients. The presence of DRB1*1501 ($p < 0.01$, odds ratio = 3.68) was the only significant factor associated with an increase in the proportion of PNH-type cells based on a univariate analysis, and a multivariate analysis confirmed this result ($p < 0.01$). The presence of DRB1*1502 was not considered to be a contributing factor.

Favorable factors affecting response to ATG plus CsA therapy
Fifty-five of 77 patients (71.4%) improved with ATG plus CsA therapy. The factors favorably affecting the response to IST in the AA patients were examined under a univariate and multivariate analysis (Table 3). Only the presence of PNH-type cells was significantly associated with the response to IST based on a multivariate analysis. After taking into account the kinetics of the response to treatment, we made Kaplan-Meier curves to determine the probability of response to IST in three different groups of patients as defined by DRB1 alleles (Fig. 3). There were significant differences in the probability of the response to IST between the DRB1*1501$^+$1502$^-$ patients and either the DRB1*1501$^-$1502$^+$ patients ($p < 0.01$) or the DR15$^-$ patients ($p = 0.01$) (Fig. 3A). However, these differences in the probability of response IST were no longer observed when the probability of response was compared in either the PNH$^+$ patients or the PNH$^-$ patients (Fig. 3B, C).

Discussion

This study demonstrated for the first time that, in addition to DRB1*1501, which is a major DRB1 allele determining

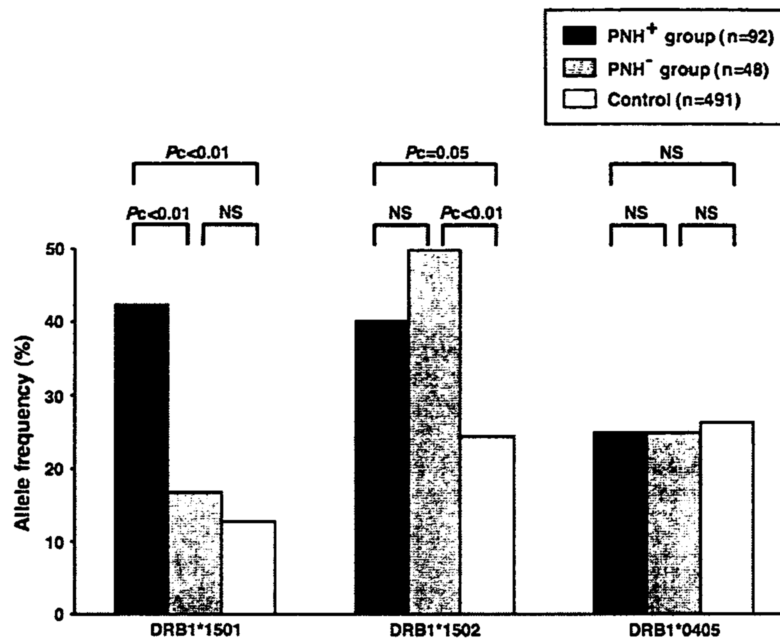

Figure 2. HLA-DRB1 allele frequencies in paroxysmal nocturnal hemoglobinuria (PNH)$^+$ and PNH$^-$ aplastic anemia (AA) patients. Frequencies of the three alleles, DRB1*1501, DRB1*1502, and DRB1*0405 are compared in the PNH$^+$ AA patients, PNH$^-$ AA patients, and controls.

the presentation of HLA-DR15 in Caucasian [2,3] and Chinese populations [4], DRB1*1502 is frequently present in Japanese AA patients. This finding, based on a large number of patients, suggests that the DR15 molecule plays a definite role in development of a subset of AA. Another novel finding in the present study was that the significantly increased frequency of HLA-DR15 was only observed in old AA patients. The frequency of HLA-DR15 reached up to 80% in AA patients 40 years of age or older. The apparent age-dependent differences in HLA-DR15 frequency suggest that the pathophysiology of AA in older patients may therefore differ from that in younger patients. Several studies of Japanese pediatric patients have revealed a relatively high incidence of MDS secondary to AA compared to adult patients [22–24]. Given the lower frequency of HLA-DR15, pediatric AA may thus display a higher proportion of bone marrow failure caused by nonimmune mechanisms than adult AA.

In contrast to the findings of previous reports, DRB1*1501 appeared to confer a better chance of response to regimens including ATG than other DRB1 alleles, including DRB1*1502. We previously demonstrated that DRB1*1501 predicts the response to CsA, but not to ATG [11]. In the previous study, only 6 of 59 ATG-treated patients received CsA. The combined use of CsA and the larger number of ATG-treated patients in the present study probably accounts for the different findings regarding the role of DRB1*1501 in predicting the response to ATG therapy. DRB1*1501 may affect the response of AA to ATG therapy only when CsA is administered in combination with ATG.

Several previous studies failed to confirm the role of HLA-DR15 in predicting the response to ATG [3,10]. Most previous studies analyzed DRB1 alleles using low-resolution methods that are unable to sufficiently distinguish DRB1*1502 from DRB1*1501. DRB1*1502 accounts for 3% to 7% of the DRB1 alleles corresponding to DR15 even in Caucasians [25], and this frequency may even be higher in AA patients, particularly among AA patients 40 years of age or older. As a result, some patients with DR15 who did not respond to ATG in previous studies may have been DRB1*1502$^+$, rather than DRB1*1501$^+$. The results of this study indicate the importance of

Table 3. Pretreatment variables associated with a response to antithymocyte globulin plus cyclosporin A therapy

	p Value	
Favorable factors	univariate*	multivariate**
Gender (male vs female)	0.32	0.47
Age (at least 40 y vs younger)	0.79	0.37
Severity (severe vs moderate)	0.61	0.86
HLA-DRB1*1501 (positive vs negative)	0.03	0.19
HLA-DRB1*1502 (positive vs negative)	0.61	0.46
PNH-type cells (positive vs negative)	<0.01	<0.01

*Fisher's exact probability test.
**Wald c^2 test for a logistic regression model.
PNH = paroxysmal nocturnal hemoglobinuria.

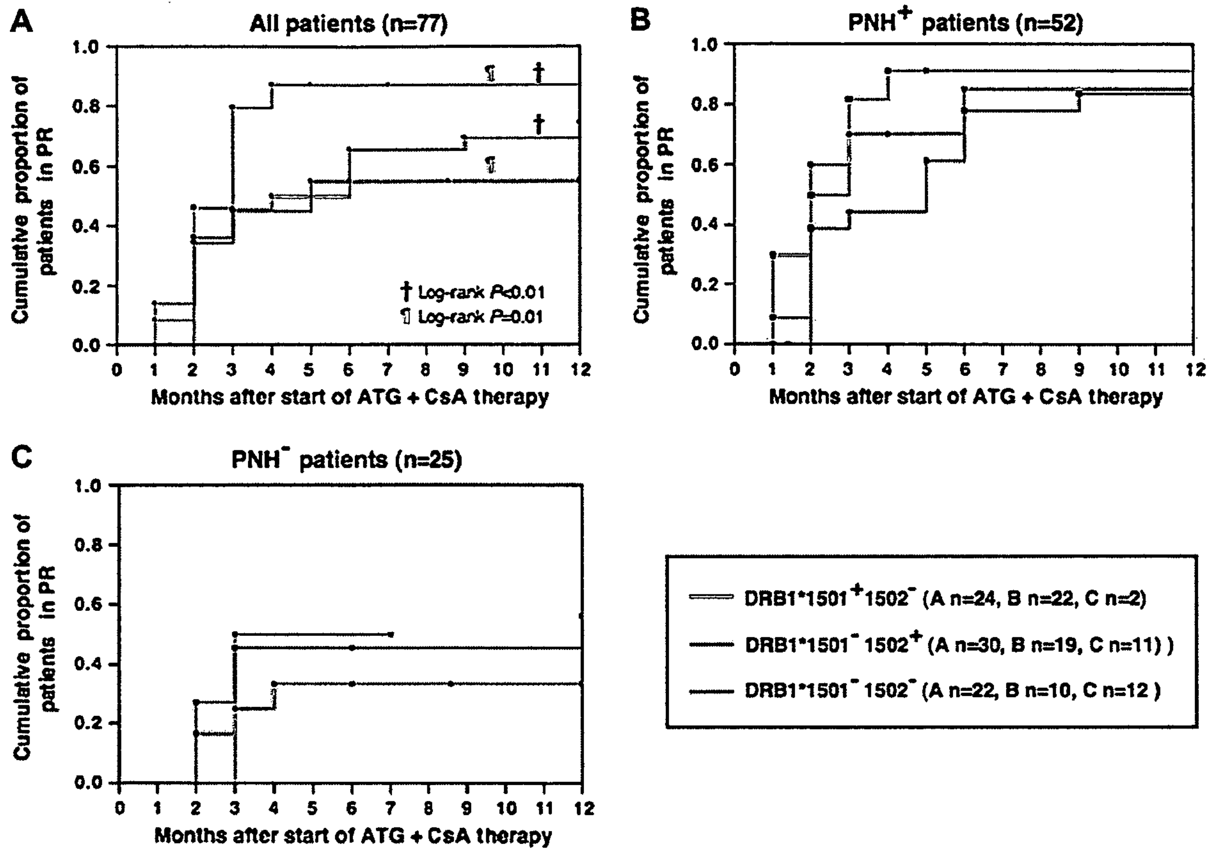

Figure 3. Kinetics of response to antithymocyte globulin (ATG) plus cyclosporin A (CsA) therapy. Kaplan-Meier curves for the response in the different groups of patients based on the DRB1 alleles are shown. DRB1*1501$^+$1502$^+$ patients were not showed in this figure because only one patient (he was paroxysmal nocturnal hemoglobinuria [PNH]$^+$) was available for the analysis. (**A**) all patients; (**B**), PNH$^+$ patients; (C), PNH$^-$ patients.

accurately determining the DRB1 alleles using high-resolution methods to clarify the role of HLA-DR15 in predicting a response to IST.

A higher frequency of HLA-DR15 among PNH$^+$ patients in comparison to PNH$^-$ patients has been reported by Maciejewsky et al. [26]. The present study confirmed this finding using a different flow cytometry assay that distinguished PNH$^+$ patients from PNH$^-$ patients using lower levels of glycosylphosphatidyl inositol-anchored protein-deficient cells than the assay used in the previous study. Our methods also identified a significant difference between DRB1*1501 and DRB1*1502 in the minimal expansion of PNH clones. The frequencies of both alleles increased in the PNH$^+$ patients in comparison to normal controls, thus supporting the preliminary results of our study of 23 patients with refractory anemia [13]. However, only DRB1*1501 represented a genetic factor significantly associated with an increase in the proportion of PNH-type cells in AA patients in the present study because the frequency of DRB1*1502 was high in both PNH$^+$ and PNH$^-$ AA patients, thus indicating that the minimal expansion of PNH clones is not affected by DRB1*1502. Together with the difference in the response rate to IST between DRB1*1501$^+$ and DRB1*1502$^+$ AA patients, all these findings suggest that DRB1*1501 and DRB1*1502, therefore, play a different role in the pathogenesis of AA.

In AA patients carrying DRB1*1501, the presentation of autoantigen by this molecule may readily induce a cell-mediated attack against hematopoietic stem cells that may be associated with minimal expansion of a PNH clones. Previous studies have demonstrated that the presence of a CD4$^+$ T-cell attack against hematopoietic stem cells allows the survival of PNH-type stem cells [27,28]. On the other hand, polymorphic gene alleles of myelosuppressive cytokines, in linkage disequilibrium with DRB1*1502 may predispose individuals with HLA-DRB1*1502 toward development of AA. In keeping with this hypothesis, a recent study on diabetes mellitus patients revealed that a haplotype of TNFa12-DRB1*1502 was, therefore, more frequent in patients likely to develop insulin-dependency than in those who do not develop insulin-dependency [29]. Several reports have demonstrated TNFa12 to be associated with a higher secretion of tumor necrosis factor-α [30].