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Table 1. Hematologic parameters of donor and recipient

	Donor		Recipient			
			Before 1st SCT	Before 2nd SCT	At ATG therapy	After 20 mo of ATG therapy
Date	Apr 2002	May 2008	Apr 2002	Jan 2006	Aug 2006	Apr 2008
WBC count, $\times 10^9$/L	7.0	5.1	1.2	1.7	5.3	4.0
Neutrophil proportions, %	77	65	0	0	17	62
RBC count, $\times 10^{12}$/L	4.21	4.43	2.20	2.75	2.07	3.04
Reticulocytes, $\times 10^9$/L	not tested	35	2	3	26	61
Hemoglobin, g/L	146	150	72	89	75	120
Platelet count, $\times 10^9$/L	261	230	19	52	22	54

$CD55^-CD59^-CD11b^+$. The *D1S80* locus was amplified from DNA of different cell populations with an AmpliFLP D1S80 PCR Amplification Kit (Perkin-Elmer Cetus, Norwalk, CT).

PIGA gene analysis

The coding regions of *PIGA* were amplified by seminested PCR or nested PCR from DNA extracted from the sorted PNH-type cells using 12 primer sets[3,4] (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), and 6 ligation reactions were used to transform competent *Escherichia coli* JM109 cells (Nippon Gene, Tokyo, Japan). Five clones were selected randomly from each group of transfectants and subjected to sequencing with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Amplification refractory mutation system PCR

On the basis of a mutant sequence detected in *PIGA* of the patient, a nested amplification refractory mutation system (ARMS) forward primer with a 3′-terminal nucleotide sequence complementary to the mutant sequence was prepared[5] (Table S1). To enhance the specificity, a mismatch at the penultimate nucleotide position of the mutation site was incorporated in the ARMS forward primer (P1).[6,7] P1 and a reverse primer (P3) were used to amplify a 127 bp fragment containing the mutant sequence from the exon 2 amplified product. PCR was conducted under the following conditions; denaturation for 30 seconds at 94°C, annealing for 60 seconds at 64°C and extension for 90 seconds at 72°C for 20 cycles. Another forward primer (P2), complementary to the wild-type *PIGA* sequence upstream of the mutation site, was used in combination with P3 to amplify an internal control according to the same condition of ARMS-PCR.

Results and discussion

PNH-type cells were not detected in the donor or the patient at the time of development of the first LGF, whereas 0.147% PNH-type granulocytes and 0.019% PNH-type RBCs were detected in the PB

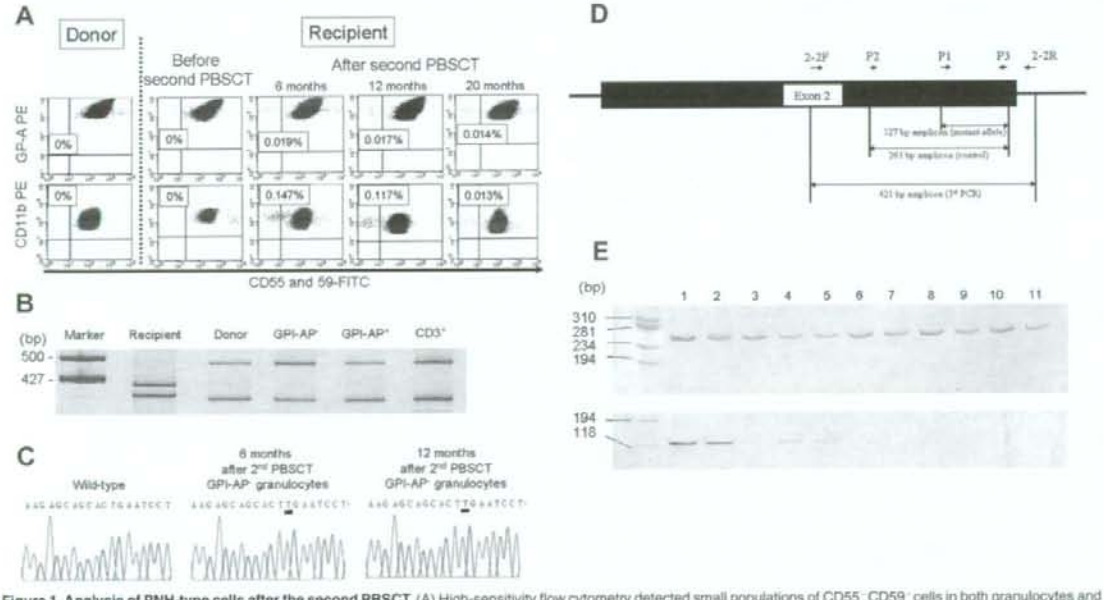

Figure 1. Analysis of PNH-type cells after the second PBSCT. (A) High-sensitivity flow cytometry detected small populations of $CD55^-CD59^-$ cells in both granulocytes and red blood cells at the development of the second LGF as well as in those obtained 6 and 12 months later, but did not detect PNH-type cells in the donor or in the recipient before the second PBSCT. The numbers denote the proportion of PNH-type cells in $CD11b^+$ granulocytes or glycophorin A^+ RBCs. (B) *D1S80* allelic patterns of sorted $GPI\text{-}AP^-$ granulocytes, $GPI\text{-}AP^+$ granulocytes, and $CD3^+$ lymphocytes. The polymerase chain reaction (PCR) products were subjected to 8% polyacrylamide gel electrophoresis and visualized by silver staining. (C) Nucleotide sequences of *PIGA* exon 2 in DNA from PNH-type granulocytes obtained 6 and 12 months after the second PBSCT. (D) A schematic illustration for ARMS-PCR is shown. Primer positions for the first, second are shown by short arrows. A black box and adjacent lines represent exon 2 and introns, respectively. (E) Amplified products of control PCR (the upper gel) and ARMS-PCR (the lower gel) were electrophoresed in 12.5% polyacrylamide gel and visualized by the silver staining. A pMD20-T vector containing the mutated exon 2 fragment was used as a positive control for ARMS-PCR. The template DNA derives from a plasmid containing the mutated exon 2 in lane 1, donor BM in lane 2, donor PB in lane 3, recipient BM in lane 4, recipient PB in lane 5, and BM from healthy individuals in lanes 6 to 11. PCR with a 5′ primer specific to the nucleotide sequence upstream of the mutated sequence amplified a 261 bp fragment from DNA of the donor and all healthy individuals.

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obtained at the time of development of the second LGF (Figure 1A). Similar percentages of PNH-type blood cells were detectable in the PB of the patient 6 and 14 months later. When PB from 51 SCT recipients was examined, none of the patients were found to have detectable PNH-type cells (data not shown). PNH-type blood cells were also undetectable in a donor PB sample obtained 21 months later.

The *D1S80* locus allelic pattern of the PNH-type granulocytes in the patient was compatible to that of the donor (Figure 1B). The emergence of donor-derived PNH-type cells and hematologic improvement after immunosuppressive therapy suggest that LGF arises as a result of de novo development of AA which affects the donor-derived hematopoietic stem cells (HSCs).

PIGA gene analysis of the DNA prepared from the sorted PNH-type cells of the patient obtained at the development of LGF and 6 months later showed an insertion of thymine at position 593 (codon 198) in 3 of 5 clones and 5 of 5 clones examined, respectively (Figure 1C). Mutations in other exons were not identified. The presence of a single *PIGA* mutation in PNH-type granulocytes and its persistence over 6 months suggest that these PNH-type cells are derived from a mutant HSC rather than from a committed granulocyte progenitor cell. Moreover, an ARMS-PCR with a 5′ primer specific to the mutated sequence amplified a 127 bp fragment from DNA of the donor BM as well as of the recipient BM and PB while it failed to amplify the same fragment in donor PB and in BM of all 10 healthy individuals (Figure 1D).

These experiments demonstrate that *PIGA*-mutant HSCs were present in the BM of the donor in a dormant state and were transplanted into the recipient and provide, for the first time, in vivo evidence that *PIGA* mutant, GPI-AP–deficient HSCs have a survival advantage in the setting of immune mediated BM injury. Similarly, relative resistance to immune injury likely accounts for the high incidence of PNH observed in association with acquired AA.

Acknowledgments

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Authorship

Contribution: K.M. and C.S. participated in designing and performing the research. Z.Q. and X.L. performed experiments. K.M., C.S., and S.N. wrote the paper. C.S., A.T., K.I., Y.K., H.Y., and H.O. provided patient care. All authors have approved the final version of the manuscript.

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Anti-Moesin Antibodies in the Serum of Patients with Aplastic Anemia Stimulate Peripheral Blood Mononuclear Cells to Secrete TNF-α and IFN-γ[1]

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Moesin is an intracellular protein that links the cell membrane and cytoskeleton, while also mediating the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane. To determine the roles of anti-moesin Abs derived from the serum of patients with aplastic anemia (AA) in the pathophysiology of bone marrow failure, we studied the expression of moesin on various blood cells and the effects of anti-moesin Abs on the moesin-expressing cells. The proteins recognized by anti-moesin mAbs were detectable on the surface of T cells, NK cells, and monocytes from healthy individuals as well as on THP-1 cells. The peptide mass fingerprinting of the THP-1 cell surface protein and the knock-down experiments using short hairpin RNA proved that the protein is moesin itself. Both the anti-moesin mAbs and the anti-moesin polyclonal Abs purified from the AA patients' sera stimulated THP-1 cells and the PBMCs of healthy individuals and AA patients to secrete 60–80% as much TNF-α as did LPS 100 ng/ml. Although the polyclonal Abs induced IFN-γ secretion from the PBMCs of healthy individuals only when the PBMCs were prestimulated by anti-CD3 mAbs, the anti-moesin Abs were capable of inducing IFN-γ secretion from the PBMCs of AA patients by themselves. Anti-moesin Abs may therefore indirectly contribute to the suppression of hematopoiesis in AA patients by inducing myelosuppressive cytokines from immunocompetent cells. *The Journal of Immunology*, **2009, 182: 703–710.**

Acquired aplastic anemia (AA)[4] is a syndrome characterized by pancytopenia and bone marrow (BM) hypoplasia (1). The T cell-mediated suppression of hematopoiesis is considered to be the most important mechanism responsible for the development of this syndrome because approximately 70% of AA patients respond to immunosuppressive therapy, such as antithymocyte globulin and cyclosporine (2, 3). In addition to a large body of evidence for T cell involvement in the pathogenesis of AA (4–7), recent studies have revealed the presence of Abs specific to self-Ags in the serum of AA patients (8–11). Although some of these Abs are directed toward Ags that are abundant in hematopoietic cells (e.g., kinectin (Ref. 8) and DRS-1 (Ref. 9)), their roles in the pathophysiology of AA are unclear.

Of the various autoAbs detected in the autoimmune diseases, some are known to exhibit stimulatory effects on the target cells rather than inhibitory effects, such as anti-thyroglobulin Abs in Basedow's disease (12) and anti-desmoglein Abs in pemphigus vulgaris (12, 13). The autoAbs specific to platelet-derived growth factor receptors in patients with scleroderma and those with extensive chronic graft-vs-host diseases trigger an intracellular loop, involving Ha-Ras-ERK 1 and 2 (ERK 1/2)-reactive oxygen species (Ha-Ras-ERK 1/2-ROS), and augment collagen gene expression as well as myofibroblast phenotype conversion of normal human primary fibroblasts (14, 15). The anti-proteinase 3 Abs detected in Wegener's granuloma stimulate monocytes through the binding of cell surface proteinase 3 to secrete IL-8 (16). The autoAbs detected in AA patients may also be involved in the pathophysiology of BM failure by way of other mechanisms than the direct toxicity against the hematopoietic cells, though there has been no evidence for such functional autoAbs in AA patients.

We previously demonstrated that Abs specific to moesin, a membrane-cytoskeleton linker protein in the cytoplasm, were detectable in approximately 40% of AA patients (11). 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 (17). On the other hand, some reports have identified molecules that were recognized by anti-moesin mAbs on the surface of blood cells such as T cells and macrophages (18, 19). Because these immune cells are an important source of myelosuppressive cytokines such as TNF-α and IFN-γ, it is conceivable that anti-moesin Abs in AA patients may bind such moesin-like molecules on these immune cells and affect the cytokine secretion from these cells.

To test these hypotheses, we studied the expression of moesin on blood cells and the effects of anti-moesin Abs on the moesin-expressing

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[4] Abbreviations used in this paper: AA, aplastic anemia; BM, bone marrow; pAb, polyclonal Ab; PB, peripheral blood.

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cells. The present study revealed a novel function of autoAbs, which may contribute to the pathophysiology of BM failure.

Materials and Methods

Study subjects (patients)

Sera were obtained from 19 patients with AA and 4 healthy individuals. BM plasma was obtained from five patients with AA and three healthy individuals. All AA patients had severe AA and were positive for anti-moesin Abs. The samples were cryopreserved at −80°C until use. Peripheral blood (PB) was obtained from 7 patients with AA and 10 healthy individuals and BM was aspirated from 3 healthy individuals. The PBMCs were isolated using lymphoprep (Nycomed). All patients and healthy volunteers provided an informed consent before sampling according to the Declaration of Helsinki. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science.

Cell lines

Molt-4, THP-1, U937, K562, Daudi, and Jurkat cell lines were purchased from the Health Science Research Resources Bank. A megakaryoblastic leukemia cell line UT-7, a myeloid leukemia cell lines OUN-1, and a myelodysplastic syndrome cell line TF-1 were provided by Dr. N. Komatsu of Jichi Medical School, Dr. M.Yasukawa of Ehime University, and Dr. S. Ogawa of the University of Tokyo, respectively.

Purification of anti-moesin Abs in the sera of patients with AA

The anti-moesin polyclonal Abs (pAbs) were purified from the patients' serum with affinity chromatography using a protein G column (mAb Trap kit, no. 17-1128-0; GE Healthcare) and recombinant moesin protein (11) fixed on an agarose-gel column (1 ml, HiTrap NHS-activated HP, no. 17-0716-01; GE Healthcare) according to the manufacturer's instruction. In brief, 20 ml of serum from AA patients was applied to the Protein G column. After washing with the binding buffer, the whole IgG was eluted with the elution buffer and neutralized with the neutralizing buffer. The purified IgG was then applied to the recombinant moesin-fixed affinity chromatography column. After washing with the binding buffer (75 mM sodium phosphate (pH 8.0)), anti-moesin pAbs were eluted with the elution buffer (100 mM glycine-HCl and 500 mM NaCl (pH 2.7)). The purified anti-moesin pAbs were dialyzed in PBS at 4°C overnight using a spectra/Por Float-A-Lyzer column (Spectrum Laboratories; no. 235118). The purity of the isolated anti-moesin pAbs was confirmed by PAGE followed by Coomassie Brilliant Blue staining.

Flow cytometry

Mouse anti-moesin mAb (clone 38/87; Neomarkers) which was labeled with FITC (Immuno-Biological Laboratories) was used in combination with mAbs specific to CD3-PE (BD Pharmingen; no. 555333), CD19-PE (BD Pharmingen; No. 555413), CD4-PE (BD Pharmingen; No. 347327), CD-8-PE (BD Pharmingen; no. 555367), CD14-PE (BD Pharmingen; No. 555398), CD11b-PE (BD Pharmingen; No. 555388), CD34-PE (BD Pharmingen; no. 348057), and CD3-Cy-Chrome (BD Pharmingen; no. 555334). Isotype-matched control mAbs (BD Pharmingen) were used as negative controls. For the detection of moesin-like molecules on leukocytes and leukemia cell lines, 1 μl of anti-moesin mAbs and 2 μl of PE-labeled mAbs were added to 50 μl of cell suspension containing 1×10^6 cells and the suspension was incubated on ice for 30 min. For the detection of moesin-like molecules by pAbs, 1×10^6 THP-1 cells were washed twice with PBS containing 1% BSA (Sigma-Aldrich; no. A8022) and resuspended in 200 μl of a PBS containing 2% FCS, 2% goat serum, and 2% BSA. The cell suspension was incubated for 30 min at 4°C. Then, 5 μg/ml anti-moesin pAbs or isotype control human IgG pAbs isolated from healthy individuals were added to the cell suspension and incubated for 1 h at 4°C. The cells were washed twice with PBS containing 1% BSA followed by incubation with a secondary Ab (goat anti-human IgG FITC-labeled Ab; Sigma-Aldrich; no. F5512) diluted 1/100 in PBS containing 2% goat serum and were incubated at 4°C for 30 min. Finally, the cells were washed twice with PBS containing 1% BSA and subjected to flow cytometry.

Stimulation of THP-1 cells with PMA/LPS

THP-1 cells (10^6) were suspended in 2 ml of RPMI 1640 containing 10% FCS and 20 ng/ml PMA (Wako Chemicals; no. 545-00261) and incubated for 24 h at 37°C in a CO_2 incubator. A total of 10 ng/ml LPS (Sigma-Aldrich; no. L2880) was added to the cell suspension and further incubated for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry using anti-moesin mAbs (clone 38/87; Neomarkers).

Isolation of monocytes and T cells

Monocytes were isolated by plastic adherence as previously described (20). In brief, 5×10^6 PBMCs/well were distributed into 12-well plates (Corning) and allowed to adhere in a 5% CO_2 incubator at 37° for 2 h in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin - 0.1 mg/ml streptomycin (Invitrogen; No.15140-148) and 10 μg/ml polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538). Nonadherent cells were removed and the remaining adherent cells on the plates were used as monocytes. T cells were purified by negative selection using the Human T Cell Enrichment Columns (R&D Systems; no. HTCC-500) following the manufacturer instructions. The purity of enriched T cells and monocytes was approximately 90% as determined by flow cytometry using anti-CD3-PE and anti-CD-14-PE mAbs, respectively.

Stimulation of THP-1 cells, PBMCs, monocytes, and T cells with anti-moesin Abs

THP-1 cells, PBMCs, monocytes, or T cells were suspended in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS. Polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538) was added at 10 μg/ml to eliminate any contaminating endotoxin. The endotoxin concentration in the pAbs and the reagents used for culture was <10 pg/ml as demonstrated by chromogenic *Limulus* amebocyte lysate assay (Seikagaku). The cells (5×10^5) were incubated for 48 h in the presence of 5 μg/ml of anti-moesin mouse mAbs (clone 38/87, IgG1; Neomarkers) or 5–10 μg/ml of pAbs isolated from the serum of AA patients as described above. Mouse mAbs (Coulter Clone; IgG1, no. 6602872) and control human IgG pAbs isolated from healthy individuals were used as negative controls. This Ab concentration was selected based on an estimated concentration of anti-moesin pAbs in the serum of an AA patient. For prestimulation of PBMCs to induce IFN-γ secretion, the cells were incubated for 1 h in the presence of 100 ng/ml of anti-CD3 mAbs (Clone OKT3) (eBioscience; functional grade no. 16-0037). For co-stimulation of isolated T cells to induce IFN-γ secretion, the cells were cultured for 48 h on a 48-well tissue culture plate that was coated with 100 ng/ml of anti-CD3 mAbs (clone OKT3) (eBioscience; functional grade no. 16-0037) overnight at 4°C and washed. Then, 100 ng/ml LPS or 10 μg/ml PHA (Sigma-Aldrich; no. L4144) was included instead of anti-moesin Abs as the positive controls for the induction of TNF-α or IFN-γ secretion, respectively, and control human IgG pAbs isolated from healthy individuals were added as a negative control.

Western blotting

Western blotting was performed using THP-1 cell lysates. The specific bands were visualized by anti-moesin mAbs, pAbs from AA patients, or control human IgG pAbs from healthy individuals as described in a previous report (11). The expression levels of α-tubulin were determined as an internal control using Western blotting with anti-α-tubulin mAbs (Sigma-Aldrich; no. T 5168).

Isolation of proteins on the surface of THP-1 cells

The THP-1 cells were treated with sulfo-NHS-SS-biotin, and the cell surface proteins were isolated with avidin-fixed columns according to the manufacturer's instructions (Pierce). Thereafter, 1×10^7 cells were washed twice with 8 ml of ice-cold PBS. The cells were suspended in 10 ml PBS containing 2.5 mg sulfo-NHS-SS-Biotin and incubated for 30 min at 4°C. Then, 500 μl of quenching solution was added to the cell suspension and the cells were washed with 10 ml TBS twice. The cell pellet was lysed in 500 μl of lysis buffer containing 60 μl of preotease inhibitor cocktail (Sigma-Aldrich; no. P-8340) and then disrupted by sonication. The biotin-labeled membrane proteins were isolated by an immobilized NeutrAvidin Gel column. The isolated membrane proteins were subjected to Western blotting and peptide mass fingerprinting.

Peptide mass fingerprinting

Mass spectrometric identification of 80- and 75-kDa proteins on the surface of the THP-1 cells was performed as previously described (21). In brief, the proteins fractionated by SDS-PAGE were visualized by Coomassie Brilliant Blue staining and the 80- and 75-kDa bands were excised from gels, followed by in-gel digestions with trypsin (Promega) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C. Molecular mass analyses of the tryptic

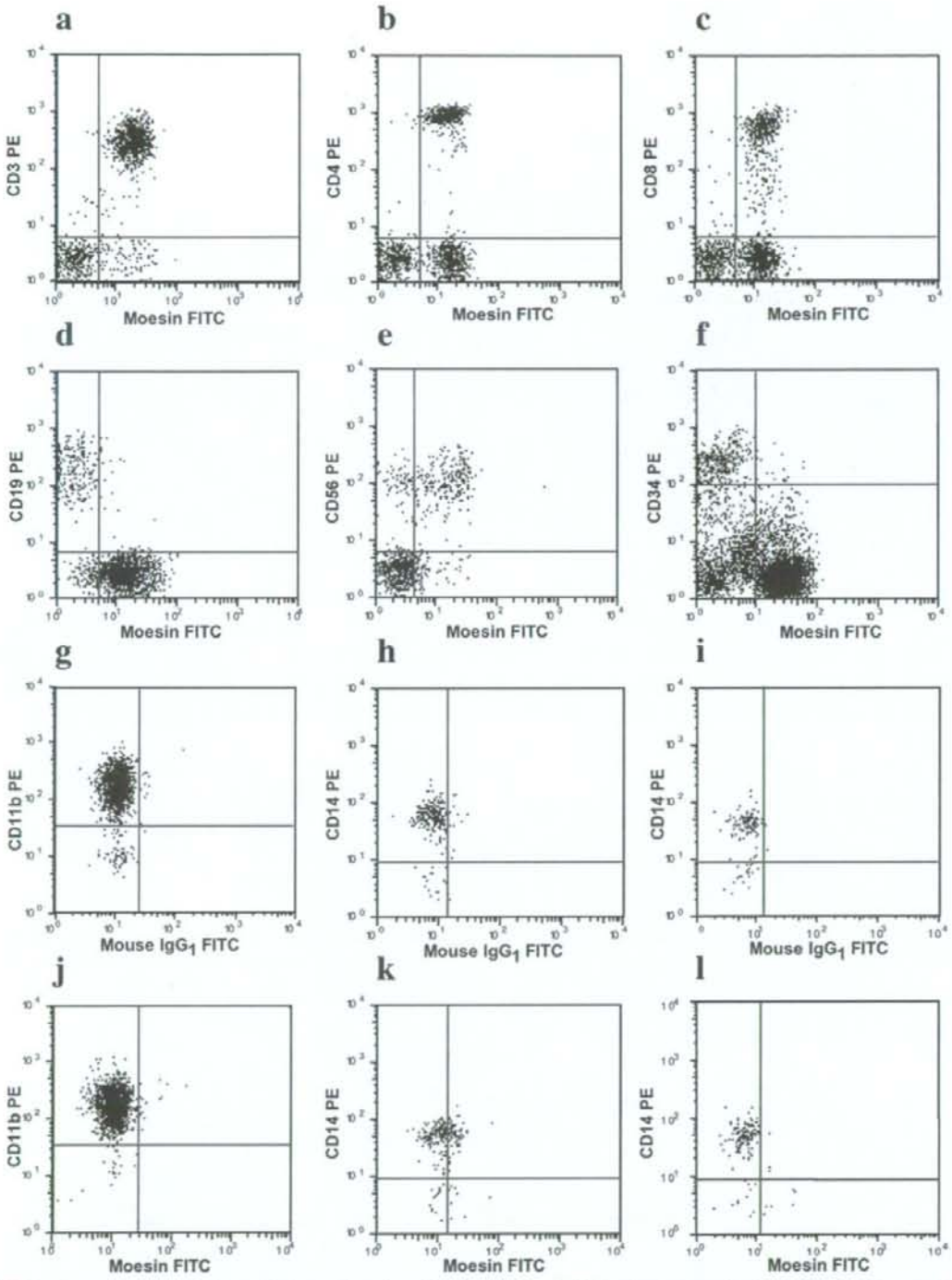

FIGURE 1. Expression of moesin-like molecules on the surface of various blood cells. PB lymphocytes, granulocytes, and monocytes, as well as BM mononuclear cells, of a healthy individual and a patient with AA were analyzed by flow cytometry. The gate was set up for lymphocytes (*a–d*), $CD3^-$ lymphocytes (*e*), granulocytes (*g* and *j*), and monocytes (*h* and *k*) derived from a healthy individual and monocytes (*i* and *l*) derived from an AA patient. BM mononuclear cells (*f*) of a healthy individual were included in the analysis. One representative result of three experiments is shown.

peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an ultraflex TOF/TOF (Bruker Daltonics). The proteins were identified by comparisons between the molecular weights determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the theoretical peptide masses of proteins registered in NCBInr.

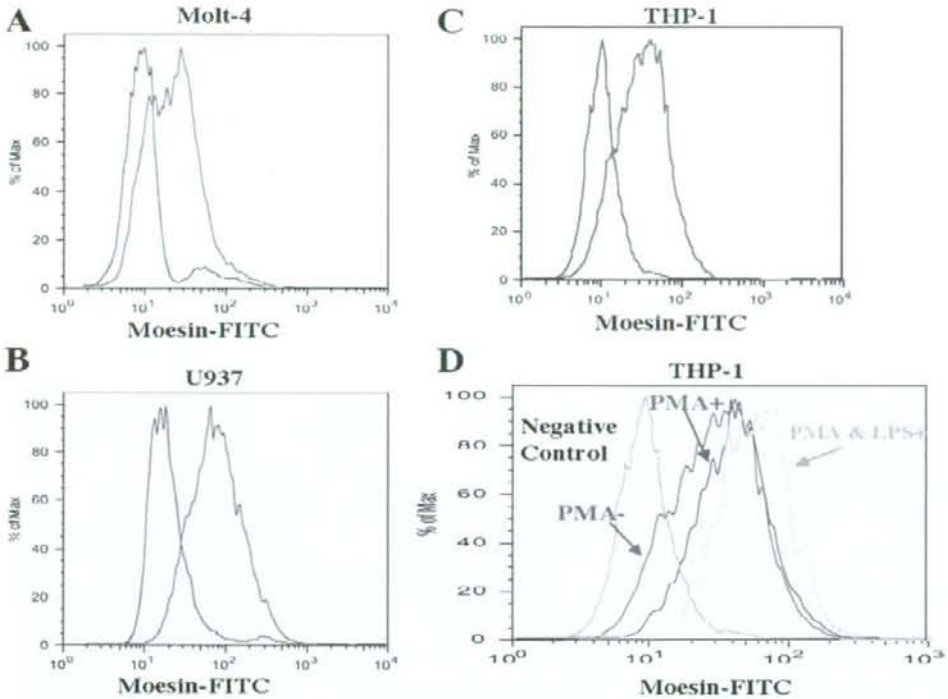

FIGURE 2. Expression of moesin-like molecules on the surface of T cell and monocytic leukemia cell lines. *A–C*, Three leukemia cell lines were examined for the cell surface expression of moesin-like molecules. Left lines, mouse IgG used as negative control; right lines, FITC-labeled anti-moesin mAbs. *D*, THP-1 cells were cultured in the presence or absence of 20 ng/ml PMA for 24 h and then the PMA-stimulated cells were further cultured in the presence of 10 ng/ml LPS for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry. One representative result is shown.

Transfection of moesin short hairpin (shRNA)

Moesin shRNA plasmid (pENTR/moesin-shRNA-264) (22) was kindly provided by Dr. G. M. Kelly of the University of Western Ontario (Ontario, Canada). THP-1 cells were transfected by electroporation using a Gene Pulser II Electroporation System (Bio-Rad). In brief, 3–5 μg of moesin shRNA plasmid or control shRNA (pENTR/U6-GW/lacZ^{shRNA}) was mixed with 800 μl of Opti-Mem I medium (Invitrogen) containing 1×10^6 THP-1 cells and incubated on ice for 10 min. The cells were electroporated in a 4-mm cuvette (Bio-Rad) at the setting of 300 V of voltage pulse and 960 μF of capacitance. Immediately after electroporation, the transfected THP-1 cells were left on ice for 10 min and then 3 ml of RPMI 1640 containing 10% FCS was added to the cell suspension followed by overnight incubation at 37°C. The cells were rinsed and cultured in 3 ml of fresh RPMI 1640 containing 10% FCS for 72 h at 37°C in a CO_2 incubator and were analyzed for the expression of moesin-like molecules by flow cytometry using FITC-labeled anti-moesin mAb (clone 38/87; Neomarkers).

ELISA

The TNF-α and IFN-γ concentration in the culture supernatant, as well as in PB serum and BM plasma was measured using ELISA kits (Mabtech;

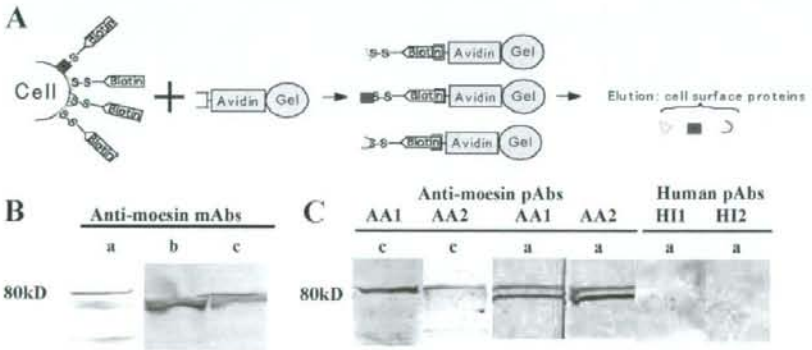

FIGURE 3. Isolation and identification of proteins on THP-1 cells recognized by anti-moesin Abs. *A*, THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns. *B*, Three different protein lysates (*a*, whole cells; *b*, cytoplasmic proteins; and *c*, surface proteins) were subjected to Western blotting with anti-moesin mAbs. *C*, THP-1 cell lysates (*a*) and surface proteins (*c*) isolated from THP-1 cells were subjected to Western blotting using anti-moesin pAbs purified from two AA patients' sera (AA1 and AA2) or non-specific control human IgG pAbs purified from two healthy individuals' sera (HI1 and HI2).

AB, No. 3510-1H-20, and Mabtech; AB, No. 3420-1H-6) according to the manufacturer's instructions. The OD absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments). For determination of cytokine levels in the PB serum and BM plasma, the following additional procedures were performed. Samples were centrifuged at 10,000 rpm for 10 min. ELISA plates were covered with 200 μl/well of TNF-α assay diluent (eBioscience; No. 00-4202-AD) or IFN-γ assay diluent (Mabtech; No. 3652-D) for 1 h at room temperature before adding samples to block nonspecific reactions. TNF-α assay diluent (eBioscience; No. 00-4202-AD) and IFN-γ assay diluent (Mabtech; No. 3652-D) were used to dilute biotinylated mAb TNF-α-II solution and biotinylated mAb 7-B6-1, respectively.

Statistics

The results are given as the mean ± SD. Comparisons were made using the paired *t* test.

Results

Expression of moesin-like molecules on the surface of various blood cells

To confirm the expression of moesin-like molecules on the PB and BM cells, various leukocyte subsets were examined using flow cytometry with anti-moesin mAbs. Fig. 1 shows the representative results of flow cytometry on one healthy individual. Moesin-like molecules were detectable on T cells, NK cells, and monocytes on their surface but not on B cells, neutrophils, and BM $CD34^+$ cells as shown in Fig. 1. All three healthy individuals and the three AA patients showed similar results except that moesin-like molecules were not detectable on monocytes derived from the three AA patients. The mean fluorescence intensity values of the monocytes from healthy individuals and AA patients were 11.5 ± 2.2 and 6.6 ± 2.1, respectively, and the difference was significant (mean fluorescence intensity ± SD, $p < 0.05$, unpaired *t* test). In addition to the leukocyte subsets from the healthy individuals, moesin-like molecules were detectable on a T cell leukemia cell line Molt-4 as well as on monocytic leukemia cell lines U937 and THP-1 (Fig. 2), while they were undetectable on myeloid leukemia cell lines such as K562, UT-7, OUN-1, and TF-1. They were either undetectable on the Burkitt lymphoma cell line, Daudi, or T cell lymphoma cell line, Jurkat (data not shown). The treatment of THP-1 with 20 ng/ml PMA for 24 h and/or 10 ng/ml LPS for 20 h augmented the expression of moesin-like molecules (Fig. 2*D*), thus, indicating an up-regulation of the moesin-like molecules associated with the differentiation of THP-1 cells into macrophages.

Identification of moesin on the surface of THP-1 cells

To identify the proteins on THP-1 cells recognized by anti-moesin Abs, the THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns (Fig. 3*A*). Western blotting of the isolated proteins with anti-moesin mAbs showed two clear bands of which the sizes were 75 and 80 kDa (Fig. 3*B*). Mass fingerprinting of the eluted protein revealed the 80 kDa protein to be moesin. The 75 kD band proved to be nucleolin and eukaryotic translation elongation factor 2. To confirm that anti-moesin pAbs in the serum of AA patients can bind to this cell surface moesin, anti-moesin pAbs were purified from the AA patients' sera (AA1 and AA2) with recombinant moesin proteins using affinity chromatography and then were used for Western blotting. As shown in Fig. 3*C*, the serum-derived anti-moesin pAbs bound to moesin derived from the surface proteins of THP-1.

Effect of moesin-specific shRNA on the expression of moesin on THP-1 cells

To further confirm the expression of moesin on the surface of THP-1 cells, the cells were transfected with moesin shRNA using electroporation. Flow cytometry showed a decrease in the moesin

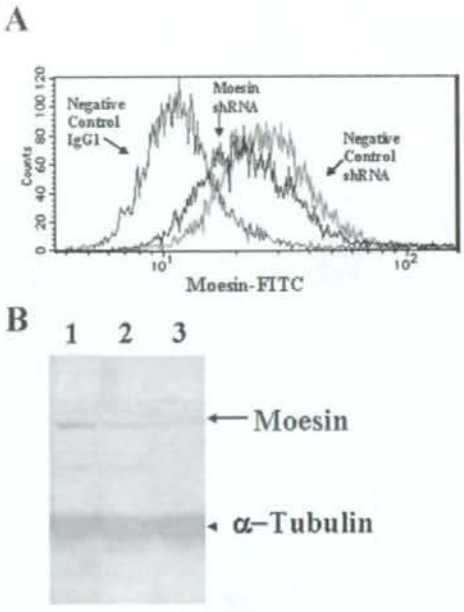

FIGURE 4. Effect of moesin shRNA transfection on the expression of moesin by THP-1 cells. *A*, THP-1 cells transfected with 5 μg of moesin shRNA or control shRNA were examined for the expression of moesin with flow cytometry. The blue line, non-transfected THP-1 cells stained with control mouse IgG1 mAbs; the green line, moesin shRNA transfected cells stained with anti-moesin IgG1 mAbs; the red line, negative control shRNA transfected cells stained with anti-moesin IgG1 mAbs. *B*, Negative control shRNA or moesin-specific shRNA transfected THP-1 cell lysates were examined by Western blotting. 1, 5 μg control shRNA; 2, 3 μg moesin shRNA; 3, 5 μg moesin shRNA.

expression level on the surface of the THP-1 cells transfected with moesin shRNA in comparison to the THP-1 cells transfected with negative control shRNA (Fig. 4*A*). When the THP-1 cells transfected with different dosages of moesin-specific shRNA were examined by Western blotting, the moesin expression by the THP-1 cells was decreased in a dose-dependent manner. The control shRNA specific to LacZ had no effect on moesin expression.

Effect of anti-moesin Abs on THP-1 cells

To determine whether anti-moesin Abs have some effects on THP-1 cells, the THP-1 cells were cultured in the presence of anti-moesin Abs or control IgG for 48 h and the TNF-α concentration of the culture supernatant was measured using ELISA. Both the anti-moesin mAbs and pAbs induced a significantly greater amount of TNF-α from the THP-1 cells than did the control IgG (Fig. 5*A*). The amount of TNF-α induced by anti-moesin pAbs (5 μg/ml) was almost comparable to that induced by LPS (100 ng/ml) (Fig. 5*A*). The anti-moesin pAbs' binding to moesin on the THP-1 cells was ascertained by flow cytometry (Fig. 5*B*).

Effect of anti-moesin pAbs on PBMCs, monocytes, and T cells from healthy individuals and AA patients

The expression of moesin on the T cells and monocytes as well as the TNF-α secretion from the THP-1 cells induced by anti-moesin pAbs suggested that anti-moesin pAbs in the AA patients' sera might also stimulate these immune cells from healthy individuals and AA patients to secrete cytokines. When the PBMCs from healthy individuals were incubated for 48 h in the presence of 5

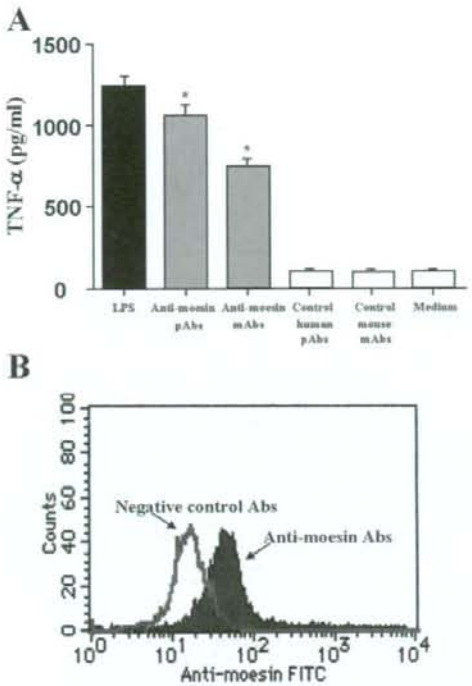

FIGURE 5. TNF-α release from THP-1 cells stimulated by anti-moesin Abs. *A*, THP-1 cells were cultured for 48 h with 5 μg/ml of anti-moesin Abs or control Abs. Anti-moesin pAbs, anti-moesin polyclonal IgG isolated from the serum of AA patients; control human pAbs, control human IgG pAbs isolated from healthy individuals; anti-moesin mAbs, anti-moesin mouse IgG1 mAbs (clone 38/87); control mouse mAbs, control mouse IgG1 mAbs. Then, 100 ng/ml LPS was used as a positive control. The data represent the mean TNF-α concentration ± SD of three experiments. *, $p < 0.01$ vs control Abs. *B*, The detection of moesin on THP-1 cells by anti-moesin pAbs purified from the serum of an AA patient.

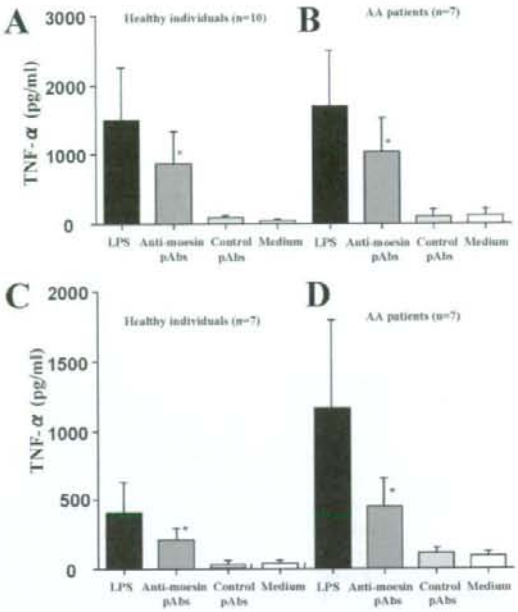

FIGURE 6. TNF-α release from PBMCs or monocytes stimulated by anti-moesin pAbs. The PBMCs or isolated monocytes were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 100 ng/ml of LPS was used as a positive control. PBMCs isolated from 10 healthy individuals (*A*) and 7 AA patients (*B*), and monocytes separated from the PBMCs of 7 healthy individuals (*C*) and 7 AA patients (*D*), were used as targets. The data represent the mean TNF-α concentration ± SD. *, $p < 0.005$ vs control Abs.

μg/ml of anti-moesin pAbs, the amount of TNF-α in the culture medium was approximately 10 times more than those of control cultures and was more than half of that of the culture stimulated by 100 ng/ml of LPS (Fig. 6*A*). The same concentration of anti-moesin pAbs induced a similar amount of TNF-α from the PBMCs from AA patients (Fig. 6*B*). On the other hand, when monocytes isolated from the PBMC of healthy individuals or AA patients were used as a target, anti-moesin pAbs induced less than half the amount of TNF-α of that induced from whole PBMCs (Fig. 6, *C* and *D*).

The unexpectedly high inducibility of TNF-α secretion from the PBMCs by the anti-moesin pAbs prompted studies on the inducibility of IFN-γ secretion from the PBMCs by the Abs. Fig. 7, *A* and *B*, shows the effect of anti-moesin Abs on the IFN-γ secretion from PBMCs. Although anti-moesin pAbs alone could not induce IFN-γ secretion from the PBMCs derived from healthy individuals, the Abs stimulated PBMCs that were prestimulated with anti-CD3 mAbs to secrete nearly as much IFN-γ as that PHA did. In contrast, the PBMCs from the AA patients could secrete IFN-γ in response to anti-moesin pAbs without the prestimulation by anti-CD3 mAbs, and the amount of IFN-γ was approximately 40% as much as that of the culture stimulated by 10 μg/ml of PHA. In contrast, T cells isolated from the PBMC of healthy individuals or AA patients could not secrete a significantly larger amount of IFN-γ in response to anti-moesin pAbs compared with that in response to control IgG pAbs (Fig. 7, *C* and *D*), and the amount of IFN-γ secreted by T cells was one-tenth as much as that by PBMCs.

When the sera of the 16 AA patients comprising 7 anti-moesin Ab-positive and 9 anti-moesin Ab-negative patients were examined using ELISA, no significant differences in TNF-α and IFN-γ concentrations were observed between the 2 groups (TNF-α: 88.0 ± 106.3 pg/ml in anti-moesin Abs-positive patients, 90.1 ± 161.3 in anti-moesin Abs-negative patients; IFN-γ: 44.6 ± 33.8 pg/ml in anti-moesin Abs-positive patients, 47.5 ± 44.9 pg/ml in anti-moesin Abs-negative patients). None of the sera derived from four healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml). On the other hand, when the BM plasma from five patients with AA was examined using ELISA, three anti-moesin Abs-positive patients showed higher levels of TNF-α (129, 338, and 349 pg/ml) compared with those of TNF-α (13 and 128 pg/ml) in two anti-moesin Abs-negative patients. IFN-γ concentrations of three anti-moesin Abs-positive patients were 29, 123, and 133 pg/ml, while those of two anti-moesin Abs-negative patients were 13 and 80 pg/ml. None of the BM plasma derived from three healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml).

Discussion

The present study revealed that the proteins recognized by the anti-moesin Abs are detectable on the surface of various leukocytes subsets including T cells, NK cells, and monocytes as well as on T lymphocytic and monocytic leukemia cell lines. Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites

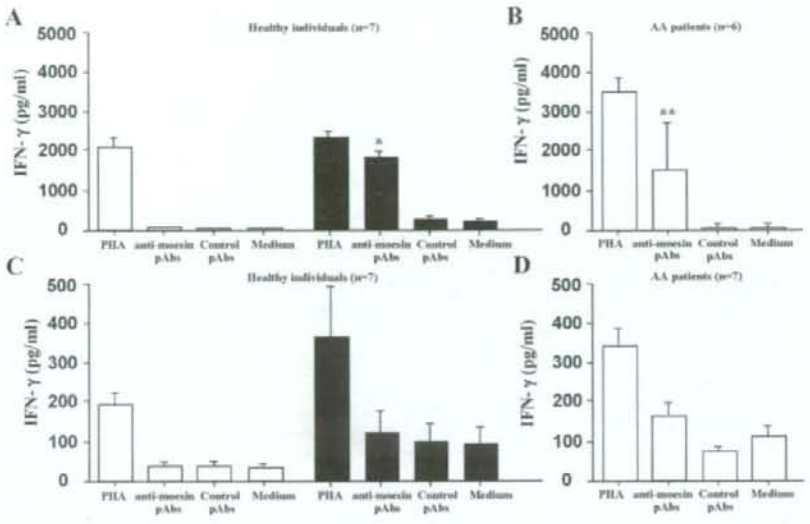

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as a positive control. Unprimed PBMCs (□) or CD3-primed PBMCs (■) were used for the culture. PBMCs were isolated from seven healthy individuals (*A*) and six AA patients (*B*). Uncostimulated T cells (□) or CD3-costimulated T cells (■) were used for the culture. T cells were isolated from the PBMCs of seven healthy individuals (*C*) and seven AA patients (*D*). The data represent the mean IFN-γ concentration ± SD. *, $p < 0.0001$ vs control Abs; **, $p = 0.04$ vs control Abs.

as well as ruffling of the cell membrane (17). This membrane-linking protein is expressed by various blood cells including megakaryocytes and granulocytes (23), but its expression was thought to be localized inside the cell membrane and not on the cell surface. Some studies revealed that anti-moesin Abs could bind to the surface of T cells (18) and macrophages (19) in keeping with our observation. However, none of the previous studies characterized the cell surface protein recognized by the anti-moesin Abs. Using biotin-labeled membrane proteins coupled with an avidin gel column and peptide massfinger printing, the present study identified the cell surface 80 kDa protein to be moesin. The decrease in the cell surface moesin induced by moesin shRNA has substantiated the presence of moesin on the cell surface of THP-1 cells.

Little is known about the function of anti-moesin Abs in vitro and in vivo. In contrast to our results, Amar et al. (24, 25) found that anti-moesin mAbs (clone 38) suppressed LPS-induced TNF-α secretion from monocytes through binding of moesin-like molecules on the cell surface. They used a different anti-moesin mAbs (clone 38) from the mAbs (clone 38/87) used in the present study. When we examined the effect of clone 38 mAbs on TNF-α secretion from THP-1 cells induced by LPS using the same condition as the one described by Amar et al. (24), a dose-dependent inhibition of TNF-α secretion was observed (data not shown). In contract to clone 38/87 mAb and pAbs from AA patients' sera, the clone 38 mAbs alone did not induce TNF-α secretion from THP-1 cells. Because the clone 38 preparation contains 1.5 mM sodium azide as a preservative, it is most likely that the dose-dependent inhibition of TNF-α secretion by clone 38 mAbs was due to toxic effect of sodium azide. Alternatively, clone 38 mAb which recognizes the C-terminal portion (554–564 amino acid residues) of moesin may exert a different effect on THP-1 cells from the effect of mAb clone 38/87 which recognizes the middle portion (317–398 amino acid residues) of moesin and from the effect of pAbs purified from AA patients' sera.

The present study revealed that both mAbs and pAbs specific to moesin stimulated the THP-1 cells to secrete TNF-α at an Ab concentration compatible to that in the serum of the AA patients. Moreover, anti-moesin pAbs were as potent as LPS in inducing TNF-α secretion from the PBMCs derived from healthy individuals and the AA patients. Our preliminary analyses on the activation of signaling pathways leading to TNF-α secretion from THP-1 cells showed the phosphorylation of ERK1/2 kinase induced by anti-moesin Abs (49th American Society of Hematology annual meeting abstract #1690, 2007 and submitted). In two patients from whom anti-moesin pAbs were purified, the Abs induced TNF-α release from autologous PBMCs. High concentrations of TNF-α were indeed present in the BM sera of two patients with high anti-moesin Ab titer. Although no difference in the serum TNF-α level was observed between anti-moesin Ab-positive and -negative patients, these findings suggest that anti-moesin Abs may induce a subtle amount of TNF-α from the monocytes or macrophages in the BM, thereby contributing to the pathogenesis of AA.

In contrast to TNF-α, IFN-γ was not induced by the anti-moesin pAbs alone from the PBMCs from healthy individuals, though anti-moesin pAbs augmented IFN-γ secretion from the PBMCs prestimulated with anti-CD3 mAbs. On the other hand, anti-moesin pAbs stimulated the PBMCs from the AA patients to secrete as much IFN-γ as did PHA. It has been shown that T cells from AA patients are in an activated state and are prone to produce IFN-γ in response to suboptimal stimuli (26). The amount of secreted TNF-α from isolated monocytes as well as the amount of secreted IFN-γ from isolated T cells was greatly reduced compared with those from unfractionated PBMCs. The inability to secrete a sufficient amount TNF-α and IFN-γ of isolated monocytes and T cells suggests that the interaction between monocytes and T cells may be required to efficiently respond to extrinsic stimuli as described by previous reports (27, 28). When the anti-moesin Abs titers in the serum were longitudinally measured in three patients, the Abs titer decreased in two patients in association with the response to immunosuppressive therapy, while the Abs titer increased in one patient who became dependent on transfusions due to the relapse of AA in comparison to the titer detected in remission (data not shown). The high titer TNF-α levels in BM plasma of patients showing high anti-moesin Abs titers and the decrease in the Ab titers in parallel with disease amelioration support the hypothesis that anti-moesin Abs are involved in the pathogenesis of AA by way of myelosuppressive cytokine induction from immunocompetent cells. One may wonder why high titer anti-moesin Abs in some AA patients do not induce hypercytokinemia. However, inability of T cell-stimulating Abs such as anti-CD3 Abs to induce IFN-γ secretion in vivo has been shown by previous reports (29, 30). There may be some regulatory mechanisms that mitigate T cell activation by stimulating Abs in vivo.

A previous study demonstrated the presence of anti-moesin Abs in 14–34% of patients with rheumatoid arthritis (11, 31), and a

case-control study on AA conducted by the International Agranulocytosis and Aplastic Anemia Study Group revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA (32). The anti-moesin pAbs derived from patients with rheumatoid arthritis also enhanced TNF-α secretion from THP-1 cells (data not shown). It is, therefore, possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin. Anti-TNF-α therapy has been successfully used for patients with rheumatoid arthritis (33–35) as well as for some patients with myelodysplastic syndrome (36, 37). Recent reports have shown the efficacy of anti-CD20 Abs in restoring hematopoietic functions of AA (38, 39). Therefore, autoAbs capable of inducing cytokine secretion like anti-moesin Abs may be a new target of therapy for AA.

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Disclosures

The authors have no financial conflict of interest.

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Long-term responses and outcomes following immunosuppressive therapy in large granular lymphocyte leukemia-associated pure red cell aplasia: a Nationwide Cohort Study in Japan for the PRCA Collaborative Study Group

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ABSTRACT

Large granular lymphocyte leukemia-associated pure red cell aplasia accounts for a significant portion of secondary pure red cell aplasia cases. However, because of its rarity, long-term responses and relapse rates after immunosuppressive therapy are largely unknown. We conducted a nationwide survey in Japan and collected 185 evaluable patients. Fourteen patients with large granular lymphocyte leukemia-associated pure red cell aplasia were evaluated. Cyclophosphamide, cyclosporine A and prednisolone produced remissions in 6/8, 1/4 and 0/2 patients respectively. Seven and 5 patients were maintained on cyclophosphamide or cyclosporine A respectively. Two patients relapsed after stopping cyclophosphamide, and 2 patients relapsed during maintenance therapy with cyclosporine A. The median relapse-free survival in the cyclophosphamide - and the cyclosporine A groups was 53 and 123 months respectively. Large granular lymphocyte leukemia-associated pure red cell aplasia showed a good response to either cyclophosphamide or cyclosporine A. Most patients continued to receive maintenance therapy and it remains uncertain whether cyclophosphamide or cyclosporine A can induce a maintenance-free hematologic response in large granular lymphocyte leukemia-associated pure red cell aplasia.

Key words: pure red cell aplasia, large granular lymphocyte leukemia, cyclophosphamide, cyclosporine.

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Introduction

Large granular lymphocyte (LGL) leukemia is the most common underlying disease of secondary pure red cell aplasia (PRCA) in a single institutional study from the United States, and the second most common cause in Japan.[1-5] LGL leukemia is also referred to as granular lymphocyte-proliferative disorders (GLPD) or lymphoproliferative disease of granular lymphocytes.[1,2,4-6] LGL leukemia is a heterogeneous disorder characterized by a persistent increase in the number of peripheral blood LGLs, and the majority of patients have a clonal rearrangement of T-cell receptors.[4,6,7] Clonal disorders of LGLs may arise from natural killer cells, and may be indolent or behave as an aggressive disease. Neutropenia is the

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most frequent cytopenia in T-cell LGL leukemia, and anemia is also caused by various mechanisms in 48% of the patients.[8,9]

LGL leukemia-associated PRCA has been primarily treated with chemotherapy, such as oral cyclophosphamide (CY) with or without prednisolone (PSL), cyclosporine A (CsA), or methotrexate.[8,9,10,11] The combination of CY and PSL is associated with a longer duration of response than PSL alone.[1,10,12] The overall response to initial CY therapy has been reported to be 66-100%[8,11] and the median duration of response is 32 months.[11] However, optimal management of LGL leukemia-associated PRCA and long-term outcome after immunosuppressive therapy are largely unknown because of the rarity of this disorder.

The efficacy and long-term outcome after immunosuppressive therapy for secondary PRCA might differ according to the underlying diseases. We, therefore, conducted a nationwide survey to investigate the current status of immunosuppressive therapy for acquired chronic PRCA based on a relatively large patient cohort in Japan. This report is a summary focusing on immunosuppressive therapy for LGL leukemia-associated PRCA.

Design and Methods

Data collection of the data and patients' characteristics

As described elsewhere,[8,13] the questionnaires were sent to 109 hematology departments in Japan, and a total of 185 evaluable patients were collected from 45 institutions. Seventy-three patients were classified as having idiopathic PRCA and 112 patients had secondary PRCA.[8] Diagnosis of LGL leukemia was based on the presence of a persistent (>6 months) increase in the number of peripheral blood LGL (>500/mL), since the normal range for peripheral blood LGL counts is 223±99/μL[14] and clonal disease has been documented in 8% of patients when absolute LGL counts are in a range from 600 to 1,000/mL.[8] A 6-month follow-up criterion was not applied when clonality was established.[4] Fourteen patients (7.6%) were found to have both LGL leukemia and PRCA (Table 1). Rearrangement of T-cell receptor (TCR) was examined in 11/14 patients. In case 12, the diagnosis of LGL-leukemia was established on morphological criteria. Case 13 has been previously reported.[15] A unique patient number was given at each participating institution to protect individual information. This study was approved by the institutional review board, and performed according to the declaration of Helsinki and the ethical guidelines for epidemiological research of the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labour and Welfare of Japan.

Definition of response and data analysis

Definition of response and data analysis has been described elsewhere.[8,13] Relapse was defined as the reappearance of transfusion requirement. In some analyses, the patients were classified according to the agent used for maintenance therapy such as the CY group or the CsA group regardless of the agents used for successful remission induction. The agents for remission induction and salvage therapy were defined as those used initially and those used either sequentially or in a later combination respectively. Survival was estimated by the Kaplan-Meier method and statistical difference was calculated by the log-rank test. Endpoints of this study were the response rate, the relapse-free survival (RFS) and overall survival (OS).

Results and Discussion

Response to the first remission induction therapy

The initial treatment for these patients included CY (n=8), CsA (n=4), and PSL (n=2). For one patient (case 2) who had been given PSL for rheumatoid arthritis before the onset of PRCA, CsA was determined as the initial agent for PRCA. CY achieved CR and PR in 2 and

Table 1. Characteristics of large granular lymphocyte leukemia-associated pure red cell aplasia.

Case	Observation period	Age/ Sex	WBC (/μL)	Lym (%)	LGL (/μL)	Hb (g/dL)	CD3 (%)	CD4/8	CD56 (%)	TCR rearrangement
1	2001-2007	85/F	7,460	68	4,849	7.2	98.4	0.12	0.6	+
2	2005-2006[a]	84/F	5,670	18	964	6.1	97.7	0.20	1.2	+
3	1999-2007	56/M	3,470	55	1,562	6.6	96	0.36	5.9	+
4	2004-2007	69/M	5,500	81	4,345	5.5	88	1.00	11.5	+
5	1996-2007	58/M	7,300	78	3,176	7.2	99	0.20	2.9	+
6	2004-2007	62/M	12,880	79	NA	7.6	NA	0.12	NA	+
7	1999-2007	71/F	2,990	52	NA	5.1	NA	0.20	NA	NA
8	1994-2007	45/M	6,500	48	1,092	5.7	97	0.12	3	+
9	1996-2007	44/F	3,400	54	993	3.4	97	0.33	4	+
10	2005-2007	65/F	3,200	73	NA	6.3	96.9	0.55	0.7	+
11	2002-2007	64/F	11,000	50	NA	9.8	NA	NA	NA	+
12	1993-2007	52/M	3,200	27	NA	5.5	92.3	0.66	18	-
13	1992-1999[b]	55/M	7,400	80	2,960	3.4	97	0.17	NA	+
14	1996-2006[a]	76/M	6,500	55	2,730	4.5	94	NA	0.6	+

NA: not available; F: female; M: male; BW: body weight; WBC: white blood cells; Lym: lymphocytes; LGL: large granular lymphocytes; Hb: hemoglobin; TCR: T-cell receptor; [a]: follow-up-end; [b]: dead.

4 patients respectively. Response rate was 75%. The median initial dose of CY for the responding patients (n=6) was 100 mg with a range of 50-100 mg. Two non-responding patients were also given 100 mg of CY. When the patients who responded to initial CY therapy were evaluated, the time for transfusion-independence from the start of therapy was 29±45 days (range 0-92 days). Four patients achieved transfusion-independence within two weeks and 5 patients within three months. One patient achieved remission later than six months from the start of CY therapy. The median duration of CY therapy (including remission induction and maintenance therapy) was 24 months with a range of 10–124 months.

CsA achieved a response in 1/4 (25%) patients. The initial dose of CsA for the responding patient (case 8) was 200 mg (3.7 mg/kg), and he achieved transfusion-independence within two weeks. Although one patient was given 450 mg (8.8 mg/kg) of CsA for 49 days, he did not respond (case 6). PSL did not produce any clinical response (n=2). In these patients, the initial doses of PSL were 0.9 mg/kg (case 9) and 0.4 mg/kg (case 13). Two out of 14 patients (14%) responded neither to CY nor CsA. There was no significant difference in the response to the first remission induction therapy between CY and CsA by the χ^2 test.

Salvage therapy for non-responders to the first remission induction therapy

Seven patients failed to respond to remission induction therapy. In 2 patients who failed to respond to the initial CY therapy for 77 (case 10) and 182 days (case 14), one patient (case 10) responded to CsA. Another patient (case 14) did not respond to the sequential salvage therapies including CsA, azathioprine and methotrexate. All 3 patients who failed to respond to the initial CsA therapy for 36 (case 2), 49 (case 6) and 176 days (case 7) responded to CY. In 2 patients who failed to respond to the initial PSL therapy, one patient (case 9) responded to CsA. Although the other patient (case 13) partially responded to anti-thymocyte globulin (ATG) after the sequential administration of CsA and CY, he died of pneumonia.

Duration of response to immunosuppressive therapy

We classified the patients with LGL leukemia-associated PRCA according to the agent used for maintenance therapy as the CY group (n=7, cases 1-7) and the Csa group (n=5, cases 8-12) (Figure 1). Four out of 12 (33%) patients relapsed and they were 2 patients (cases 1 and 3) of the CY group and 2 patients (cases 9 and 12) of the CsA group. Estimated median duration of the RFS in the CY group (53 months) was shorter than that

Case	BW (kg)	a) Agents in order or combination	b) Initial Dose (mg)				c) TI-dep. Period (days)	d) RFS1	e) Maintenance (mg)	f) Relapse	g) RFS2
Cyclophosphamide-group (n=7)											
1	50	CY	50				0	9.7	Off	Yes	21.0
2	47	CsA/CY	140	50			113	4.0	Off	No	11.1+
3	64	CY	100				60	14.5	Off	Yes	38.5
4	NE	CY	50				0	16.1	Off	No	6.1+
5	NE	CY-MTX	100	2.5			0	31.4	Off	No	0.0+
6	51	CsA/CY	450	50			196	22.7+	CY 25	No	
7	38	CsA/CY	200	50			20	33.0+	CY 50	No	
Cyclosporine-group (n=5)											
8	54	CsA	200				0	119.8+	CsA 100	No	
9	66	PSL/CsA	60	200			115	77.1	CsA 100	Yes	
10	NE	CY/CsA	100	200			92	14.7+	CsA 50	No	
11	66	CY/CsA	100	150			0	60.3+	CsA 150	No	
12	NE	CY-AZP/CsA	100	50	200		941	122.8	CsA 150	Yes	
Non-responders											
13	48	PSL-CsA/CY/ATG	20	350	100	ND	175	4.0	PSL 10	Yes	
14	41	CY/CsA/AZP/MTX	100	300	50	ND	3318+	NE	Off	NE	NE

CR PR NR

Figure 1. Response to immunosuppressive therapy and relapse of anemia in large granular lymphocyte-associated pure red cell aplasia. NE: not evaluable, a) Agents are listed in order, (/); in sequential administration, (-); in combination later on, b) The initial dose and response to the agents; the order of agents corresponds to that shown in column, c) Transfusion-dependent period (days) after the initiation of remission induction therapy, d) RFS1; relapse-free survival (months) estimated as transfusion-free survival is shown as the period before the discontinuation of maintenance therapy, e) Off; tapered off, f) Relapse was defined as reappearance of transfusion requirement, g) RFS2; RFS after the discontinuation of maintenance therapy.

Case	BW (kg)	a) Agents in order or combination	b) Initial Dose (mg)				c) TI-dep. Period (days)	d) RFS1	e) Maintenance (mg)	f) Relapse	g) RFS2
1	50	CY	25				0	21.0	Off	Yes	18.0
3	64	CY/CsA	50	200			253	8.8	Off	Yes	15.0
9	66	CsA-CY	200	50			20	47.8+	CsA 200	No	
12	NE	CY/MTX/CsA	50	2.5	150		47	4.7+	CsA 125	No	

CR PR NR

Figure 2. Immunosuppressive therapy for relapsing patients. See Figure 1 for abbreviations.

of the CsA-group (123 months) with statistical significance (p=0.0423). Maintenance therapy was discontinued in 5 patients in the CY-group, and 2 patients relapsed at 21 and 39 months after the discontinuation of CY. Three patients have still maintained remission after the discontinuation of CY, but the RFS after discontinuation of CY was only 0, 6 and 11 months (RFS2 in Figure 1). Although all 5 patients in the CsA group were still on maintenance therapy, 2 patients relapsed.

Immunosuppressive therapy for relapsing patients

Two patients in the CY group, who relapsed after the discontinuation of maintenance CY therapy, again responded to CY and were maintained in remission with CY for 21 (case 1) and nine months (case 3) (Figure 2). However, they relapsed again at 18 (case 1) and 15 months (case 3) after the discontinuation of CY, but later responded to CsA (*data not shown*). Two patients in the CsA-group relapsed during maintenance CsA therapy. Trough levels of CsA in case 9 and 12 when their anemia relapsed were 93.0 ng/mL and unknown respectively. One patient (case 9) partially responded to CsA and completely responded to the latter in combination with CY. The other patient (case 12) did not respond to the sequential administration of CY and methotrexate, but again responded to CsA (Figure 2).

Mortality and overall survival

One patient (case 13) died of infection following ATG therapy at 85 months after the onset of PRCA. The estimated median overall survival time has not yet been reached with the median observation period of 87 months (from 19 to 170 months) and the estimated 10-year overall survival (OS) after the onset of PRCA was 86%.

Despite a relatively small number of patients, we have demonstrated that the overall response rate to initial CY therapy is 75% in LGL leukemia-associated PRCA. CY seemed to have a better activity in remission induction of LGL leukemia-associated PRCA than CsA, but this was not statistically significant. In contrast, CsA has been shown to be the most effective agent for idiopathic and thymoma-associated PRCA[3,13] so the efficacy of these agents may differ depending upon the sub-types of PRCA.

Although remission induction can be achieved in the majority of patients with LGL leukemia-associated PRCA, a further problem is concern over how many patients treated with CY or CsA achieve a sustained remission and how many relapse, and whether or not there is need for maintenance treatment. We have reported that the discontinuation of maintenance CsA therapy is strongly correlated with relapse in idiopathic PRCA patients,[3] and that thymoma-associated PRCA may also be CsA-dependent.[13] In the present study, there were 3 patients who maintained remission after the discontinuation of CY; however, each relapse-free period after the discontinuation was still only 0, 6 and 11 months. Considering that a relapse can occur even 39 months after the discontinuation of CY, these observation periods may be insufficient to conclude that LGL-leukemia can be cured by CY.

There is general agreement that alkylating agents are the most powerful medications for treating autoimmune disease.[16] Although CY seems to be a key drug for remission induction of LGL leukemia-associated PRCA, the duration of the maintenance therapy is one of the major concerns considering the late toxicity of CY.[17] The risk of toxicity from alkylating agents is related to the cumulative dose of the medication and the duration of therapy.[17-19] Bladder cancer and myelodysplastic syndrome are the most common malignancies associated with daily CY therapy.[17-21] Therefore, strategies that reduce the duration of exposure can minimize the long-term risks. In a series of 7 patients with T-cell-LGL leukemia-associated PRCA, all patients were successfully treated with oral CY monotherapy.[5] Therapeutic responses began after eight weeks, and clinical CRs were obtained after six months. Clinical remission was associated with the disappearance of TCR rearrangement, which suggests that the disappearance of TCR rearrangement may be an indicator for the discontinuation of CY.

No secondary malignancy has been reported up to now in the present patient cohort. The median duration of maintenance CY therapy was 16 months with a range of 4–33 months, suggesting some difficulty in stopping CY while trying to maintain remissions. Interestingly, 2 patients who responded to CY could be maintained with CsA for 60 and 123 months (cases 11 and 12 respectively). We speculate that CY is of limited value as a maintenance agent due to its late toxicity, which may be the reason why the actual median RFS in the CY group (53 months) was shorter than that of the CsA group (123 months).

It remains uncertain whether immunosuppressive agents can induce maintenance-free hematologic response, as is the case with idiopathic[3] or thymoma-associated PRCA.[13] Considering the recurrent nature of LGL leukemia-associated PRCA, CsA may be an effective alternative to prevent relapse of anemia following successful remission induction with CY. The median OS of all patients has not yet been reached with a median observation period of 90 months and an estimated 10-year OS of 86%, which suggests that LGL leukemia-associated PRCA has a relatively good prognosis.

In conclusion, we have demonstrated for the first time that most patients with LGL-associated PRCA are still receiving maintenance therapy and may be CY/CsA-dependent. Effective and less toxic maintenance therapy to prevent relapse of anemia needs to be established.

Appendix

The following institutions participated in the Collaborative Study Group: Aichi Medical School, Akita University, Asahikawa Medical School, Chiba University, Dokkyo Medical School, Ehime University, Fujita Health University, Fukui University, Fukui National Hospital, Fukuoka University, Fukushima Medical University, Gifu University, Gunma University, Hamamatsu Medical School, Hirosaki

University, Hiroshima University, Hokkaido University, Hyogo Medical University, Iwate Medical School, Jichi Medical School, Jikei University, Juntendo University, Kagawa Children's Hospital, Kagawa University, Kagoshima University, Kanazawa University, Kanazawa Medical School, Kansai Medical University, Kawasaki Medical School, Keio University, Kinki University, Kitazato University, Kobe University, Kochi University, Kumamoto University, Kurume University, Kyoto Prefectural University, Kyoto University, Kumamoto Medical Center, Kyushu University, Mie University, Nagasaki University, Nagoya City University, Nagoya Medical Center, Nagoya University, Nara Medical University, National Cancer Center, National Institute of Infectious Diseases, Niigata University, Nishi Sapporo National Hospital, Nippon Medical School, Nippon University, NTT Kanto Medical Center, Oita University, Okayama Medical Center, Okayama University, Osaka City University, Osaka Medical School, Osaka National Hospital, Osaka University, Ryukyu University, Saga University, Saitama Medical School, Sapporo Medical School, Sendai Medical Center, Shimane University, Shinsyu University, Showa University, St. Marianna University, Teikyo University, Toho University, Tohoku University, Tokai University, Tokushima University, Tottori University, Tokyo Medical Center, Tokyo Medical School, Tokyo Medical and Dental University, Tokyo University, Tokyo Women's Medical School, Tsukuba University, University of Occupational and Environmental Health, Wakayama Medical University, Waseda University, Yamagata University, Yamaguchi University, Yamanashi University, Yokohama City University.

Authorship and Disclosures

NF: designed the research, analyzed data and wrote the paper; KS and MH: designed the research, analyzed data and contributed to writing the paper; KO, KS, AM, MT, MK, AA, YY, SN, AU, MO, and KO: designed the research and helped organize this collaborative study.

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Acquired pure red cell aplasia: updated review of treatment

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Summary

Pure red cell aplasia (PRCA) is a syndrome characterized by a severe normocytic anaemia, reticulocytopenia, and absence of erythroblasts from an otherwise normal bone marrow. Primary PRCA, or secondary PRCA which has not responded to treatment of the underlying disease, is treated as an immunologically-mediated disease. Although vigorous immunosuppressive treatments induce and maintain remissions in a majority of patients, they carry an increased risk of serious complications. Corticosteroids were used in the treatment of PRCA and this has been considered the treatment of first choice although relapse is not uncommon. Cyclosporine A (CsA) has become established as one of the leading drugs for treatment of PRCA. However, common concerns have been the number of patients treated with CsA who achieve sustained remissions and the number that relapse. This article reviews the current status of CsA therapy and compares it to other treatments for diverse PRCAs.

Keywords: pure red cell aplasia, corticosteroids, cyclosporine A, cyclophosphamide, alemtuzumab, rituximab.

Pure red cell aplasia (PRCA), a disorder first characterized in 1922 (Kaznelson, 1922), is a syndrome characterized by severe normochromic, normocytic anaemia associated with reticulocytopenia and absence of erythroblasts from an otherwise normal bone marrow. PRCA may appear as a congenital disorder or occur as an acquired syndrome. The acquired form of PRCA presents either as an acute self-limited disease, predominantly seen in children, or as a chronic illness that is more frequently seen in adults. It may present as a primary haematological disorder in the absence of any other disease, or secondary to parvovirus infection, collagen vascular disease, leukaemia, lymphoma, thymoma, solid tumors, treatment with recombinant human erythropoietin (EPO) or other drugs, ABO-incompatible haematopoietic stem cell transplantation and pregnancy. Depending on the cause, the course can be acute and self-limiting or chronic with rare spontaneous remissions (Dessypris, 1988; Dessypris & Lipton, 2004).

Primary, or secondary PRCA not responding to treatment of the underlying diseases, is treated as an immunologically-mediated disease, based on a number of studies implicating a pathological role of serum auto-antibodies, natural killer (NK) cell-mediated or T lymphocyte-mediated effects impairing various stages and mechanisms of erythropoiesis as extensively reviewed by Fisch *et al* (2000). The major objective in the treatment of PRCA is to induce a remission with the recovery of erythropoiesis, thus providing relief from transfusions and avoiding transfusion-associated problems. The therapeutic plan usually focuses on the sequential use of various immunosuppressive therapies until a remission is obtained. Remissions have been achieved by treatment with corticosteroids (CS), cyclophosphamide (CY), cyclosporine A (CsA), antithymocyte globulin (ATG), splenectomy, and plasmapheresis (Dessypris & Lipton, 2004). More recently, the efficacies of the anti-CD20 monoclonal antibody, rituximab (Zecca *et al*, 2001), and anti-CD52 monoclonal antibody, alemtuzumab (Willis *et al*, 2001), to induce remissions of therapy-resistant PRCA have also been reported.

In general, remission can be easily achieved in the majority of patients. To date, the efficacy of CS, CY and CsA for patients with primary or secondary PRCA has been reported to be between 30–62%, 7–20% and 65–87%, respectively (Clark *et al*, 1984; Dessypris, 1988; Raghavachar, 1990; Marmont, 1991; Lacy *et al*, 1996; Mamiya *et al*, 1997). The efficacy of a combination of CY and CS for refractory patients has been reported to be between 40–60% (Clark *et al*, 1984; Dessypris, 1988, Mamiya *et al*, 1997). Since the initial cases were successfully treated by Totterman *et al* (1984), CsA has established itself as one of the leading drugs for the treatment

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of PRCA. However, concern has centred around the precise number of patients treated with CsA who achieve a sustained remission and the number who relapse. In 1988, Dessypris pointed out that treatment of PRCA with CsA appeared to be very promising, but that such treatment should be considered still experimental, and that further studies were necessary to determine the effectiveness of this drug, the optimal and least toxic dosage, the minimum duration of therapy for induction of remission, and whether or not there was a need for maintenance treatment (Dessypris, 1988). An advantage of CsA therapy for PRCA has long remained unclear, as comparing one therapeutic approach to another has been almost impossible because the disease is so rare that controlled studies could not be performed. However, the number of patients treated with CsA has accumulated over two decades, which made it possible to conduct an analytical study. The present paper reviews the current status of CsA therapy, comparing it to other treatments for the diverse types of acquired PRCA except for transient erythroblastopenia of childhood.

Diagnosis and initial evaluation

Pure red cell aplasia in adults can be easily diagnosed when isolated anaemia, in the presence of normal white cell and platelet counts, is associated with a marrow of normal cellularity in which there is an almost complete absence of erythroblasts but normal myeloid cells and megakaryocytes (Dessypris & Lipton, 2004). The classification of the clinical course (acute or chronic) and pathogenesis, such as secondary or idiopathic (no definite underlying disease) is essential to select the optimal therapeutic modality. Evaluations for the possible causes of PRCA should include a previous history of drug use and toxins or infections, liver and kidney functions, immunological examination including auto-antibodies, a bone marrow examination including morphology, chromosome and rearrangement of T cell receptor (TCR) analysis, peripheral-blood flow cytometry, virological examination including parvovirus B19 DNA, and computed tomography and/or magnetic resonance imaging examinations to rule out the presence of thymoma and neoplasms.

Today, a careful assessment of the increase of large granular lymphocytes (LGLs) is especially critical and an analysis of immunophenotype and TCR rearrangement of lymphocytes may be essential for ruling out LGL leukaemia, also referred to as granular lymphocyte proliferative disorders (GLPD) (Oshimi *et al*, 1993) or lymphoproliferative disease of granular lymphocytes (LDGL) (Go *et al*, 2001). LGL leukaemia was the most common underlying disease of secondary PRCA in a single institutional study from the United States, and the second most common cause in Japan (Lacy *et al*, 1996; Mamiya *et al*, 1997; Sawada *et al*, 2007). Since the diagnosis of LGL leukemia is somewhat difficult in patients without lymphocytosis, this group of patients can be misdiagnosed as idiopathic PRCA although LGL leukemia-associated PRCA may require a different treatment for the primary disease. It is a heterogeneous disorder characterized by a persistent increase in the number of peripheral blood LGLs, and the majority of patients have a clonal rearrangement of T-cell receptors (Oshimi *et al*, 1993; Semenzato *et al*, 1997; Chan *et al*, 2001). Clonal disorders of LGLs arise from either mature T lymphocytes or NK cells, and may be indolent or behave as an aggressive disease. T-cell LGL leukaemia is the most common form of clonal LGL disorders and most cases behave in an indolent fashion. Neutropenia is the most frequent cytopenia in T-cell LGL leukaemia, and anaemia occurs in 48% of the patients (Loughran, 1993; Lamy & Loughran, 1998, 2003).

The evidence of a granular lymphocytosis greater than 2×10^9/l lasting for more than 6 months has been regarded as the criteria for defining the disease (Loughran & Starkebaum, 1987; Semenzato *et al*, 1987; Oshimi, 1988). However, the normal range for peripheral blood LGL counts is $0{\cdot}223 \pm 0{\cdot}099 \times 10^9$/l (Loughran *et al*, 1987) and clonal disease has been documented in 8% of patients when absolute LGL counts are between 0·6 to $1{\cdot}0 \times 10^9$/l (Loughran, 1993). Thus, an expansion of a restricted LGL subset demonstrates the diagnosis of LGL-leukaemia and a 6-month follow-up criterion is not necessary when clonality is established (Semenzato *et al*, 1997). The characteristic finding is the presence of increased numbers of LGL, usually identified by a greater size than normal lymphocytes, abundant pale cytoplasm, and prominent azurophilic granules. However, these features may vary, even among cells from the same patient (Loughran, 1993). The granulation can range from fine to coarse, and some cells may have otherwise characteristic features but lack granules (sometimes called large agranular lymphocytes) (Bassan *et al*, 1986). Occasionally, clonally expanded lymphocytes with a characteristic $CD3^+$, $CD57^+$ phenotype may not have LGL morphology on a peripheral smear (Ahern *et al*, 1990) but may represent *in vivo* antigen-activated cytotoxic effector T cells. An increase of $CD3^+/CD56^-$ or $CD3^-/CD56^+$ cells by peripheral-blood flow cytometry and/or an inverted $CD4^+/CD8^+$ cell ratio (<1·0) suggests the existence of LGL leukaemia (Gonzales-Chambers *et al*, 1992).

Initial management

During the initial evaluation, red cell transfusions can be given as necessary. In cases supposed to be primary idiopathic PRCA, it would be preferable to wait for at least a month before instituting specific treatment, with the rationale that 10–12% of PRCA patients run a short and self-limited course (Dessypris, 1988). If, after such a waiting period, no signs of recovery of erythropoiesis appear, specific treatment should be instituted. Many of the secondary PRCAs are due to drugs and disappear when the drug is stopped. Those secondary to parvovirus B19 can be treated with intravenous immunoglobulin. Secondary PRCA not responding to treatment of the underlying diseases and primary PRCA are treated as immunologically-mediated diseases.

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Immunosuppressive therapy

Corticosteroids

Corticosteroids (CS) were the first immunosuppressive drugs used in the treatment of PRCA and so far have been considered the treatment of first choice, especially in young adults (Clark *et al*, 1984; Dessypris, 1988; Charles *et al*, 1996; Dessypris & Lipton, 2004). The details of CS therapy are described elsewhere (Dessypris, 1988; Dessypris & Lipton, 2004). In an era when CsA was not yet available, Clark *et al* (1984) reported the largest series of PRCA patients receiving immunosuppressive therapy and showed that 10/27 (37%) patients with acquired PRCA responded to CS within a mean period of 2·5 weeks. Comparable results of CS treatment were obtained at other institutions, ranging from 30–62% (Dessypris, 1988; Raghavachar, 1990; Marmont, 1991; Lacy *et al*, 1996; Mamiya *et al*, 1997). One of the important drawbacks of CS is that relapse is not uncommon: 80% of patients relapsed, as the dosage was tapered, during the 24 months after remission (Clark *et al*, 1984). The principal reason for discontinuing the drug, despite subsequent recurrence of anaemia, was the presence of unacceptable side effects, such as myopathy, infection, hyperglycemia, and compression fractures at the dose required to maintain remission. Treatment of relapses was successful, with 10/13 (77%) patients entering a second or third remission, and the median survival in patients with primary idiopathic PRCA was 14 years (Clark *et al*, 1984). Cytotoxic drugs administered in combination with CS were the most effective form of treatment in this study, producing 18/32 (56%) remissions. Although such vigorous immunosuppressive treatment is capable of inducing and maintaining remission in a majority of patients, it carries increased risks of serious infections, malignancy, and sterility (Clark *et al*, 1984). Thus, an individualized approach to management of PRCA has been widely accepted, i.e. escalating therapy in proportion to the severity of the disease for those patients who have failed CS therapy.

Cyclosporin A

Raghavachar (1990) reviewed the treatment of PRCA, focusing on the results of cyclosporin A (CsA) therapy, in 43 patients. He showed that the overall response rate to CsA is excellent (65%) and proposed that CsA should be the first drug to be given in acquired PRCA. Of note is that a high dosage was used in order to obtain these results (12 mg/kg per day). Comparable results of CsA treatment have been obtained at other institutions, ranging from 65–87% (Dessypris, 1988; Marmont, 1991; Means *et al*, 1991; Lacy *et al*, 1996; Mamiya *et al*, 1997; Sawada *et al*, 2007). Mamiya *et al* (1997) reviewed the clinical features of 150 patients with acquired PRCA in Japan. In their surveillance, CsA was given to 38 patients in a daily dose of 200–600 mg (most often 200–300 mg) and 31 (82%) showed haematological recovery. The response rate to CsA was 87% in the patients with primary PRCA and 73% in those with secondary PRCA, which encouraged them to recommend CsA therapy as first-line therapy for this disease.

Recently, The Japan PRCA Collaborative Study Group conducted a nationwide survey in Japan between 1990 and 2006 (Sawada *et al*, 2007). From a total of 185 patients, consisting of 73 primary idiopathic and 112 secondary PRCA cases, 62 patients with primary idiopathic PRCA were evaluated, which is the largest and the longest follow-up study so far. Although a retrospective one, this study, for the first time, answered many of the unknown questions concerning CsA therapy. The remission induction therapies for these patients by CsA and CS produced remissions in 74% and 60% of patients, respectively. The initial dose of CsA for the responding patients was 4·8 ± 1·2 mg/kg (mean ± SD, $n = 23$) with a range of 2·9–7·6 mg/kg body weight. Patients treated with CsA alone ($n = 23$) became transfusion-independent by 82 ± 200 d, with a range up to 910 d, after the start of therapy. Fifteen patients (65%) achieved transfusion-independence within 2 weeks, 17 patients (74%) within 1 month and 18 patients (78%) within 3 months. Salvage immunosuppressive treatment achieved remissions in 58 patients (94%). Forty-one and 15 patients were maintained on CsA ± CS (CsA-containing group) or CS alone (CS-group), respectively. The median relapse-free survival (RFS; estimated as transfusion-free survival) in the CsA-containing group was 103 months, significantly longer ($P < 0·01$) than that seen in the CS-group (33 months). Thus, combined CsA therapy can sustain a longer duration of initial remission than CS, however, discontinuation of maintenance therapy was strongly correlated with relapse ($P < 0·001$) and caused relapses with a median of 3 months with a range of 1·5–40 months. In contrast, 88% of relapses in the CS-group occurred during maintenance prednisolone (PSL) therapy (Sawada *et al*, 2007).

Tötterman *et al* (1989) also reported that PRCA patients did not remain in remission after CsA was stopped. An important question is whether or not the maintenance of patients in remission may have a beneficial influence on survival. One study (Sawada *et al*, 2007) reported an estimated median OS of the CsA-containing group of 12 years, which was not significantly different than the CS-group, while the 10-year OS in all patients was 95% and the median OS had not yet been reached in all patients. Of importance is the fact that the CsA is required to maintain remissions (Sawada *et al*, 2007) and the decreased probability of relapse and requirement of red cell transfusions reduces the dangers of hemolysis, infections and iron overload with possible superoxide damage to body tissues. CsA is more expensive than CS and requires renal function to be monitored, but it seems to be important to prevent relapses and to sustain remissions in primary idiopathic PRCA. Although higher doses of CsA, such as 12 mg/kg per day have been used for patients with PRCA (Raghavachar, 1990), this dosage has been toxic for Japanese PRCA patients. Since cytochrome P-450 isoenzymes, involved in CsA metabolism, have a variable frequency of a reduced function allele depending on race and each individual (Bladford, 2004), the

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most important therapeutic index should be trough CsA levels. Caucasian patients with anti-EPO antibody-related PRCA have been successfully treated with CsA alone at a dose of 200 mg/d (or 100 mg twice daily) (Verhelst *et al*, 2004; Rossert *et al*, 2005). Since organ transplantations have shown that long-term immunosuppression is associated with post-transplant malignancies (Cattran *et al*, 1995; Young *et al*, 2006), continuous and careful follow-up is required for patients receiving long-term CsA therapy.

Cytotoxic immunosuppressive drugs

Patients with an absolute contraindication for CsA or patients refractory to CsA may be treated with CS or a combination of CS and other immunosuppressives. Cyclophosphamide (CY) has been the principal alkylating agent utilized as an immunosuppressive drug in PRCA. The details of CY therapy are described elsewhere (Dessypris, 1988; Dessypris & Lipton, 2004). The initial dosage of CY is 50 mg/d p.o. and PSL at a dose of 20–30 mg/d is added in the absence of any contraindication. If the white blood cell and platelet counts allow, it is increased by 50 mg weekly or biweekly to a maximum of 150 mg daily until remission occurs or bone marrow suppression develops. The mean time to response is approximately 11 to 12 weeks with an overall response rate of 40 to 60%. When response occurs, the dose of PSL is tapered, and then the dose of CY is progressively decreased and eventually discontinued after 3–4 months from the time of normalization of haematocrit.

The duration of remission induced by CY seems to be prolonged as compared to remissions induced by CS (Clark *et al*, 1984; Firkin & Maher, 1988; Go *et al*, 2001). LGL leukaemia-associated PRCA has been primarily treated with chemotherapy, such as CY with or without CS, CsA, CS or methotrexate (Dhodapkar *et al*, 1994; Loughran *et al*, 1994; Lacy *et al*, 1996; Yamada *et al*, 1997; Sood *et al*, 1998; Hamidou *et al*, 2000; Go *et al*, 2001; Battiwalla *et al*, 2003; Osuji *et al*, 2006). The combination of CY plus CS is associated with a longer duration of response than CS alone (Dhodapkar *et al*, 1994; Lacy *et al*, 1996; Go *et al*, 2001). The overall response to initial CY ± CS therapy has been reported to be 66 to 100 % (Yamada *et al*, 1997; Go *et al*, 2003) and the median duration of response is 32 months (Go *et al*, 2003).

In one study, none of the patients with a response to cytotoxic agents had relapses (Lacy *et al*, 1996), but the other studies reported that a substantial number of patients relapsed when the CY was withdrawn (Zaentz *et al*, 1976; Clark *et al*, 1984). Maintenance CY therapy may prevent relapse, however, recognition of a variety of toxicities, particularly concerns about the long-term risk of malignancy and gonadal toxicity, often lead clinicians to consider less toxic alternative medications whenever possible (Csuka *et al*, 1986; Hoffman *et al*, 1992). These toxicity risks from alkylating agents are related to the cumulative dose of the medication (Reinhold-Keller *et al*, 2000) and the duration of therapy (Radis *et al*, 1995). Thus, the best role of CY therapy for PRCA might be to induce remissions using oral treatment lasting not longer than six months, with a switch to less toxic medication, such as CsA, for maintenance, but no controlled studies exist and this is purely speculative. It has been reported that crossover to azathioprine was effective in patients initially unresponsive to CY and vice versa (Firkin & Maher, 1988).

Anti-thymocyte globulin

In the largest series of nine PRCA patients treated with anti-thymocyte globulin (ATG) at a dose of 15 mg/kg per day for 10 d, six responded to therapy, five with normal haematocrits and one with a stable haematocrit of 32% (Abkowitz *et al*, 1986). Three remained in complete remission and two relapsed, which suggests that ATG is an effective form of treatment. However, this is an expensive and confining therapy that requires hospitalization because of a possible anaphylactic reaction.

Alemtuzumab

The anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) is a humanized $IgG_{1\kappa}$ monoclonal antibody directed against the CD52 antigen, present on B and T lymphocytes, NK cells, monocytes/macrophages, dendritic cells and eosinophils but not on human haemopoietic stem cells (Hale, 2001). The rationale behind the use of alemtuzumab in refractory cytopenias is that T-lymphocytes are thought to play an important role in the pathogenesis of autoimmune cytopenias, as they are involved in the control of expansion of immunoglobulin-producing, auto-reactive B-lymphocyte clones (Willis *et al*, 2001). Willis *et al* (2001) reported the effect of treatment with alemtuzumab followed by CsA administration in 21 patients with severe and life-threatening autoimmune cytopenias including four patients with PRCA. A response was seen in 2/4 patients with PRCA, although one patient relapsed at 7 months when the CsA blood level was suboptimal. One patient with PRCA in association with low-grade non-Hodgkin lymphoma died from high-grade transformation at 16 months while still in remission from the PRCA. Ru and Liebman (2003) reported two patients with chronic lymphocytic leukaemia (CLL) and a CD8 T-LGL leukaemia who were refractory to multiple treatments for PRCA. When both patients were treated with alemtuzumab, there was a rapid increase in the reticulocyte count that occurred as early as the third infusion. At the time reporting, both patients had been in PRCA remission for 9 and 5 months, respectively. These results indicated that alemtuzumab is an alternative option in the treatment of patients with refractory PRCA, however, relapse can occur and a significant number of patients need maintenance therapy with CsA after alemtuzumab treatment. Therefore, it remains uncertain whether alemtuzumab can induce a maintenance-free haematological response in PRCA. The use of alemutuzumab should be limited to patients with PRCA who are refractory to conventional immunosuppressive

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therapy because of the limited information and high risk of infections.

Rituximab

Rituximab is a genetically engineered chimeric mouse/human monoclonal antibody that targets the CD20 molecule present on mature B cells, which are the precursors of autoantibody-producing plasma cells. Rituximab can selectively deplete B-cells by mechanisms including antibody-dependent cell-mediated cytotoxicity, complement-mediated cytotoxicity and inhibition of cell proliferation with direct induction of B-cell apoptosis (Smith, 2003). Rituximab has been found to be useful in treating primary autoimmune hemolytic anaemia and thrombocytopenia (Quartier *et al*, 2001; Stasi *et al*, 2001) and several reports have shown that PRCA was successfully treated with rituximab in patients with B-cell lymphoproliferative disorders mainly consisting of CLL (Batlle *et al*, 2002; Ghazal, 2002; Gupta *et al*, 2002; Hegde *et al*, 2002; Ru & Liebman, 2003; Pantelidou *et al*, 2004; Narra *et al*, 2006). The maximum time for onset of response was 4 weeks. In two cases, where PRCA and the primary disorder were simultaneously diagnosed, rituximab led to response in both PRCA and the primary disorder (Narra *et al*, 2006). One patient had a response to rituximab lasting only 4 weeks, but then responded to alemtuzumab (Ru & Liebman, 2003). No significant side effects were reported in any of these patients treated with rituximab. Dungarwalla *et al* (2007) have shown that the results of rituximab therapy in patients with severe, resistant and life-threatening PRCA refractory to conventional immunosuppression are disappointing. In their pilot study, all three of these patients with idiopathic PRCA did not respond to a conventional dose of rituximab (375 mg/m^2) weekly for 4 weeks.

Other therapeutic options

Intravenous immunoglobulin

In immunocompromised hosts, such as recipients of organ transplantation (Wong *et al*, 1999), patients infected with human immunodeficiency virus (HIV) (Frickhofen *et al*, 1990) or receiving chemotherapy (Song *et al*, 2002; Isobe *et al*, 2004), acute or chronic anaemia can develop following parvovirus B19 infection due to the lack of the production of specific antibodies. Chronic B19 infection-related PRCA is a treatable anaemia and demonstration of the virus DNA in blood by the polymerase chain reaction or dot-blot hybridization assays is essential. Intravenous immunoglobulin (IVIG) contains neutralizing antibody against parvovirus B19 and has been reported to be effective for chronic B19 infection-related anaemia in immunocompromised hosts. Recurrence of anaemia is common in HIV-infected patients with low CD4$^+$ T-cell counts ($<0{\cdot}08 \sim 0{\cdot}1 \times 10^9$/l) and requires additional IVIG (Ramratnam *et al*, 1995). Anti-retroviral therapy may resolve chronic anaemia in HIV-infected patients (Mylonakis *et al*, 1999). IVIG may also be effective for PRCA due to parvovirus B19 in patients treated with rituximab (Sharma *et al*, 2000; Song *et al*, 2002). Alemtuzumab causes prolonged, severe CD4 and CD8 lymphopenia (Keating *et al*, 2002) and PRCA due to parvovirus B19 infection in a patient with cutaneous T-cell lymphoma treated with alemtuzumab has been reported (Herbert *et al*, 2003). Tacrolimus (FK506) is often associated with chronic B19 infection-associated anaemia in organ transplant recipients and cessation of tacrolimus or replacement with other immunosuppressants results in an improvement of anaemia (Wong *et al*, 1999).

Thymectomy

Surgical resection of thymomas has been recommended as the initial treatment of thymoma-associated PRCA, with an expected haematological response rate of 25–30% (Zeok *et al*, 1979). In recent reports, resection of the thymoma by itself was effective in remitting the anaemia in only a small percentage of patients (Mamiya *et al*, 1997; Thompson & Steensma, 2006) and a significant fraction of patients developed PRCA after thymectomy (Suzuki *et al*, 2003; Thompson & Steensma, 2006; Hirokawa *et al*, 2008). Although the pathogenesis of thymoma-associated PRCA remains to be elucidated, there exist two potential mechanisms. Thymoma itself alters the subset and/or the repertoires of T lymphocytes, leading to the production of autoaggressive T-cell clones (Hoffacker *et al*, 2000). Another intriguing possibility is that thymectomy may represent a risk for the development of systemic autoimmune disorders over years (Gerli *et al*, 1999).

Thompson and Steensma (2006) reported that surgical resection of thymoma was insufficient for normalization of erythropoiesis in all 13 patients so treated, but immunosuppressive therapy was effective as an adjuvant treatment. Immunosuppressive therapy including CS, CY and CsA has been reported to be useful in cases with thymoma-associated PRCA (Marmont *et al*, 1975; Garcia Vela *et al*, 1993; Charles *et al*, 1996; Thompson & Steensma, 2006), but optimal management of this disorder has remained unclear. Recently, it has been reported that thymoma-associated PRCA showed an excellent response to CsA and CsA-containing regiments were effective in preventing relapse (Hirokawa *et al*, 2008).

Splenectomy, plasmapheresis, and bone marrow transplantation have been used on rare occasions and can be tried if all else fails.

Peptide-based EPO receptor agonist

Krantz and Kao (1967), for the first time, reported that plasma from a patient with PRCA inhibited haem synthesis by the patient's own bone marrow cells *in vitro*. The serum of patients with anti- EPO antibody-related PRCA also inhibited the growth of erythroid progenitor cells *in vitro* (Casadevall *et al*, 2002). PRCA due to autoantibodies against endogenous EPO occurs but is rare in patients who have never been treated with

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