antibodies to these clones using a phage plate assay, only the DRS-1 clone was recognized in 2 patients. Focus was thus placed on DRS-1 for further studies.

Detection of specific antibodies to DRS-1 in AA patients

To confirm the presence of antibodies specific to DRS-1 in the sera of AA patients, a recombinant His-tag DRS-1 protein was prepared in addition to His-tag and DRS-1 proteins. Figure 1A shows the results of Western blotting using these recombinant proteins. Serum of a PNH^+ AA patient displayed both His-tag DRS-1 and DRS-1, but not His-tag, whereas serum of a healthy individual did not display any of these recombinant proteins (Figure 1A).

Figure 1B shows results of the same Western blotting for selected patients with AA or MDS and healthy individuals. Clear bands indicating the presence of DRS-1 Abs were produced by the sera of several PNH^+ AA patients but not by the sera of AA patients without PNH-type cells (PNH^- AA patients) or those of PNH^- MDS patients.

Measurement of DRS-1 Ab titers with ELISA in patients with AA and MDS

To measure titers of DRS-1 Abs in serum, we established an ELISA using recombinant DRS-1 protein. Figure 2 shows antibody titers in the sera of different groups of patients. AA and MDS patients were divided into 2 groups based on the presence of increased PNH-type cells, which represent a marker for immune pathophysiology in AA.[12,13] Twenty-seven (38.0%) of the 71 PNH^+ AA patients and 5 (38.5%) of 13 PNH^+ MDS patients showed antibody titers greater than the cutoff value, which were significantly higher than that of PNH^- AA (6.3%, 2 of 32) and PNH^- MDS (0 of 42) patients, but there was no significant difference between PNH^+ AA and PNH^+ MDS patients ($P = .976$). All 5 MDS patients with DRS-1 Abs had RA without karyotypic abnormalities. None of 5 patients with florid PNH were positive for DRS-1 Abs (data not shown).

Response to IST in AA patients with DRS-1 Abs

To determine if the presence of DRS-1 Abs reflects immune pathophysiology in AA, we selected 22 patients whose sera were tested for DRS-1 Abs before IST and compared response rates to IST between DRS-1 Ab^+ patients and DRS-1 Ab^- patients. Response to IST was evaluated at 6 months after therapy according to the response criteria of Camitta.[22]

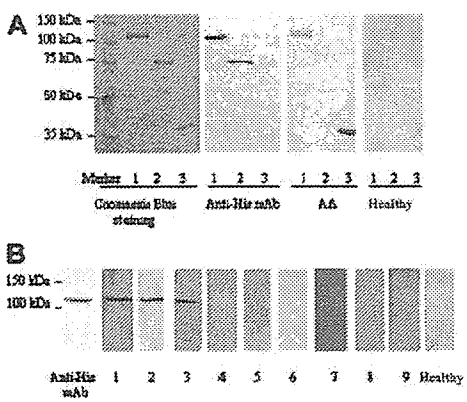

Figure 1. Specific antibody to recombinant DRS-1 in the serum of an AA patient. (A) Purified His-tag DRS-1, His-tag, and native DRS-1 were loaded in lanes 1, 2, and 3, respectively. Proteins were visualized using Coomassie blue staining. Blotted membranes were incubated with anti-His mAb, serum of a PNH^+ AA patient, and serum of a healthy individual for detection of DRS-1 Abs. (B) An equal amount of purified His-tag DRS-1 protein was used to detect antibodies specific to DRS-1 in sera from 3 PNH^+ AA patients (lanes 1-3), 3 PNH^- AA patients (lanes 4-6), 3 PNH^- MDS patients (lanes 7-9), and a healthy individual (Healthy).

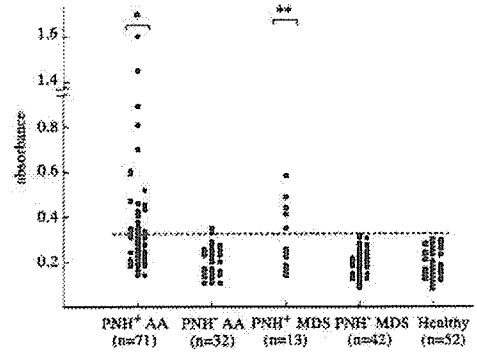

Figure 2. Titration of DRS-1 Abs in sera of patients using ELISA. Antibody titers against purified His-tag DRS-1 in the sera of 71 PNH^+ AA patients, 32 PNH^- AA patients, 13 PNH^+ MDS patients, 42 PNH^- MDS patients, and 52 healthy individuals were determined using sera diluted at a 1:1000 dilution. The dotted line denotes a cutoff value defined as mean + 2 SD for absorbance in 52 healthy individuals. Asterisks indicate a prevalence of DRS-1 Ab titers significantly higher than that of PNH^- AA patients, PNH^- MDS patients, and healthy individuals ($^*P < .001$, $^{**}P < .05$).

All 11 (100%) DRS-1 Ab^+ patients responded to ATG + CsA (4) or CsA (7), whereas 6 (55%) of the 11 DRS-1 Ab^- patients improved with ATG + CsA (4) or CsA (2). Several factors that may influence response to IST were analyzed using multivariate analysis. The presence of DRS-1 Abs ($P = .0026$) was significantly associated with a good response to IST, whereas age ($P = .2439$), sex ($P = .3852$), severity of AA ($P = .4159$), and increased PNH-type cells ($P = .7389$) did not affect response to IST in the 22 patients.

Expression of DRS-1 gene by hematopoietic cells

Although DRS-1 gene expression is reportedly ubiquitous, expression of the gene by hematopoietic cells has not been studied in detail. We studied DRS-1 gene expression in various leukemia cell lines using RPA. Myeloid leukemia cell lines such as K562, UT-7, KU812, and KH88 displayed high expression of the DRS-1 gene (Figure 3A). Conversely, lymphoid or monocytoid leukemia cell lines did not display detectable levels of DRS-1 mRNA. When expression of the DRS-1 gene was quantified using real-time PCR, DRS-1/GAPDH ratios of K562, UT-7, and bone marrow $CD34^+$

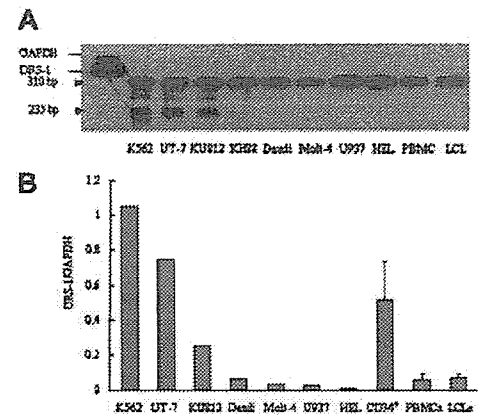

Figure 3. DRS-1 gene expression in hematopoietic cells. (A) Total RNA (20 μg) from each cell line was subjected to ribonuclease protection assay using biotin-labeled DRS-1 RNA probe and GAPDH RNA probe. The protected GAPDH probe and DRS-1 probe were visualized at 310 bp and 235 bp, respectively. (B) The same amounts of cDNA derived from each cell line or $CD34^+$ cells were used to amplify DRS-1 or GAPDH, respectively. Relative expression levels of DRS-1 to GAPDH were determined as DRS-1/GAPDH. The levels for $CD34^+$, PBMCs, and LCLs represent mean + SD of 3, 6, and 7 healthy individuals, respectively.

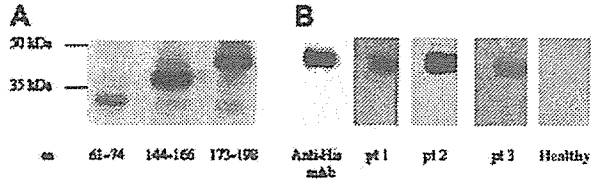

Figure 4. Mapping of antibody epitopes in DRS-1 protein. (A) Lysates of transformed *E coli* expressing 3 different DRS-1 fragments (aa's 61-74, aa's 144-166, aa's 173-198) were tested for reactivity to the original serum from patient 1 (pt 1). (B) Recombinant proteins derived from one epitope clone aa's 173 to 198 were purified and subjected to Western blotting using the sera of AA patients who exhibited antibodies specific to the native DRS-1 protein. Patient 2 (pt 2) and patient 3 (pt 3) represent other PNH+ AA patients who showed high titers of DRS-1 Abs.

cells from healthy individuals were 1.62, 0.75, and 0.51, respectively, which were 35-, 16-, and 11-fold higher than DRS-1/GAPDH ratios for PBMCs from healthy individuals (Figure 3B). Other leukemia cell lines such as Daudi, Molt-4, U937, and HEL, as well as LCLs derived from healthy individuals, displayed expression levels similar to those of normal PBMCs.

Antibody epitopes of DRS-1 protein

To determine whether there is a common epitope recognized by antibodies derived from different AA patients, randomly cut fragments of DRS-1 were ligated into pSCREEN T-Vector, and DRS-1 fragments derived from ligated plasmids were examined for reactivity to sera that were positive for DRS-1 Abs. Immunoblotting using the original serum revealed 3 antibody epitopes corresponding to aa's 61 to 74, aa's 144 to 166, and aa's 173 to 198 of DRS-1 (Figure 4A). Among these 3 epitopes, only aa's 173 to 198 were recognized by sera from other patients including patients 2 and 3 carrying HLA-DR15 (Figure 4B). Antibodies to this epitope were present in 7 (53.8%) of the 13 DRS-1 Ab+ patients.

T-cell responses to endogenous DRS-1 protein

To determine if DRS-1 can elicit T-cell responses to antigen-presenting cells (APCs) expressing DRS-1, we looked for peptides that can be presented by HLA-DR15 in the aa sequence of DRS-1 and identified 2 putative CD4+ T-cell epitopes, peptide 1 and peptide 2. Interestingly, peptide 1 (aa's 191-204) was found to overlap with the common antibody epitope aa's 173-198. PBMCs from patient 2 stimulated by peptide 1 were examined for reactivity to 1501-L cells transduced with the DRS-1 gene. Primed CD4+ T cells exhibited significantly higher proliferative response to 1501-L cells transduced with DRS-1 gene than to nontransduced 1501-L cells or to 0101-L cells transduced with DRS-1 gene ($P < .05$; Figure 5A). DRS-1–specific CD4+ T cells also killed DRS-1–transduced L cells in a dose-dependent fashion. Cytotoxicity against DRS-1–transduced 1501-L cells reached 52.8% at an effector-target ratio of 20, significantly higher than that against DRS-1–transduced 0101-L cells or nontransduced 1501-L cells ($P < .001$; Figure 5B). These findings suggest that DRS-1 can be processed in APCs, and the DRS-1 peptide presented by HLA-DR15 may provoke T cells to attack APCs expressing the DRS-1 gene.

T-cell precursors specific to DRS-1 peptides in peripheral blood of DRS-1 Ab+ patients

In order to determine the frequency of T-cell precursors specific to DRS-1 peptides in peripheral blood T cells of DRS-1 Ab+ AA patients, another peptide (peptide 3) with low affinity to HLA-DR15 was prepared, and PBMCs in patient 2 and patient 3 were examined using ELISPOT assay. Stimulation of PBMCs from patient 2 with peptide 1 and peptide 2 induced a higher number of INF-γ spots than stimulation with peptide 3 (Figure 6A). Such high induction of INF-γ spots was not induced in PBMCs of a DRS-1 Ab− AA patient (patient 4) and a healthy individual. Stimulation with 0101-L cells pulsed with peptide 1 and peptide 2 also failed to induce INF-γ spots from the same PBMCs. In patient 3, only peptide 1 induced as many as INF-γ spots as phorbol myristate acetate (PMA) plus ionomycin did (Figure 6B).

Discussion

DRS-1 was identified as a novel candidate for autoantigen in AA, using the SEREX method with a cDNA library derived from UT-7 and serum from an AA patient possessing increased PNH-type cells and HLA-DRB1*1501. Several findings in the present study suggest that DRS-1 is involved in the immune pathophysiology of AA. These include a high prevalence of DRS-1 Abs in PNH+ AA and PNH+ RA patients, high rate of response to immunosuppressive therapy in patients with this antibody, high expression of DRS-1 gene by myeloid leukemia cell lines and CD34+ cells from healthy individuals, inducibility of specific T cells recognizing APCs that express the DRS-1 gene, and the presence of T-cell precursors specific to DRS-1 in peripheral blood from AA patients displaying DRS-1 Abs.

DRS-1 is identical to monofunctional peroxisomal Δ^3 Δ^2-enoyl-CoA isomerase (*PECI*).[23] ECI is unique in that its activity is essential for the β-oxidation of all unsaturated fatty acids,[24,25] and presence of this enzyme has been demonstrated in mammalian peroxisomes and mitochondria.[23] This gene is abundantly expressed in various tissues, including the heart, lung, brain, and liver, but not in peripheral blood leukocytes.[23] Expression of *PECI* may be increased in immature hematopoietic cells because they require active utilization of fatty acids as an energy source.[23] Indeed, the results of our RPA and real-time PCR indicate that *DRS-1* is highly expressed by immature myeloid cells, including CD34+ cells, supporting our hypothesis that DRS-1 could represent a target antigen of immune system attack directed against hematopoietic progenitor cells in AA. Although DRS-1 is ubiquitously expressed, only hematopoietic progenitor cells may be vulnerable to

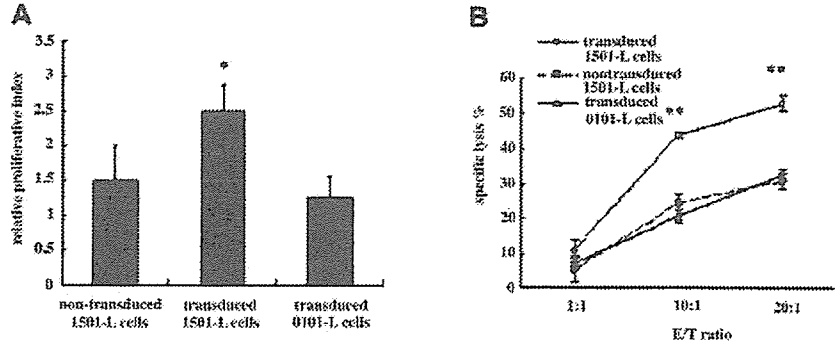

Figure 5. Response of DRS-1–specific T cells to APCs with DRB1*1501 expressing the DRS-1 gene and immature myeloid cells. (A) ^{3}H-thymidine incorporation of DRS-1–specific T cells to DRS-1–transduced 1501-L cells and 0101-L cells, as well as nontransduced 1501-L cells. Values represent mean + SD of triplicate cultures from 4 different experiments. (B) Cytotoxicity of DRS-1–specific T cells against DRS-1–transduced 1501-L cells and 0101-L cells, as well as nontransduced 1501-L cells. L-cell transfectants were incubated with differing numbers of DRS-1–specific T cells in a 4-hour cytotoxicity assay. Values represent mean ± SD of duplicate cultures from 3 different experiments. Asterisks in Figure 5A-B indicate values significantly different from nontransduced 1501-L cells or transduced 0101-L cells (*$P < .05$, **$P < .001$). E/T indicates effector-target ratio.

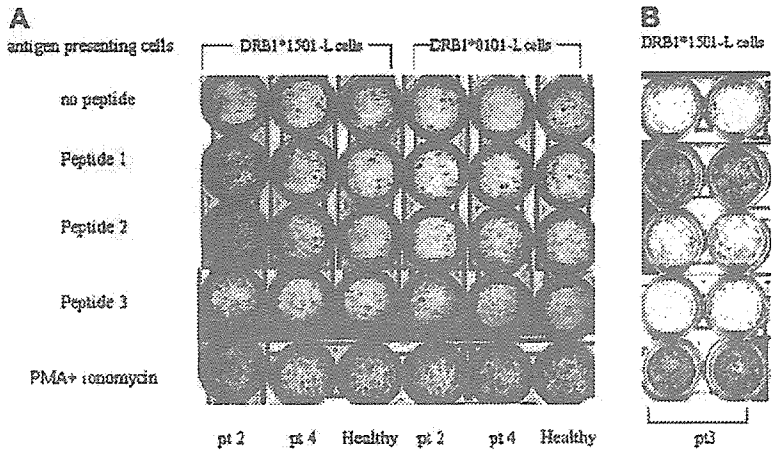

Figure 6. Frequency of T-cell precursors specific to the DRS-1 epitope. (A) PBMCs of a DRS-1 Ab$^+$ patient (pt 2), a DRS-1 Ab$^-$ patient (patient 4; pt 4), and a healthy individual were subjected to ELISPOT assay using different combinations of APCs and DRS-1–derived peptides. (B) Another DRS-1 Ab$^+$ patient (pt 3) was subjected to ELISPOT assay. The figure shows results of duplicate culture.

cellular immune responses to DRS-1 due to the expression of functional HLA-DR molecules.[26]

Antibodies to DRS-1 have been detected in patients with autoimmune diabetes,[27] breast cancer,[28] renal cancer,[29] and hepatocellular carcinoma[30] using the SEREX method. However, the significance of antibodies in the pathophysiology of these diseases has remained unclear due to the low prevalence ($\leq$ 8%) of antibodies in these diseases. ELISA in the present study revealed significantly higher titers of DRS-1 Abs compared with healthy controls in 38.0% of PNH$^+$ AA patients and 38.5% of PNH$^+$ MDS patients, both of which are considered to have immune-mediated bone marrow failure.[12,13,31] As for MDS patients, DRS-1 Abs were detected only in PNH$^+$ RA patients, supporting the significance of a small number of PNH-type cells as a marker for immune pathophysiology. Antibody production against DRS-1 is not a secondary phenomenon associated with destruction of PNH-type cells because none of 5 patients with florid PNH displayed DRS-1 Abs. In PNH$^+$ AA patients who do not show increased titers of DRS-1 Abs, antigens other than DRS-1 may be involved in immune pathogenesis of AA. Multivariate analysis identified presence of DRS-1 Abs as a factor predicting a good response to IST. PNH$^+$ bone marrow failure is thus the first disease where antibody response to DRS-1 has been implicated in an autoimmune pathophysiology.

Overlap of immunodominant T- and B-cell epitopes has been observed in pyruvate dehydrogenase complex for primary biliary cirrhosis,[32-35] myelin basic protein[36,37] and proteolipid protein for multiple sclerosis,[38-41] and glutamic acid decarboxylase 65 for IDDM,[42-44] suggesting that this is a common theme for autoimmune diseases. The hot spot of the antibody epitope we identified in epitope mapping was shared by 53.8% of DRS-1 Ab$^+$ AA patients, and the C-terminal half of the epitope sequence overlapped with a deduced CD4$^+$ T-cell epitope (aa's 191-204) presented by HLA-DR15. Colocalization of the immunodominant T- and B-cell epitopes of DRS-1 may be important in the development of autoimmune responses against DRS-1 in AA. Antigen-specific B cells play important roles as APCs by way of uptake of antigens via surface immunoglobulin.[45,46] Antibody binding to specific antigen modulates antigen processing by human B lymphoblastoid cells.[46] In antigen-presentation of tetanus toxoid, a single bound antibody or associated F(ab) fragment can enhance the presentation of one T-cell determinant while strongly suppressing the presentation of a different T-cell determinant. Both suppressed and boosted determinants fall within an extended domain of antigen stabilized by the antibody during proteolysis.[47]

Some intracellular proteins can be processed in the cytoplasm and chaperoned to HLA class II molecules by the invariant chain peptide.[48] Whether proteosome proteins like DRS-1 can take this pathway to be presented by HLA-DR is unknown. The specific response of DRS-1–specific T cells to HLA-DRB1*1501-L cells transfected with DRS-1 gene strongly suggests that the cytoplasmic protein can be processed by APCs and the cells can be targeted through recognition of the DRS-1 peptide–HLA-DR15 complex by specific T cells. Several studies have shown that HLA-DR molecules in hematopoietic progenitor cells bind some intracellular proteins such as tubulin β-chain, prolidase, thrombospondin 1, and granzyme.[49] ELISPOT assay in the present study demonstrated that T-cell precursors specific to peptide 1 were increased in 2 AA patients carrying HLA-DR15 and DRS-1 Abs. The DRS-1 epitope may thus stimulate T cells to raise both antibodies and CD4$^+$ T cells specific to DRS-1. Although we could not examine the effect of DRS-1–specific T cells on the growth of hematopoietic progenitor cells due to the unavailability of autologous CD34$^+$ cells, these findings suggest that DRS-1–specific CD4$^+$ T cells may contribute to the development of AA by directly killing hematopoietic progenitor cells. The high prevalence of immune response to DRS-1 in PNH$^+$ AA patients appears to support this hypothesis, as CD4$^+$ T cells specific to certain antigens on hematopoietic cells allow expansion of PNH clones.[14]

Hirano et al[50] recently identified kinectin as a possible antigen in AA, using the SEREX method with a cDNA library derived from fetal liver cells. They induced kinectin-specific CD8$^+$ T cells from PBMCs of HLA-A2–positive healthy donors and demonstrated that T cells inhibited in vitro growth of hematopoietic progenitor cells in an HLA-A2–restricted fashion. Although the number of AA patients they studied was very low, CD8$^+$ T cells specific to endogenous proteins like kinectin may conceivably play a role in bone marrow failure for some AA patients. However, the high incidence of HLA-DR15 and increased PNH-type cells in immune-mediated AA suggest the importance of CD4$^+$ T cells rather than CD8$^+$ T cells in the development of AA.[13] Our study demonstrated for the first time that immune responses to a protein abundantly expressed in hematopoietic progenitor cells by both T and B cells are operative in immune-mediated AA. Identification of DRS-1–specific T cells with HLA class II tetramers and subsequent functional analysis would further clarify the roles of DRS-1 in the pathogenesis of AA.

Acknowledgment

We would like to thank Dr Haruo Tsuchiya for his helpful discussion and ideas.

References

1. Young NS. Pathophysiology II: immune suppression of hematopoiesis. In: Young NS, Alter BP, eds. Aplastic Anemia Acquired and Inherited. Philadelphia, PA: Saunders; 1994:68-99.
2. Bacigalupo A, Broccia G, Corda G, et al. Antilymphocyte globulin, cyclosporin, and granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): a pilot study of the EBMT SAA Working Party. Blood. 1995;85:1348-1353.
3. Rosenfeld SJ, Kimball J, Vining D, Young NS. Intensive immunosuppression with antithymocyte globulin and cyclosporine as treatment for severe acquired aplastic anemia. Blood. 1995;85:3058-3065.
4. Viale M, Merli A, Bacigalupo A. Analysis at the clonal level of T-cell phenotype and function in severe aplastic anemia patients. Blood. 1991;78: 1268-1274.
5. Zoumbos NC, Gascon P, Djeu JY, Trost SR, Young NS. Circulating activated suppressor T lymphocytes in aplastic anemia. N Engl J Med. 1985;312:257-265.
6. Herrmann F, Griffin JD, Meuer SG, Meyer zum Buschenfelde KH. Establishment of an interleukin 2-dependent T cell line derived from a patient with severe aplastic anemia, which inhibits in vitro hematopoiesis. J Immunol. 1986;136:1629-1634.
7. Lieberman SM, Evans AM, Han B, et al. Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. Proc Natl Acad Sci U S A. 2003;100:8384-8388.
8. Viglietta V, Kent SC, Orban T, Hafler DA. GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes. J Clin Invest. 2002; 109:895-903.
9. Gran B, Rostami A. T cells, cytokines, and autoantigens in multiple sclerosis. Curr Neurol Neurosci Rep. 2001;1:263-270.
10. Markovic-Plese S, McFarland HF. Immunopathogenesis of the multiple sclerosis lesion. Curr Neurol Neurosci Rep. 2001;1:257-262.
11. Harrison LC, Honeyman MC, DeAizpurua HJ, et al. Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes. Lancet. 1993;341:1365-1369.
12. Wang H, Chuhjo T, Yasue S, Omine M, Nakao S. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. Blood. 2002;100: 3897-3902.
13. Sugimori C, Chuhjo T, Wang H, Feng X, Nakao S. Roles of HLA-DR15 in the immune mechanisms of acquired aplastic anemia: methods of contribution differ between DRB1*1501 and DRB1*1502 [abstract]. Blood. 2003;102:508a.
14. Murakami Y, Kosaka H, Maeda Y, et al. Inefficient response of T lymphocytes to glycosylphosphatidylinositol anchor-negative cells: implications for paroxysmal nocturnal hemoglobinuria. Blood. 2002;100:4116-4122.
15. Takami A, Zeng W, Wang H, Matsuda T, Nakao S. Cytotoxicity against lymphoblastoid cells mediated by a T-cell clone from an aplastic anaemia patient: role of CD59 on target cells. Br J Haematol. 1999;107:791-796.
16. Araten DJ, Nafa K, Pakdeesuwan K, Luzzatto L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. Proc Natl Acad Sci U S A. 1999;96:5209-5214.
17. Wang H, Chuhjo T, Yamazaki H, et al. Relative increase of granulocytes with a paroxysmal nocturnal haemoglobinuria phenotype in aplastic anaemia patients: the high prevalence at diagnosis. Eur J Haematol. 2001;66:200-205.
18. Sahin U, Tureci O, Schmitt H, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci U S A. 1995;92:11810-11813.
19. Nakao S, Takami A, Takamatsu H, et al. Isolation of a T-cell clone showing HLA-DRB1*0405-restricted cytotoxicity for hematopoietic cells in a patient with aplastic anemia. Blood. 1997;89: 3691-3699.
20. Takami A, Nakao S, Tatsumi Y, et al. High inducibility of heat shock protein 72 (hsp72) in peripheral blood mononuclear cells of aplastic anaemia patients: a reliable marker of immune-mediated aplastic anaemia responsive to cyclosporine therapy. Br J Haematol. 1999;106:377-384.
21. Sturniolo T, Bono E, Ding J, et al. Generation of tissue-specific and promiscuous HLA ligand database using DNA microarrays and virtual HLA class II matrices. Nat Biotechnol. 1999;17:555-561.
22. Camitta BM. What is the definition of cure for aplastic anemia? Acta Haematol. 2000;103:16-18.
23. Geisbrecht BV, Zhang D, Schulz H, Gould SJ. Characterization of PECI, a novel monofunctional Δ^3, Δ^2-enoyl-CoA isomerase of mammalian peroxisomes. J Biol Chem. 1999;274:21797-21803.
24. Geisbrecht BV, Zhu D, Schulz K, et al. Molecular characterization of *Saccharomyces cerevisiae* Δ^3, Δ^2-enoyl-CoA isomerase. J Biol Chem. 1998;273: 33184-33191.
25. Gurvitz A, Mursula AM, Firzinger A, et al. Peroxisomal Δ^3-cis-Δ^2-trans-enoyl-CoA isomerase encoded by ECI1 is required for growth of the yeast *Saccharomyces cerevisiae* on unsaturated fatty acids. J Biol Chem. 1998;273:31366-31374.
26. Harris PE, Maffei A, Colovai AI, Kinne J, Tugulea S, Suciu-Foca N. Predominant HLA-class II bound self-peptides of a hematopoietic progenitor cell line are derived from intracellular proteins. Blood. 1996;87:5104-5112.
27. Suk K, Kim YH, Hwang DY, Ihm SH, Yoo HJ, Lee MS. Molecular cloning and expression of a novel human cDNA related to the diazepam binding inhibitor. Biochim Biophys Acta. 1999;1454:126-131.
28. Scanlan MJ, Gout I, Gordon CM, et al. Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. Cancer Immun. 2001;1:4.
29. Scanlan MJ, Gordan JD, Williamson B, et al. Antigens recognized by autologous antibody in patients with renal-cell carcinoma. Int J Cancer. 1999;83:456-464.
30. Wang Y, Han KJ, Pang XW, et al. Large scale identification of human hepatocellular carcinoma-associated antigens by autoantibodies. J Immunol. 2002;169:1102-1109.
31. Ishiyama K, Chuhjo T, Wang H, Yachie A, Omine M, Nakao S. Polyclonal hematopoiesis maintained in patients with bone marrow failure harboring a minor population of paroxysmal nocturnal hemoglobinuria–type cells. Blood. 2003;102: 1211-1216.
32. Coppel RL, Gershwin ME. Primary biliary cirrhosis: the molecule and the mimic. Immunol Rev. 1995;144:17-49.
33. Shimoda S, Nakamura M, Ishibashi H, Hayashida K, Niho Y. HLA DRB4 0101-restricted immunodominant T cell autoepitope of pyruvate dehydrogenase complex in primary biliary cirrhosis: evidence of molecular mimicry in human autoimmune diseases. J Exp Med. 1995;181:1835-1845.
34. Kita H, Lian ZX, Van de Water J, et al. Identification of HLA-A2-restricted CD8 (+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells. J Exp Med. 2002;195:113-123.
35. Shimoda S, Van de Water J, Ansari A, et al. Identification and precursor frequency analysis of a common T cell epitope motif in mitochondrial autoantigens in primary biliary cirrhosis. J Clin Invest. 1998;102:1831-1840.
36. Tsuchida T, Parker KC, Turner RV, McFarland HF, Coligan JE, Biddison WE. Autoreactive CD8+ T-cell responses to human myelin protein-derived peptides. Proc Natl Acad Sci U S A. 1994; 91: 10859-10863.
37. Wucherpfennig KW, Catz I, Hausmann S, Strominger JL, Steinman L, Warren KG. Recognition of the immunodominant myelin basic protein peptide by autoantibodies and HLA-DR2-restricted T cell clones from multiple sclerosis patients: identity of key contact residues in the B-cell and T-cell epitopes. J Clin Invest. 1997;100: 1114-1122.
38. Pelfrey CM, Trotter JL, Tranquill LR, McFarland HF. Identification of a novel T cell epitope of human proteolipid protein (residues 40-60) recognized by proliferative and cytolytic CD4+ T cells from multiple sclerosis patients. J Neuroimmunol. 1993;46:33-42.
39. Pelfrey CM, Tranquill LR, Vogt AB, McFarland HF. T cell response to two immunodominant proteolipid protein (PLP) peptides in multiple sclerosis patients and healthy controls. Mult Scler. 1996;1: 270-278.
40. Honma K, Parker KC, Becker KG, McFarland HF, Coligan JE, Biddison WE. Identification of an epitope derived from human proteolipid protein that can induce autoreactive CD8+ cytotoxic T lymphocytes restricted by HLA-A3: evidence for cross-reactivity with an environmental microorganism. J Neuroimmunol. 1997;73:7-14.
41. Dressel A, Chin JL, Sette A, Gausling R, Hollsberg P, Hafler DA. Autoantigen recognition by human CD8 T cell clones: enhanced agonist response induced by altered peptide ligands. J Immunol. 1997;159:4943-4951.
42. Panina-Bordignon P, Lang R, van Endert PM, et al. Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. J Exp Med. 1995;181:1923-1927.
43. Wicker LS, Chen SL, Nepom GT, et al. Naturally processed T cell epitopes from human glutamic acid decarboxylase identified using mice transgenic for the type 1 diabetes-associated human MHC class II allele, DRB1*0401. J Clin Invest. 1996;98:2597-2603.
44. Reijonen H, Elliott JF, van Endert P, Nepom G. Differential presentation of glutamic acid decarboxylase 65 (GAD65) T cell epitopes among HLA-DRB1*0401-positive individuals. J Immunol. 1999;163:1674-1681.
45. Lanzavecchia A. Antigen-specific interaction between T and B cells. Nature. 1985;314:537-539.
46. Lanzavecchia A. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. Annu Rev Immunol. 1990;8:773-793.
47. Simitsek PD, Campbell DG, Lanzavecchia A, Fairweather N, Watts C. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. J Exp Med. 1995;181:1957-1963.
48. Stumptner-Cuvelette P, Benaroch P. Multiple role of the invariant chain in MHC class II function. Biochim Biophys Acta. 2002;1542:1-13.
49. Papadopoulos KP, Suciu-Foca N, Hesdorffer CS, Tugulea S, Maffei A, Harris PE. Naturally processed tissue- and differentiation stage-specific autologous peptides bound by HLA class I and II molecules of chronic myeloid leukemia blasts. Blood. 1997;90:4938-4946.
50. Hirano N, Butler MO, Von Bergwelt-Baildon MS, et al. Autoantibodies frequently detected in patients with aplastic anemia. Blood. 2003;102: 4567-4575.

Bone Marrow Transplantation (2004) 34, 703–709

www.nature.com/bmt

Graft-versus-tumor effects

Expansion and activation of minor histocompatibility antigen HY-specific T cells associated with graft-versus-leukemia response

A Takami[1], C Sugimori[1], X Feng[1], A Yachie[2], Y Kondo[1], R Nishimura[2], K Kuzushima[3], T Kotani[1], H Asakura[1], S Shiobara[1] and S Nakao[1]

[1]*Department of Cellular Transplantation Biology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan;* [2]*Department of Pediatrics, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; and* [3]*Laboratory of Viral Oncology, Aichi Cancer Center Research Institute, Nagoya, Japan*

Summary:

The immune system of females is capable of recognizing and reacting against the male-specific minor histocompatibility antigen (mHA), HY. Thus, cytotoxic T-lymphocytes (CTLs) recognizing this antigen may be useful in eradicating leukemic cells of a male patient if they can be generated *in vivo* or *in vitro* from a human leukocyte antigen (HLA)-identical female donor. The HLA-A*0201-restricted HY antigen, FIDSYICQV, is a male-specific mHA. Using HLA-A2/HY peptide tetrameric complexes, we reveal a close association between the emergence of HY peptide-specific CD8$^+$ T cells in peripheral blood and molecular remission of relapsed BCR/ABL$^+$ chronic myelogenous leukemia in lymphoid blast crisis in a patient who underwent female-to-male transplantation. Assessment of intracellular cytokine levels identified T cells that produce interferon-γ in response to the HY peptide during the presence of HY tetramer-positive T cells. These results indicate that transplant with allogeneic HY-specific CTLs has therapeutic potential for relapsed leukemia, and that expansion of such T cells may be involved in the development of a graft-versus-leukemia response against lymphoblastic leukemia cells.

Bone Marrow Transplantation (2004) **34**, 703–709. doi:10.1038/sj.bmt.1704583
Published online 23 August 2004
Keywords: minor histocompatibility antigen; HY; cytotoxic T-lymphocytes

Minor histocompatibility antigens (mHAs) are immunogenic peptides derived from polymorphic cellular proteins.[1] These peptides bind to human leukocyte antigen (HLA) and are recognized by allogeneic T cells. Following stem cell transplantation (SCT) with HLA-matched donor cells, graft-versus-host disease (GVHD) can arise through disparities in mHAs between the donor and the recipient. Using tetrameric HLA class I–mHA complexes, Mutis *et al*[2] demonstrated that a limited number of mHA-specific T cells expand in peripheral blood (PB) in parallel with increasing GVHD severity. It is believed that donor-derived T cells specific for mHAs play a significant role in the development of graft-versus-leukemia (GVL) effect as well as GVHD after allogeneic SCT. However, there is only limited evidence for the killing of leukemic cells by mHA-specific T cells.

Kern *et al*[3] reported that human cytomegalovirus peptide-specific CD8$^+$ T cells can be detected by flow cytometry in samples whose HLA is known. Kuzushima *et al*[4] showed that the frequency Epstein–Barr virus-specific CD8$^+$ T-cell frequencies are detectable irrespective of HLA typing when PB lymphocytes are incubated with an autologous lymphoblastic cell line (LCL). Both methods are based on multiparameter flow cytometric assays that detect rapid intracellular accumulation of interferon (IFN)γ after *in vitro* antigen stimulation in the presence of an intracellular transport blocker. By assessing the frequency of IFNγ-producing cells, the presence of functional T cells reactive with target antigens and target cells can be detected.

One male-specific mHA is the HLA-A*0201-restricted peptide, FIDSYICQV, from the male-specific antigen HY.[5] Cytotoxic T-lymphocytes (CTLs) recognizing this peptide may be useful in eradicating leukemic cells of a male patient with HLA-A2 if they can be generated *in vivo* or *in vitro* from an HLA-identical female donor. By HLA-A2/HY peptide tetramer staining and intracellular IFNγ assessment, we provide the first evidence that the emergence and activation of transferred HY-specific female CTLs contributes to molecular remission of chronic myelogenous leukemia (CML) in a male patient in lymphoid crisis.

To date, a small number of studies describe a clear dominance in T-cell receptor (TCR) variable (V)-gene segment usage in the recognition of certain HLA class I/peptide complexes in humans after SCT.[6,7] We examined PB CD8$^+$ T cells producing intracellular IFNγ of a male patient with CML in lymphoid crisis for the emergence of clonal T-cell proliferation by analyzing the T-cell repertoire

Correspondence: Dr A Takami, Department of Cellular Transplantation Biology, Kanazawa University Graduate School of Medicine, 13-1 Takaramachi, Kanazawa 920-8641, Japan.
E-mail: takami@med3.m.kanazawa-u.ac.jp
Received 20 October 2003; accepted 13 April 2004
Published online 23 August 2004

as well as *in vitro*-generated HY-specific CTLs. TCR BV spectratyping showed a similar peak with same size in some BV family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific $CD8^+$ T cells. These findings suggest the circulation of a functional T-cell clone capable of eradicating lymphoblastic leukemia cells.

Materials and methods

Case report

A patient was a 15-year-old Japanese male (HLA-A*0201-positive) with chronic-phase CML. He had experienced no GVHD after receiving busulfan- and cyclophosphamide-conditioned bone marrow from his HLA-identical sister. At 40 months after bone marrow transfer, lymphoid blast crisis suddenly developed. After treatment with cyclophosphamide, adriamycin, vincristin and prednisolone, the hematologic relapse persisted. At 2 months after chemotherapy, the patient underwent PB stem cell transplantation (PBSCT) from the same donor following conditioning with cytarabine, cyclophosphamide and total body irradiation. An unmanipulated PB stem cell graft including a total of 5.5×10^6 CD34-positive cells/kg was infused. Cyclosporine, which is used to prevent GVHD, was withdrawn on day 21 to induce GVL effect. At 3 weeks after PBSCT, conversion to full donor chimerism was obtained. The patient developed grade II acute GVHD on day 29 after PBSCT, and the disease progressed to extensive chronic GVHD on day 80. At 20 weeks after PBSCT, no BCR/ABL transcripts were detected, and he remained in molecular remission until a relapse at 13 months after PBSCT. The patient died of veno-occlusive disease shortly after the third transplantation with cytarabine- and idarubicin-conditioned bone marrow from the same donor.

Peptide

An HLA-A201-restricted HY peptide was synthesized using a semiautomatic multiple peptide synthesizer based on the reported sequence.[5] The purity of the peptide was checked by reverse-phase high-pressure liquid chromatography.

Cell preparation

Cells were obtained from the post transplant patient and his stem cell donor. Peripheral blood mononuclear cells (PBMC) were prepared using density-gradient centrifugation. For the establishment of Epstein–Barr virus (EBV)-transformed LCLs, PBMC from the donor were depleted of T cells using the rosette formation method. A total of $2–3 \times 10^6$ non-T cells were incubated in RPMI 1640 (GIBCO, Grand Island, NY, USA) medium containing 10% fetal calf serum (FCS; GIBCO) containing 10% culture medium from an EBV-producing cell line, B95-8, at 37°C for 2 h. The EBV-infected cells were cultured for 3 weeks until transformed LCL cells grew. LCL cells were maintained by changing the medium every 4 to 5 days.

Tetrameric HLA-A2/mHA HY peptide complexes

The generation of HLA-A2/mHA HY tetramers and tetramer labeling of HY-specific T cells was performed as described previously.[8]

Generation of dendritic cells (DCs) from monocytes

PB monocyte-derived DCs were generated as described previously.[9] Briefly, monocytes were isolated by adherence of donor PBMCs to plastic for 2 h. Monocytes were cultured in RPMI 1640 medium supplemented with 10% pooled AB serum, 10 ng/ml recombinant human interleukin-4 (IL-4) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (a gift from Kirin Brewery, Tokyo, Japan). On day 5, 100 U/ml recombinant human tumor necrosis factor (TNF)α was added. On day 8 or 9, the cells were harvested for use as monocyte-derived DCs for antigen presentation. Cultured cells expressed DC-associated antigens, such as CD1a, CD80, CD83, CD86 and HLA class I and class II.

Induction of HY peptide-specific CTLs

DCs were pulsed with an HY peptide for 90 min at 37°C in serum-free RPMI 1640. After washing, 1.0×10^6 peptide-(40 nM) pulsed DCs and 1.0×10^7 donor-derived (autologous) PBMC were cultured together in 24-well culture plates. The culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, minimal essential amino acids, sodium pyruvate and ampicillin (all from GIBCO) plus 10% autologous plasma. The cells were kept at 37°C in a humidified, 5% CO_2–air mixture. At days 7, 14 and 21, responder cells were restimulated with peptide-pulsed autologous DCs. From day 21, cultured T cells were suspended in 100 U/ml IL-2- (a gift from Shionogi, Osaka, Japan) containing culture medium and were restimulated weekly with peptide-pulsed autologous monocytes or DCs. T cells were harvested at day 35 and used for the cytotoxicity assay and RNA extraction for T-cell repertoire analysis.

Cytotoxicity (^{51}Cr release) assays

Donor-derived LCL cells and DCs as well as fibroblasts and leukemic cells of the patient were used as target cells in the standard 4-h ^{51}Cr release assay.[10] Specific lysis was calculated using the following formula: 100 × (cpm experimental release–cpm spontaneous release)/(cpm maximal release–cpm spontaneous release).

Preparation of target cells

Patient fibroblasts were isolated from a biopsy specimen and cultured in RPMI 1640 plus 10% FCS for 4 weeks. Single-cell suspensions were prepared by trypsinization. Donor-derived LCL cells and DCs and the patient's fibroblasts were suspended in 100 μl of ^{51}Cr solution containing an HY peptide at a concentration of 4 nM. In some experiments, concentrations of the peptide were changed as noted. Bone marrow mononuclear cells containing 98% BCR/ABL^+ cells were obtained from the patient

just before the second transplantation and were cryopreserved for use as leukemic cells. Thawed leukemic cells were cultured in RPMI 1640 plus 10% pooled AB serum for 24 h before use as a target in the CTL assay.

Blocking of cytotoxicity by monoclonal antibodies (MoAbs)

Polyclonal antibodies (control) or purified MoAbs were added to cultures of HY peptide- (4 nM) pulsed DCs in 96-well plates at a concentration of 10 μg/ml, and CTLs were immediately added to each well. MoAbs were HU-4 (anti-HLA-DR; kindly provided by Dr Akemi Wakisaka, Hokkaido University, Japan) and W6/32 (anti-HLA class I; American Type Culture Collection, Rockville, MD, USA).

Identification and isolation of IFNγ-producing $CD8^+$ T cells by flow cytometry

To detect circulating $CD8^+$ T-lymphocytes that recognize HY peptide, intracellular IFNγ was assessed by flow cytometry as described previously with slight modifications.[3,4] Briefly, donor-derived LCL cells were incubated for an hour with or without HY peptide. PBMCs were taken from the patient 12 weeks after the second transplantation. The $CD8^+$ T-lymphocytes were isolated from PBMCs using magnetic beads coated with an anti-CD8 monoclonal antibody (mAb) according to the manufacturer's instructions (Dynal AS, Oslo, Norway). A total of 10^6 $CD8^+$ cells were mixed with 10^6 autologous LCL cells in a culture tube in RPMI 1640 medium and cultured in a humidified 5% CO_2 incubator at 37°C for 1 h. Brefeldin A (Sigma, St Louis, MO, USA) was added at a final concentration of 10 μg/ml and the cells were cultured for an additional 5 h. After incubation, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with phosphate-buffered saline, cells were permeabilized with IC Perm (Biosourse International, Camarillo, CA, USA) and stained with PE-labeled anti-CD8 (Coulter, Miami, FL, USA) and FITC-labeled anti-human IFNγ (Biosourse International) MoAbs. Stained cells were analyzed and sorted on a FACScan (Becton Dickinson, San Jose, CA, USA).

RNA extraction and cDNA preparation

Total RNA was extracted from PBMCs or CTLs using a technique described elsewhere,[11] and was reverse transcribed into cDNA in a reaction primed with oligo(dT) using SuperScript II reverse transcriptase as recommended by the manufacturer (BRL, Bethesda, MD, USA).

Spectratyping of complementarity-determining region 3 (CDR3)

Conditions for the generation of the CDR3 size spectratyping have been reported previously.[6] Briefly, cDNA was polymerase chain reaction (PCR) amplified through 35 cycles (95°C for 1 min, 55°C for 1 min and 72°C for 1 min) with a fluorescent BC primer and primers specific to 24 different BV subfamilies. Analyses of the pseudogenes BV10 and BV19 were excluded from this study.[12] A measure of 1 μl of each amplified products was mixed with 2.01 100% formamide, heated at 90°C for 3 min and electrophoresed on a 6.75% denaturing polyacrylamide gel. The distribution of CDR3 size within the amplified product of each BV subfamily was analyzed with an automatic sequencer (Applied Biosystems Division, Foster City, CA, USA) equipped with a computer program allowing the determination of the fluorescence intensity of each band. The results are given as peaks corresponding to the intensity of the fluorescence. Expansion of a limited number of T cells was judged when a prominent peak appeared in the CDR3 pattern with or without a reduced number of peaks (five peaks). Given the BV-NDN-BJ sequence of the identical CDR3 size between in HY-specific $CD8^+$ cells in PB and the CTLs, a more specific primer covering CDR3 and different BJ subfamilies[13] was designed specifically to amplify cDNA of the $BV22^+$ T-cell clone in both CTLs.

Direct sequencing of PCR products

BV22-BJ PCR products were purified and sequenced as described previously.[14]

Results

Cytolytic activity of in vitro*-generated HY-specific CTLs*

Cultured T female donor cells stimulated with autologous HY peptide-pulsed DCs pulsed were able to lyse HY peptide-pulsed autologous DCs and patient fibroblasts, but could not lyse untreated DCs (Figure 1a). Of note, the CTLs lysed nonpeptide pulsed leukemic cells of the patient more efficiently than his fibroblasts. CTLs showed cytotoxicity to HY peptide-loaded autologous DCs, in a peptide concentration-dependent manner (Figure 1b). Cytotoxicity mediated by the CTLs against HY peptide-pulsed autologous DCs was blocked to a similar degree by the addition of MoAb either against anti-class I or anti-CD3, but was not affected by the addition of anti-HLA-DR (Figure 1c). In addition, the CTLs could effectively lyse LCL cells of unrelated males who shared HLA-A*0201. In contrast, apparent cytotoxic activity was neither observed against allogeneic LCL cells that did not possess HLA-A2 nor against LCL cells of unrelated females (Figure 1d).

Tetramer staining of HY-specific CTLs

The patient in lymphoid crisis with CML relapse developed acute GVHD shortly after the second transplant, which progressed to extensive chronic GVHD. He achieved a molecular remission at 4 months after PBSCT despite the fact that leukemic cells accounted for more than 98% of his bone marrow cells when the conditioning regimen was started. This unusual clinical course appeared to suggest the induction of GVL reactions associated with GVHD.

Other than the HY antigens, there was no disparity in the minor histocompatibility alleles, including HA-1, HA-2,

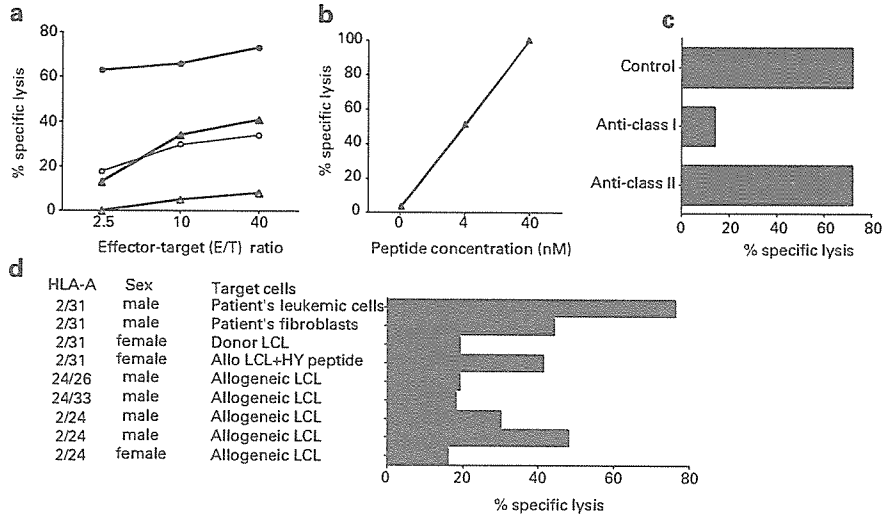

Figure 1 Cytotoxic activity of *in vitro*-generated HY-specific CTLs. (a) Cytotoxicity of cultured T cells stimulated by autologous DCs pulsed with HY peptide. The amount of peptide utilized in (a, b and c) was 4 nM. Target cells: autologous DCs without HY peptide (Δ); autologous DCs pulsed with HY peptide (▲); fibroblasts of the patient (○); leukemic cells of the patient (●). In (b, c, and d), cytotoxicity was determined at an E/T ratio of 10:1. (b) Effect of concentration of HY peptide on cytotoxicity of CTLs. Cytotoxicity of CTLs to autologous DCs loaded with various concentration of HY peptide was tested. (c) Antibody blockade of cytotoxicity against autologous DCs pulsed with HY peptide. Polyclonal antibodies (control), anti-class I MoAb or an anti-class II MoAb were added to cultures for testing blockade of cytotoxicity. (d) Cytotoxicity of HY peptide for 6 h. HY-specific CTLs against LCL cells of unrelated males who share HLA-A2 (A*0201).

CD31, CD49b and CD62L,[15,16] between the donor and the recipient. Tetramer staining demonstrated the expansion of HY peptide-specific T cells from undetectable prior to PBSCT to 15.9% of the circulating CD8$^+$ T cells 12 weeks after PBSCT (Figure 2a). Thereafter, frequencies of HY tetramer-positive T cells declined and disappeared from the PB, coinciding with molecular relapse. The *in vitro*-generated HY-specific CTLs were 90% HY tetramer-positive CD8$^+$ T cells (Figure 2b).

Detection of HY-reactive CD8$^+$ T cells by intracellular cytokine assessment

To demonstrate the HY peptide-driven expansion of CD8$^+$ T cells, we assessed intracellular accumulation of IFNγ in PB CD8$^+$ T cells by flow cytometry. At 12 weeks after PBSCT, 6.8% of PB CD8$^+$ T cells produced intracellular IFNγ in response to HY peptide-pulsed autologous LCL cells (Figure 3a), while IFNγ production was negligible in CD8$^+$ T cells in response to autologous LCL cells without the HY peptide (Figure 3b). These findings indicate that *in vivo* expansion and activation of HY peptide-reactive T cells occurred after the second transplantation. At that time, the proportion of circulating CD8$^+$ cells positive for HY-tetramer staining was 15.9% as show in Figure 2a. Although inducibility of IFNγ in HY-tetramer-positive CD8$^+$ T cells was not examined, there could be overlapping between CD8$^+$ T cells producing intracellular IFNγ in response to HY peptide and CD8$^+$ T cells stained with HY tetramer, because HY-tetramer staining must detect functional T cells reactive with HY peptide.

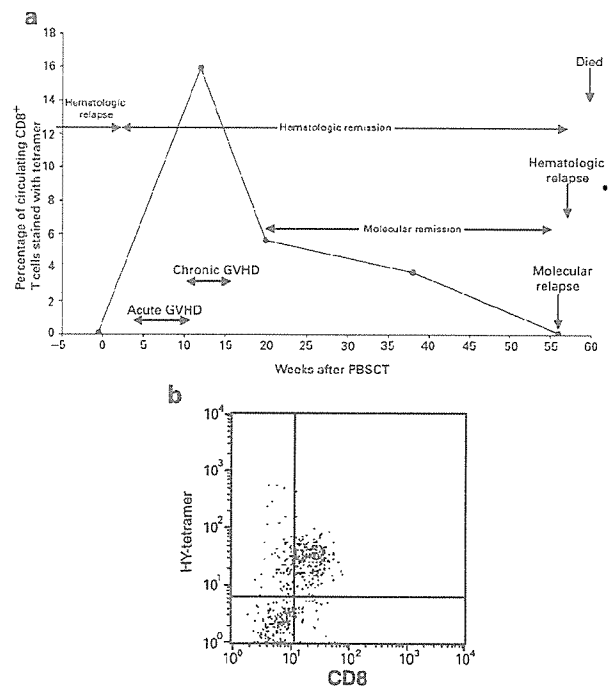

Figure 2 Monitoring and generation of HY-specific CD8$^+$ T cells. (a) Correlation between frequency of circulating HY-tetramer-positive CD8$^+$ T cells and clinical events. (b) HY-tetramer staining of the *in vitro*-generated HY-specific T cells, showing FITC-conjugated anti-CD8 antibody (*x*-axis) and PE-conjugated HY-tetramers (*y*-axis). Appropriate gates were set on vital lymphocytes according to their typical forward- and side-scattering characteristics.

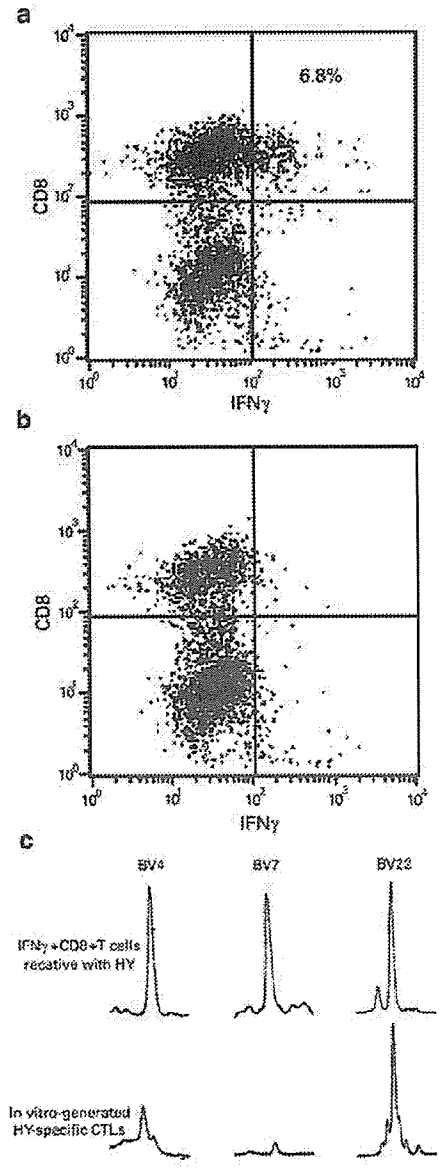

Figure 3 HY-specific IFNγ-producing CD8$^+$ T cells in PB. (a) PB CD8$^+$ T cells were incubated with autologous LCL cells pulsed with after fixation and permeabilization, the cells were stained for CD8 and IFNγ. The frequency of CD8$^+$ T cells producing IFNγ in response to HY peptide is shown as a percentage of total CD8$^+$ cells. (b) IFNγ production was negligible in CD8$^+$ T cells stimulated by autologous LCL cells not pulsed with the HY peptide. (c) CD8$^+$ IFNγ^+ T cells detected in Figure 3a were selected by fluorescence-activated cell sorting, and spectratyping was performed. CDR3 sizes of TCR BV subfamilies from CD8$^+$ IFNγ^+ T cells are shown together with the data from *in vitro*-generated HY-specific CTLs.

CDR3 size distribution of TCR BV cDNA of HY-specific T cells

Spectratyping of the TCR BV region was performed on *in vitro*-generated HY-specific CTLs and PB CD8$^+$ T cells that were stained with intracellular IFNγ MoAb in response to HY peptide stimulation and sorted as shown in Figure 3a. PB HY-reactive CD8$^+$ cells showed prominent skewing within BV4, BV7, BV22 and BV24, and the *in vitro*-generated HY-specific CTLs showed skewing within BV4, BV7, BV12, BV16 and BV22. The two T-cell populations shared the usage of BV4, BV7 and BV22 (Figure 3c), but only the BV22$^+$ T cells from *in vitro*-generated HY-specific CTLs and HY-responsive PB CD8$^+$ T cells had a similar peak with the same CDR3 size distribution.

Deduced amino-acid sequence of CDR3 of BV22 cDNA

To determine if HY-specific CTLs circulate in the patient, we subcloned the amplified cDNA of the *in vitro*-generated BV22$^+$ CTLs and BV22$^+$ T cells that produced intracellular IFNγ in response to HY peptide stimulation as shown in Figure 3a and determined the CDR3 sequence (Table 1). One of three N-D-N sequences of HY-specific CD8$^+$ cells from PB was identical to one of those of the CTLs. These findings indicate that the same cells isolated from the donor are expanding *in vivo*, and suggest that HY-specific CD8$^+$ T cells in PB of the patient have cytotoxic activity against leukemic cells.

Discussion

In mHAs HA-1- and HA-2-matched stem cell transfer between a female donor and a male recipient, we have observed the emergence of HY peptide-specific CTLs that result in the durable remission of relapsed leukemia. The present study utilized tetramer staining to show that HLA-A*0201-restricted HY peptide-specific CD8$^+$ T cells were present in the PB after the development of GVHD and led to the eradication of BCR/ABL transcript-positive leukemic cells. Further, these cells disappeared upon molecular relapse of disease. Assaying for the frequency IFNγ-producing cells by intracellular cytokine staining, we demonstrated the emergence of functional T cells in PB that were reactive with the HY peptide during the presence of HY tetramer$^+$ T cells. The data imply that HY-specific CTLs may have therapeutic potential as adoptive immunotherapy for relapsed leukemia after allogeneic SCT.

In this patient, leukemic relapse had occurred as full-blown disease of CML in lymphoid blast crisis. Although a beneficial effect of donor immunity was expected in the control of leukemic relapse, it would take months to start to work. Thus, we had chosen multiple transplants instead of donor leukocyte infusion (DLI) to reduce leukemic cells sufficiently.

Recently in mHAs HA-1 and/or HA-2 incompatible donor–recipient pairs an association between the emergence of HA-1 or HA-2 tetramer-positive CTLs and the complete disappearance of BCR/ABL$^+$ cells or of myeloma cells was reported.[17] Of the three reported patients, one who underwent female-to-male transplantation experienced an increase in HLA-B7-restricted HY-specific T cells as well as an increase in HA-2-specific T cells, but not that of

Table 1 Junctional amino-acid sequence of TCR BV22 of HY-specific T cells

V	*N-D-N*	*J*	*BJ family*
(a) BV22$^+$ T cells from in vitro-generated HY-specific CTLs			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	REGGRS	GYTFCSGTKLTV	1.2
CASS	KQKGNPPPI	SPLHFGNGTRLTVT	1.6
(b) Functional BV22$^+$ T cells from HY-reactive CTLs circulating in PB			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	RQSQGS	GYTFCSGTKLTV	1.2
CASS	RQGRGVSEF	SPLHFGNGTRLTVT	1.6

HLA-A2-restricted HY-specific T cells. However, the cell population(s) contributing to the GVL effect were unable to be identified. It is worth noting that this reported case with lymphoid blast crisis of BCR/ABL transcript-positive CML obtained molecular remission after the development of GVHD and the emergence of HY-specific CTLs as seen in the present case.

The *in vitro*-generated HY peptide-specific CTLs efficiently lysed leukemic cells of the patient, and were also to a lesser extent cytotoxic to the nonhematopoietic cells such as fibroblasts of the patient. Gratwohl *et al*[18] reported that male recipients with CML of female blood or marrow stem cell grafts are at a high risk of GVHD, but benefit from reduced incidence of disease recurrence. These findings provide evidence that HY-specific CTLs may be commonly induced in male patients given a stem cell graft from a female donor, leading to the development of GVL reactions and GVHD. This implies that the availability of a female blood or marrow graft may be beneficial to a leukemic male recipient at high risk of relapse.

In contrast to the ubiquitous expression of HY, HA-1 and HA-2 are exclusively expressed on hematopoietic cells.[1] *In vitro*-generated HA-1- and HA-2-specific CTLs specifically lyse leukemic cells, but not nonhematopoietic cells in a ^{51}Cr release assay.[1,19] Thus, upon HA-1- or HA-2-mismatched SCT and adoptive immunotherapy such as DLI, a low risk of GVHD would be expected. However, HA-1 disparity between a patient and a donor has been associated with the development of GVHD without reducing a rate of relapse.[15,20] Marijt *et al*[17] demonstrated the emergence of HA-1- and HA-2-specific CD8$^+$ T cells in PB of three patients after DLI preceding complete remission of relapsed leukemia. Relapse was associated with the development of GVHD in all three patients. A recent report showed that GVHD does not require alloantigen expression on host epithelium, and its development is primarily mediated by inflammatory cytokines such as TNFα and IL-1.[21] This may account for discrepancies between *in vitro* behavior of HA-1- and HA-2-specific CTLs and clinical observations. Based on these findings, we believe that in mHA-oriented allogeneic immunotherapy the ability of mHAs to induce powerful immune reactions is more important than restriction of mHAs to hematopoietic tissue, and so far it appears that GVHD is an inevitable consequence. In the future, selective blockade of cytokines mediating GVHD[21] may be a strategy to preserve GVL, while reducing toxicity of GVHD after mHA-oriented immunotherapy.

TCR BV spectratyping showed a similar peak with same size in a BV22$^+$ family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific CD8$^+$ T cells, and one shared N-D-N sequence. These findings suggest the expansion of a functional T-cell clone that participates in eradicating lymphoblastic leukemia cells positive for BCR/ABL transcripts, although we were not able to provide direct evidence demonstrating antileukemic activity of HY-specific CD8$^+$ T cells taken from PB of the patient. It would have been beneficial to sort the HY-tetramer-positive cells detectable in PB of the patient, to expand these cells using HY peptide-pulsed LCL cells, and to test their cytotoxicity against the leukemic targets. Restricted TCR BV usage for HA-1-specific CTLs has also been described.[7] Spectratyping could be beneficial in monitoring HY-specific CTLs *in vivo*, because spectratype analysis is more sensitive than tetramer analyses, and can be performed using as little as 500 cells.[6]

Compared with tetramer staining, a flow cytometric assay assessing intracellular IFNγ levels can be used to screen a large number of allogeneic peptides with a relatively little effort. In allogeneic SCT, this approach should be useful for initial screening of candidates for mHAs derived from polymorphic cellular proteins. Moreover, intracellular IFNγ assessment between CD8$^+$ T cells during the GVL effect or GVHD as a responder and hematopoietic cells or nonhematopoietic cells of a host as a stimulator may enable the detection of undefined mHAs-specific CTLs. As the IFNγ capture assay enables isolation of live T cells stained for surface-associated IFNγ,[22] further studies with regard to the function of responding effector T cells could elucidate their putative target antigens.

Another advantage of the flow cytometric cytokine production assay is that it is possible to assess the production of multiple cytokines on an antigen-specific, single-cell basis. It has already been demonstrated by Nazaruk *et al*[23] that a subset of EBV-specific CD8$^+$ T-cell lines produce IL-4 or IL-13 in addition to IFNγ upon stimulation with phorbol myristate acetate and ionomycin. Such a technique could be utilized in determining the cytokine production capabilities of mHA-specific CTLs in PBSCT.

In conclusion, the present data provide evidence that the emergence and activation of HY-specific CD8$^+$ T cells may participate in eradicating lymphoblastic leukemic cells. This implies that *in vitro*-generated HY-specific CTLs may have therapeutic potential for relapsed leukemia after allogeneic SCT.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare. We wish to thank Megumi Yoshii for the excellent technical assistance as well as her patience in preparation of the manuscript.

References

1 Mutis T, Goulmy E. Hematopoietic system-specific antigens as targets for cellular immunotherapy of hematological malignancies. *Semin Hematol* 2002; **39**: 23–31.
2 Mutis T, Gillespie G, Schrama E *et al.* Tetrameric HLA class I-minor histocompatibilty antigen peptide complexes demonstrate minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease. *Nat Med* 1999; **5**: 839–842.
3 Kern F, Surel IP, Brock C *et al.* T-cell epitope mapping by flow cytometry. *Nat Med* 1998; **4**: 975–978.
4 Kuzushima K, Hoshino Y, Fujii K *et al.* Rapid determination of Epstein–Barr virus-specific $CD8^+$ T-cell frequencies by flow cytometry. *Blood* 1999; **94**: 3094–3100.
5 Meadows L, Wang W, den Haan JM *et al.* The HLA-A*0201-restricted H-Y antigen contains a post translationally modified cysteine that significantly affects T cell recognition. *Immunity* 1997; **6**: 273–281.
6 Kondo Y, Shiobara S, Nakao S. Identification of T-cell clones showing expansion associated with graft-versus-leukemia effect on chronic myelogenous leukemia *in vivo* and *in vitro*. *Exp Hematol* 2001; **29**: 471–476.
7 Verdijk RM, Mutis T, Wilke M *et al.* Exclusive TCRVbeta chain usage of *ex vivo* generated minor histocompatibility antigen HA-1 specific cytotoxic T cells: implications for monitoring of immunotherapy of leukemia by TCRBV spectratyping. *Hematol J* 2002; **3**: 271–275.
8 Kuzushima K, Hayashi N, Kudoh A *et al.* Tetramer-assisted identification and characterization of epitopes recognized by HLA A*2402-restricted Epstein–Barr virus-specific $CD8^+$ T cells. *Blood* 2003; **101**: 146–1468.
9 Redchenko IV, Rickinson AB. Accessing Epstein–Barr virus-specific T-cell memory with peptide-loaded dendritic cells. *J Virol* 1999; **73**: 334–342.
10 Nakao S, Takami A, Takamatsu H *et al.* Isolation of a T-cell clone showing HLA-DRB1*0405-restricted cytotoxicity for hematopoietic cells in a patient with aplastic anemia. *Blood* 1997; **89**: 3691–3699.
11 Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate phenol–chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
12 Currier JR, Deulofeut H, Barron KS, Kehn PJ, Robinson MA. Mitogens, superantigens, and nominal antigens elicit distinctive patterns of TCRB CDR3 diversity. *Hum Immunol* 1996; **48**: 39–51.
13 Waase I, Kayser C, Carlson PJ *et al.* Oligoclonal T cell proliferation in patients with rheumatoid arthritis and their unaffected siblings. *Arthritis Rheum* 1996; **39**: 904–914.
14 Casanova JL, Pannetier C, Jaulin C, Kourilsky P. Optimal conditions for directly sequencing double-stranded PCR products with sequenase. *Nucleic Acids Res* 1990; **18**: 4028.
15 Goulmy E, Schipper R, Pool J *et al.* Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med* 1996; **334**: 281–285.
16 Maruya E, Saji H, Seki S *et al.* Evidence that CD31, CD49b, and CD62L are immunodominant minor histocompatibility antigens in HLA identical sibling bone marrow transplants. *Blood* 1998; **92**: 2169–2176.
17 Marijt WA, Heemskerk MH, Kloosterboer FM *et al.* Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci USA* 2003; **100**: 2742–2747.
18 Gratwohl A, Hermans J, Niederwieser D *et al.* Female donors influence transplant-related mortality and relapse incidence in male recipients of sibling blood and marrow transplants. *Hematol J* 2002; **2**: 363–370.
19 Mutis T, Verdijk R, Schrama E *et al.* Feasibility of immunotherapy of relapsed leukemia with *ex vivo*-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood* 1999; **93**: 2336–2341.
20 Gallardo D, Arostegui JI, Balas A *et al.* Disparity for the minor histocompatibility antigen HA-1 is associated with an increased risk of acute graft-versus-host disease (GvHD) but it does not affect chronic GvHD incidence, disease-free survival or overall survival after allogeneic human leucocyte antigen-identical sibling donor transplantation. *Br J Haematol* 2001; **114**: 931–936.
21 Teshima T, Ordemann R, Reddy P *et al.* Acute graft-versus-host disease does not require alloantigen expression on host epithelium. *Nat Med* 2002; **8**: 575–581.
22 Becker C, Pohla H, Frankenberger B *et al.* Adoptive tumor therapy with T lymphocytes enriched through an IFN-gamma capture assay. *Nat Med* 2001; **7**: 1159–1162.
23 Nazaruk RA, Rochford R, Hobbs MV, Cannon MJ. Functional diversity of the $CD8^+$ T-cell response to Epstein–Barr virus (EBV): implications for the pathogenesis of EBV-associated lymphoproliferative disorders. *Blood* 1998; **91**: 3875–3883.

Polyclonal hematopoiesis maintained in patients with bone marrow failure harboring a minor population of paroxysmal nocturnal hemoglobinuria–type cells

Ken Ishiyama, Tatsuya Chuhjo, Hongbo Wang, Akihiro Yachie, Mitsuhiro Omine, and Shinji Nakao

Although a minor population of paroxysmal nocturnal hemoglobinuria (PNH)–type blood cells is often detected in patients with aplastic anemia (AA) and refractory anemia (RA), the significance of such cells in the pathophysiology of bone marrow (BM) failure remains obscure. We therefore examined clonality in peripheral blood granulocytes from 118 female patients with AA or myelodysplastic syndrome using the X chromosome inactivation pattern. Clonality, defined as a clonal population accounting for 35% or more of total granulocytes, was confirmed in 22 of 68 (32.4%) AA patients, in 13 of 44 (29.5%) RA patients, in all 4 RA with excess blasts (RAEB) patients, and in 4 patients with PNH. When the frequency of patients with granulocyte clonality was compared with respect to the presence of increased PNH-type cells, the frequency was significantly lower in AA patients with (PNH^+; 21.2%) than without (PNH^-; 42.9%) increased numbers of PNH-type cells ($P = .049$). Clonality was absent in granulocytes from the 15 PNH^+ RA patients but present in 13 of 29 (44.8%) PNH^- RA patients ($P = .0013$). The absence of clonality in AA and RA patients before treatment was strongly associated with positive response to immunosuppressive therapy (without clonality, 74.4%; with clonality, 33.3%; $P = .0031$) in all patients as well as in PNH^+ patients (without clonality, 96.2%; with clonality, 66.6%, $P = .026$). These results suggest that AA and RA with a minor population of PNH-type cells are benign types of BM failure with immune pathophysiology that have little relationship to clonal disorders such as RAEB or acute myeloid leukemia. (Blood. 2003;102:1211-1216)

Introduction

Aplastic anemia (AA) and myelodysplastic syndrome (MDS) are hematopoietic dyscrasias characterized by pancytopenia and inappropriate production of mature blood cells from the bone marrow. They differ in terms of disease definition: AA is basically benign bone marrow (BM) failure due to extrinsic insult to hematopoietic stem cells, while MDS is a clonal disorder derived from a defective stem cell.[1-3] However, to differentiate AA from refractory anemia (RA) of MDS in clinical practice can be difficult, as a diagnosis of RA depends largely on a subjective judgment of morphologic abnormalities in mature blood cells, and a laboratory marker that can discriminate between them remains unknown.[4,5]

We recently demonstrated that a minor (< 1%) population of $CD55^-CD59^-$ granulocytes or red blood cells (RBCs) can be detected in numerous AA patients[6] and in about 20% of RA patients.[7] RA patients with a subtle increase in such paroxysmal nocturnal hemoglobinuria (PNH)–type cells (PNH^+ patients) had distinct clinical features compared with RA patients without increased PNH-type cells (PNH^- patients), such as lower rates of karyotypic abnormality and higher probability of response to cyclosporine (CyA) therapy. The presence of PNH-type cells therefore appeared to represent a marker for benign types of BM failure. However, several studies contradict this hypothesis. Some reports described AA patients with PNH clones who later developed acute myeloid leukemia (AML) with the non-PNH phenotype.[8,9] According to another report, a non-PNH stem cell developed into MDS in a PNH patient.[10] These case reports suggest that non-PNH stem cells tend to mutate in patients with BM failure who have an increased number of PNH-type cells. On the other hand, Maciejewski et al detected karyotypic abnormalities at similar frequencies in patients with PNH^+ BM failure and in those without PNH-type cells, although their definition of an increase in PNH-type cells differed from ours.[11] Whether a minor population of PNH-type cells actually represents a benign pathophysiology in BM failure therefore remains unclear.

One method to assess the pathophysiology of marrow failure is to determine whether the patient has a clonal population in granulocytes.[12-16] If a PNH^- AA or RA patient has a pathophysiology similar to that of RA with excess of blasts (RAEB) or will tend to evolve into RAEB or AML, complete or partial clonal granulopoiesis should be detected by recognizing skewed inactivation of the human androgen receptor (HUMARA) gene.[17-19] Whereas clonal hematopoiesis is considered to reflect not only the presence of defective stem cells, but also hematopoietic stem cell depletion,[20-22] it has predicted evolution to RAEB or AML in some AA patients.[23,24] If an increase of PNH-type cells in patients with AA or RA represents a benign type of BM failure, then clonal granulopoiesis may be less frequent in PNH^+ than in PNH^- patients.

In order to test these hypotheses, we first investigated the presence of clonality in granulocytes from patients with BM failure and then analyzed the relationship between clonality and increases

From Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science; the Preventive Environment Unit, Kanazawa University Hospital; the School of Health Sciences, Faculty of Medicine, Kanazawa University, Ishikawa; and the Division of Hematology, Fujigaoka Hospital, Showa University School of Medicine, Yokohama, Kanagawa, Japan.

Submitted December 5, 2002; accepted March 25, 2003. Prepublished online as *Blood* First Edition Paper, April 3, 2003; DOI 10.1182/blood-2002-12-3706.

Reprints: Shinji Nakao, Cellular Transplantation Biology, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan; e-mail: snakao@med3.m.kanazawa-u.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

in PNH-type cells. We used an improved HUMARA assay with a capillary sequencer that could reliably detect clonal populations at a ratio of 35% or more. This study revealed a lower incidence of clonal hematopoiesis in PNH^+ patients than in PNH^- patients and a significant association between the absence of clonality with a favorable response to immunosuppressive therapy (IST). These data supported our hypothesis that AA and RA harboring a minor population of PNH-type cells represent benign types of BM failure with immune pathophysiology.

Patients, materials, and methods

Study subjects

We retrospectively analyzed 68 patients with acquired AA and 44 with RA who were heterozygous for the HUMARA gene. Table 1 summarizes patient characteristics. At least 2 independent hematologists reviewed all bone marrow slides and diagnosed only patients who had greater than 1% neutrophils with the Pseudo-Pelger-Hüet anomaly and who had micromegakaryocytes in addition to signs of erythroid dysplasia such as megaloblastic changes and multinuclearity as having RA.[3,7] Among 50 AA patients and 27 RA patients examined, karyotypic abnormalities were seen in one AA patient (46, XX,9q+,9q−) and in 6 RA patients (1 with 47, XX, +8; 1 with 47, XX, +8, $inv^9[11q13]$; 2 with 46, XX, $del^5[q13q31]$; 1 with 45, X, [−X]; 1 with 46, XX, $dup^1[q32q12]$). Included as controls were 4 patients with RAEB, 4 with PNH, 4 with AML, and 111 healthy females aged 21 to 98 who were all heterozygous for the HUMARA gene. All participants provided written, informed consent to all procedures associated with the study, which was approved by the ethical committee at our institution (application no. 225 entitled "Detection of clonal hematopoiesis in bone marrow failure patients by utilizing the X chromosome inactivation pattern").

Sample preparation

Obtained from each patient was 2 to 10 milliliters heparinized peripheral blood, which was mixed with saline containing 2% dextran. After sedimentation, RBC-poor plasma was collected and sedimented using Ficoll-Hypaque (Lymphoprep; Axis-shield, Oslo, Norway). Granulocytes were collected from the pellet after lysing RBCs. Mononuclear cells (MNCs) were removed from the interface layer, suspended in RPMI 1640, and incubated for 1 hour in a CO_2 incubator at 37°C in plastic culture plates. Nonadherent cells were collected, and T cells were separated by rosette formation with sheep RBCs. The purity of each cell fraction tested using Cytospin preparations was greater than 95%. We extracted DNA using the GENERATION DNA extraction kit (Gentra Systems, Minneapolis, MN). X chromosome inactivation patterns (XCIP) were analyzed after digesting DNA (50 ng) from granulocytes and T lymphocytes with the methylation-sensitive endonuclease *Hha*I (Takara, Kyoto, Japan) before amplification.

Amplification of the HUMARA gene and assessment of clonality

The HUMARA gene was amplified as described by Karasawa et al with some modifications.[25] Since part of the HUMARA gene assessed contained a GC-rich region, we used the GC-RICH PCR System (Roche Diagnostics, Indianapolis, IN) to avoid misreading the nucleotide sequence during amplification. We amplified 20 ng DNA in a 20-μL reaction mixture according to the manufacturer's recommendations. The forward primer was 5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′, and the reverse primer was 5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′. The 5′ end of the forward primer was labeled with a 6-FAM fluorescent dye (Applied Biosystems, Foster City, CA). Following denaturation for 5 minutes at 94°C, samples were amplified for 35 cycles (denaturation, 94°C for 1 minute; annealing, 58°C for 1 minute; extension, 72°C for 1 minute) using a Touchdown thermal cycler (Hybaid Limited, Ashford, United Kingdom). A final extension (72°C for 10 minutes) was added after the amplification cycles. The amplified products (0.15-0.5 μL) were mixed with 0.2 μL of the internal size standard Gene-Scan 500 ROX and 12 μL formamide (Applied Biosystems) and denatured at 95°C for 2 minutes. After cooling on ice, samples were separated by capillary electrophoresis using POP-4 polymer on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and analyzed with GeneScan software (Applied Biosystems).

To correct an inequality of amplification efficiency between the 2 HUMARA gene alleles, we determined the ratios of both allele areas before (lower allele/higher allele: A/B) and after (lower allele/higher allele: A′/B′) *Hha*I digestion using a C value calculated by (A/B)÷(A′/B′) as a marker of skewing in granulocytes (C_G) and T lymphocytes (C_L).[18,26-28] Skewing was judged evident when the C value was far from 1.0 or when 1 of 2 alleles completely disappeared after *Hha*I digestion.

Flow cytometry

To detect PNH-type granulocytes, phycoerythrin (PE)–labeled anti-CD11b monoclonal antibodies (MoAb, Becton Dickinson, Mountain View, CA), fluorescein isothiocyanate (FITC)–labeled anti-CD55 (clone IA10, mouse IgG2a, Pharmingen, San Diego, CA), and FITC-labeled anti-CD59 (clone p282, mouse IgG2a, Pharmingen) were combined with isotype-matched control MoAbs as described.[6] To analyze PNH-type RBCs, we used PE-labeled anti–glycophorin A MoAb (clone JC159, DAKO, Glostrup, Denmark) instead of anti-CD11b MoAb.[7] Fresh peripheral blood was diluted to 3% with phosphate-buffered saline, and 50 mL diluted blood was incubated with PE-labeled anti–glycophorin A MoAb, FITC-labeled anti-CD55, and anti-CD59 MoAb on ice for 25 minutes. A total of at least 10^5 $CD11b^+$ granulocytes and glycophorin A^+ RBCs within each corresponding gate were analyzed using a FACScan flow cytometry (Becton Dickinson). Based on the analytical results from 68 healthy individuals, the presence of more than 0.003% $CD11b^+$ granulocytes and 0.005% glycophorin A^+ RBCs was judged abnormal.[6,7]

Southern blotting

We performed Southern blotting on the DNA of T cells with extremely skewed XCIP using a T-cell–receptor β chain probe as described.[29]

Immunosuppressive therapy (IST)

Antithymocyte globulin (ATG; Lymphoglobulin, Pasteur Mérieux, Lyon, France; 15 mg/kg/d, 5 days) in combination with cyclosporine A (CyA, Novartis, 6 mg/kg/d) was administered to 29 patients (28 with AA and 1 with RA), whereas CyA alone was administered to 28 patients (14 with AA and 14 with RA), respectively. Response was evaluated at 6 months after therapy. Response criteria included resolution of requirement for transfusions and a 2 g/dL or more rise of hemoglobin levels.

Statistics

Differences in clinical characteristics between PNH^+ and PNH^- patients and relationships between the presence of clonality and an increase in the percentage of PNH-type cells were assessed using Mann-Whitney *U* test and Fisher exact probability test or the chi-square test. A *P* value of less than .05 was considered statistically significant.

Table 1. Patient characteristics

Diagnosis	Number of patients	Age, y (median)
Aplastic anemia	68	12-86 (53)
Moderate	42	12-77 (46)
Severe	26	23-86 (62)
Myelodysplastic syndrome	48	17-88 (70)
Refractory anemia	44	17-88 (71)
Refractory anemia with excess of blasts	4	37-78 (65)
Acute myeloid leukemia	4	29-65 (46)
Paroxysmal nocturnal hemoglobinuria	4	37-51 (46)

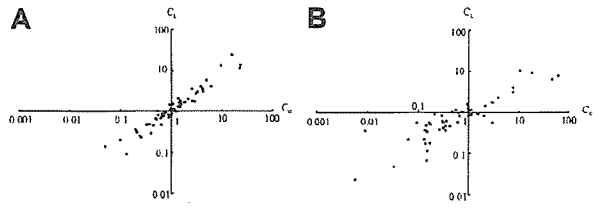

Figure 1. HUMARA gene analysis and its interpretation. Correlations between C_G and C_L in healthy younger women (A, 20-69 years old) and elderly women (B, 70-98 years old). Correlation coefficient was 0.862 in younger women and 0.741 in elderly women.

Results

Criteria for a diagnosis of clonality

To estimate constitutional and age-related skewing of XCIP in granulocytes and T lymphocytes, C_G and C_L values were plotted for healthy individuals. Figures 1A-B show correlations between C_G and C_L in younger women (A, age < 70 years) and in elderly women (B, age ≥ 70 years). In the younger women, C_G correlated well with C_L, indicating that constitutional and age-related skewing of XCIP in granulocytes occurs in parallel with that in T cells. Therefore, the influence of physiological and age-related skewing on the assessment of clonality in granulocytes was considered offset by using the absolute values of log (C_G/C_L) (S value) as a marker of clonality in granulopoiesis. S values of most younger women fell into the range 0-0.30, while those of most elderly women (age ≥ 70 years) fell into a larger range (0-0.40). Due to acquired skewing associated with age,[17,30-33] normal ranges of S values were established for each of the 2 age groups: the normal range of S values was arbitrarily defined as below 0.3 for the younger women, which included S values from 95% of healthy individuals. In elderly women, the normal range was defined as below 0.4, which included S values from 90% of healthy individuals.

The C_L values of 4 of the 111 healthy individuals were extremely high (> 10) or low (< 0.1). To exclude the possibility that acquired skewing of XCIP was due to the clonal proliferation of mature T cells, we performed Southern blotting on T cells from these patients using a T-cell–receptor β chain gene probe. Results from all 4 samples were negative for rearrangement bands (data not shown).

Validity and sensitivity of the HUMARA assay in detecting clonal populations

Figure 2A shows an apparent clonal pattern of leukemic cells from an AML patient. The S value of granulocytes obtained at remission was normal (0.149), indicating that polyclonal granulopoiesis had been restored by chemotherapy. We tested the sensitivity of the HUMARA assay in detecting a clonal population as follows: We mixed DNA derived from leukemic cells of the patients whose allele B'_{Blast} completely disappeared after *Hha*I digestion with various ratios of DNA derived from granulocytes at remission and determined the C_G values using the HUMARA assay. Assuming that the C_L values of the patient were identical before and after chemotherapy, the $|\log C_G|$ value would be identical to the S value. The C_G/C_L values obtained from each mixture accorded well with predicted C_G/C_L values. According to this nomogram, a C_G/C_L value of 0.5 (= 0.3 as an S value) in one patient indicated the presence of about a 35% clonal population, and a C_G/C_L value of 0.4 (= 0.4 as an S value) in another corresponded to a clonal population of approximately 45%.

Clonality in patients with BM failure

Table 2 summarizes results of the HUMARA assay of samples from patients with BM failure. Patients were divided into 2 groups according to age, and S values were compared with the normal range of each group. We detected abnormal S values in 22 of 68 (32.4%) AA patients and in 13 of 44 (29.5%) RA patients. The S values of granulocytes from all RAEB and PNH patients were abnormal. These results indicated that the incidence of clonal hematopoiesis was similar between AA and RA patients. All 7 patients (1 with AA and 6 with RA) with karyotypic abnormalities were judged to have clonality from abnormal S values ranging from 0.526 to 1.231 in 6 patients and a complete loss of one allele after *Hha*I digestion in one patient.

Ratios of PNH-type cells in AA and RA patients

The results of flow cytometry detecting $CD55^-CD59^-$ granulocytes are summarized in Figure 3. The number of $CD55^-CD59^-CD11b^+$ granulocytes was increased (≥ 0.003%) in 33 of 68 (48.5%) AA patients, whereas that of PNH-type granulocytes was increased in 15 of 44 (34.1%) RA patients ($P = .094$). All PNH^+ patients harbored 0.005% or more $CD55^-CD59^-$ glycophorin$^+$ RBCs (data not shown). PNH^- patients included most patients with abnormal S values, indicated as open circles. There were no significant differences in the disease duration (months [median], 0-22.4 [0.4] vs 0-226 [0.4] in AA and 0-84 [13.7] vs 0-182.3 [13.4] in RA) and neutrophil count ($\times 10^9$/L [median], 0.02-2.6 (0.69) vs 0.02-2.2 [1.0] in AA and 0.24-1.5 [1.0] vs 0.23-2.6 [1.28] in RA) between PNH^+ and PNH^- patients. All 7 patients with karyotypic abnormalities were PNH^-.

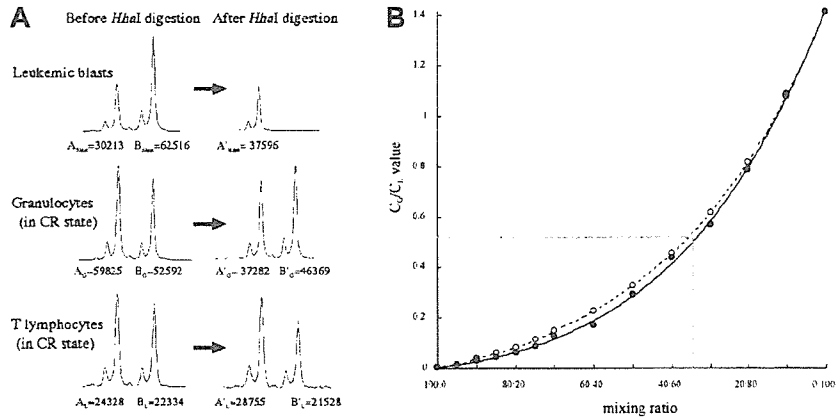

Figure 2. Sensitivity and accuracy of HUMARA assay in detecting clonal populations. Leukemic cell DNA from an AML patient (A, M5a) was mixed with DNA from mature granulocytes from the same patient at varying ratios, and C_G/C_L values were determined in each mixture. Closed circles indicate calculated C_G/C_L values; open circles, predicted C_G/C_L values.

Table 2. Percentage of patients showing abnormal S values

Diagnosis	Younger than 70 years		70 years or older	
AA	18 of 51	(35%)	4 of 17	(24%)
MDS-RA	4 of 22	(18%)	9 of 22	(41%)
MDS-RAEB	2 of 2	(100%)	2 of 2	(100%)
PNH	4 of 4	(100%)	—	

— indicates not applicable.

S values in subsets of patients defined by the presence of a few PNH-type cells

Figure 4 shows the distribution of S values among patient groups that were classified according to the presence of PNH-type cells. S values were abnormal in 3 of 19 (15.8%) and in 15 of 32 (46.9%) of the younger PNH^+ and PNH^- AA patients, respectively ($P = .023$, Figure 4A). A difference in the ratio of PNH^+ and PNH^- patients with abnormal S values also was evident in RA patients. None of 10 PNH^+ and 4 of 12 (33.3%) PNH^- patients displayed abnormal S values ($P = .044$). The difference in the frequency of patients with abnormal S values between PNH^+ and PNH^- elderly patients also was significant in RA ($P = .034$), but not in AA ($P = .29$, Figure 4B). For all patients in the 2 age groups, the frequency of abnormal S values in those who were PNH^+ was significantly lower than those who were PNH^- both in the AA (21.2% vs 42.9%, $P = .049$) and RA (0% vs 44.8%, $P = .0013$) groups.

Rates of response to immunosuppressive therapy and evolution into AML

A total of 57 patients (42 with AA and 15 with RA) were treated with ATG + CyA or CyA after assessment of clonality and PNH-type cells. Figure 5 summarizes the rates of response to IST in various subsets of patients. The absence of clonality was significantly associated with favorable response to ATG + CyA (83.3% vs 36.4%). The same trend also was seen in the rate of response to CyA therapy (66.7% vs 28.6%). Among PNH^+ patients, almost all patients without clonality responded to IST, whereas only two thirds of patients with clonality responded, as shown in Figure 5B. PNH^- patients without clonality also showed a better response to IST than those with clonality, although the difference was not statistically significant.

Over the 23-month observation period, none of the 77 AA or RA patients without clonality developed AML, whereas 5 of 35 patients with clonality developed AML 5 to 14 months after examination of clonality. All evolved patients were PNH^-.

Changes in S values over time after ATG therapy in AA patients

We examined clonality in the peripheral blood of 6 AA patients before and after ATG therapy. Of 2 patients with abnormal S values, one attained partial remission, whereas the other was unresponsive to therapy. Their S values remained unchanged over time. Among 4 patients with normal S values, 3 responded to ATG and became free from the need for transfusions for 3-4 months after therapy. S values remained within the normal range in 2 of the 3 responders and surpassed the normal range in one patient 3 months after therapy. In the other patient who was refractory to ATG, the S values remained normal.

Discussion

Clonality analysis using XCIP of the HUMARA gene has been applied to characterize hematopoiesis of various hematologic diseases.[18,19,34-38] HUMARA assays used in previous reports[16,19,30-36,39,40] could not assess clonality when the T-cell population was obviously skewed, since this cell type represents an internal control for assessing clonality in granulocytes. Accurate measurement of the allelic quantity using our HUMARA assay allowed this problem to be circumvented. Skewing of XCIP in the HUMARA gene is age related in T cells in association with that in granulocytes.[17,30-33] Our results from healthy individuals supported these findings (Figure 1). We therefore assumed that the C_G value relative to the C_L value could serve as a marker for skewing in granulocytes. When the $|\log(C_G/C_L)|$ (S) values of patients with RAEB or PNH were determined and compared with those of age-matched healthy controls, all results were positive, thus validating the use of S values as a marker for clonality. The HUMARA assay reliably detected a clonal population of 35% or more in 32.4% of AA patients and in 29.5% of RA patients. These ratios were lower[13-15,24] and higher[12,38-41] than those of some previous studies, all of which used inaccurate methods to assess skewing. Studies in which the ratios were lower or higher probably overestimated the incidence of clonality due to a lack of appropriate controls, and underestimated the incidence due to the inability of insensitive methods to detect relatively low ratios of clonal populations.

Several studies have emphasized the importance of longitudinal analysis when clonality is assessed because XCIP of granulocytes can vary from time to time[20] and may be affected by IST.[12] However, a recent study of elderly individuals presents evidence against such variance of XCIP over time.[33] Our results also indicated that the skewing of XCIP in granulocytes is relatively stable over time, even in patients whose hematopoietic function improved with ATG. The numbers and types of hematopoietic stem cells contributing to hematopoiesis may not change easily if assessed by the improved HUMARA assay. These results appear to justify our analysis of patients with heterogeneous backgrounds regarding disease duration and treatment.

We recently demonstrated that the clinical features of RA with a minor population of PNH-type cells are benign, like those of AA.[7] However, the quality of hematopoietic stem cells in patients with BM failure harboring PNH-type cells might be a concern, since a small percentage of patients with increased numbers of PNH-type cells developed AML derived from non-PNH-type cells.[8,9] The

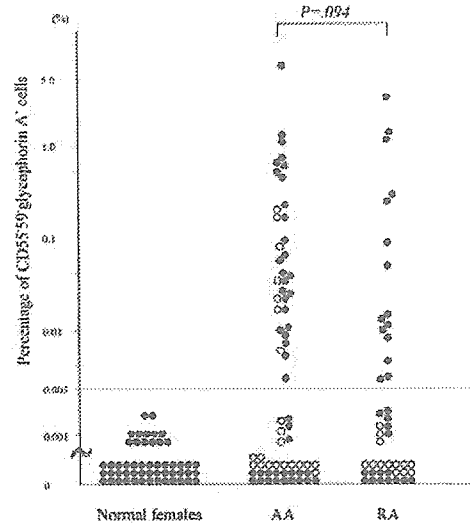

Figure 3. Distribution of PNH-type granulocytes in healthy females and patients with BM failure. Percentage of $CD55^-CD59^-$ cells in $CD11b^+$ cells was determined on 52 healthy females, 68 AA, and 44 RA patients. Closed circles indicate patients showing normal S values; open circles, patients showing abnormal S values.

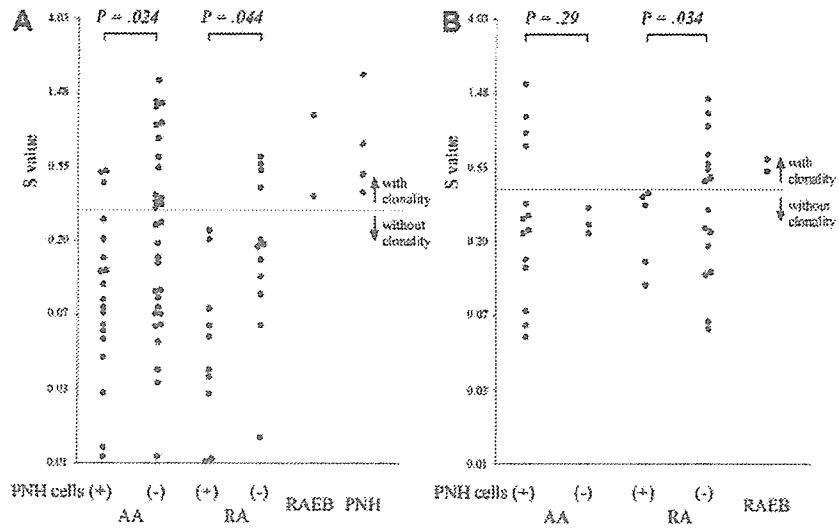

Figure 4. Distribution of S values in patients with BM failure. (A) Patients younger than 70 years. (B) Patients older than 70 years. (+) indicates patients with increased PNH-type cells; (–), patients without increased PNH-type cells. Not included in these figures are 2 PNH$^-$ patients with clonal hematopoiesis (one is a 59-year-old AA patient and another is a 76-year-old RA patient), because 1 of 2 alleles completely disappeared after digestion of their granulocyte DNA.

present study found that the incidence of clonal hematopoiesis in PNH$^+$ patients was significantly lower than that in PNH$^-$ patients despite the fact that the small numbers of PNH-type cells were clonal, as demonstrated by subcloning and sequencing the *PIG-A* gene in our previous study.[7] The difference in the incidence of clonality between PNH$^+$ and PNH$^-$ patients could be attributable to differences in the severity of BM failure or in the disease duration; more severe or prolonged depletion of stem cells due to extrinsic factors may lead to a higher incidence of clonal dominance in PNH$^-$ than in PNH$^+$ patients.[20-22] However, these mechanisms are unlikely because the hematologic parameters such as leukocyte counts and disease duration did not significantly differ between PNH$^+$ and PNH$^-$ patients. Our previous study[7] showed that the response to CyA therapy was significantly lower by PNH$^-$ than PNH$^+$ RA patients. Therefore, the high incidence of clonality in PNH$^-$ AA or RA patients appears to have relevance to nonimmune mechanisms of BM failure, such as an intrinsic defect of hematopoietic stem cells.[4,42] In these patients, the absence of immune pressure upon hematopoietic stem cells may not allow PNH-type stem cells to expand.[43] Although the implication of clonality in BM failure has not yet been established, clonal hematopoiesis in some AA patients has been implicated in the development of RAEB or AML.[23,24] The higher incidence of polyclonal hematopoiesis in PNH$^+$ patients suggests that non–PNH-type stem cells in these patients are largely healthy.

Few studies have focused on the relationship between the presence of clonality and response to IST.[44] Aivado et al showed using the HUMARA and phosphoglycerate kinase–1 clonality assays that low-risk MDS patients with a nonclonal pattern of XCIP favorably responded to ATG. We demonstrated, based on a much larger number of patients and a more accurate HUMARA assay, that the absence of clonality in AA and RA patients is strongly associated with favorable response to IST. Relatively poor response to IST in patients with clonality supports our hypothesis that nonimmune mechanisms of BM failure, such as intrinsic defects in hematopoietic stem cells, may underlie BM failure with clonality.

The results of the present study have important implications for the management of patients with BM failure. When the HUMARA assay fails to detect clonality in PNH$^+$ AA or RA patients, IST is highly recommend.[7] Conversely, when the assay reveals clonality in PNH$^+$ AA or RA patients before treatment, they may benefit more from other treatments, such as allogeneic stem cell transplantation. Because PNH$^-$ patients with clonality are less likely to respond to IST and appear to have a propensity toward developing AML, allogeneic stem cell transplantation also may need to be considered for these patients. These implications of detecting PNH-type cells and clonality in patients with BM failure should be confirmed by a prospective study on a large number of AA and RA patients. A nationwide effort addressing this is currently underway.

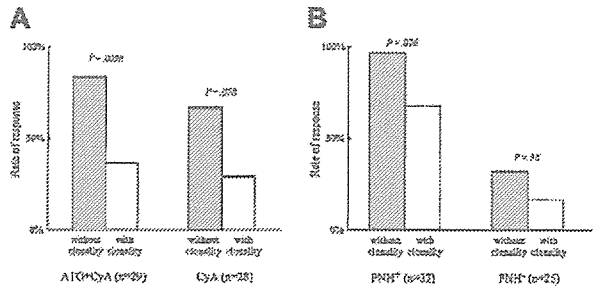

Figure 5. Response to immunosuppressive therapy in various patient subsets defined by clonality and PNH-type cells. (A) Rates of response to ATG + CyA and CyA in 57 patients (42 with AA and 15 with RA). (B) Rates of response to either ATG + CyA or CyA in PNH$^+$ and PNH$^-$ patients.

Acknowledgments

We thank the following people of Kanazawa University for their helpful comments and technical assistance: Dr S. Harada of the Center for Biomedical Research and Education, School of Medicine; Dr K. Saijoh of the Department of Hygiene, Graduate School of Medical Sciences; Ms K. Okada of the Division of Laboratory Service; and Ms M. Yoshii of Cellular Transplantation Biology. We are indebted to the following doctors for providing us with patient samples and clinical information: A. Urabe of NTT Kanto Hospital; M. Saito of NTT Kanazawa Hospital; N. Tsukamoto and M. Karasawa of Gunma University Hospital; T. Yamada of Gifu University Hospital; H. Yamazaki of Kouseiren Takaoka Hospital; Y. Kawachi of Takamatsu Red Cross Hospital; H. Yamauchi of Kurobe City Hospital; A. Matsuda and H. Yagasaki of Saitama Medical School Hospital; T. Matsunaga of Sapporo Medical University Hospital; E. Ohmoto of Yamagata University Hospital; Y. Seki of Niigata Prefectural Shibata Hospital; M. Ueda of Ishikawa Prefectural

Central Hospital; N. Ichikawa of Nagano Red Cross Hospital; T. Hattori of Teikyo University Hospital; K. Koizumi of Tonan Hospital; M. Teramura of Tokyo Women's Medical College Hospital; T. Nakamoto of Tokyo University Hospital; T. Tanaka of Yatsuo General Hospital; K. Shigeno of Hamamatsu University Hospital; A. Sawasaki of Fukuiken Saiseikai Hospital; Y. Terasaki of Toyama City Hospital; T. Yoshida of Toyama Prefectural Central Hospital; and O. Tabata of Okanoue Hospital.

References

1. Young NS. Acquired aplastic anemia. Ann Intern Med. 2002;136:534-546.
2. Heaney ML, Golde DW. Myelodysplasia. N Engl J Med. 1999;340:1649-1660.
3. Bennett JM. World Health Organization classification of the acute leukemias and myelodysplastic syndrome. Int J Hematol. 2000;72:131-133.
4. Barrett J, Saunthararajah Y, Molldrem J. Myelodysplastic syndrome and aplastic anemia: distinct entities or diseases linked by a common pathophysiology? Semin Hematol. 2000;37:15-29.
5. Brunning RD, Bennett JM, Flandrin G, et al. Myelodysplastic syndromes. In: Jaffe ES, Harris NL, Stein H, and Vardiman JW, eds. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2001: 61-74.
6. Wang H, Chuhjo T, Yamazaki H, et al. Relative increase of granulocytes with a paroxysmal nocturnal haemoglobinuria phenotype in aplastic anaemia patients: the high prevalence at diagnosis. Eur J Haematol. 2001;66:200-205.
7. Wang H, Chuhjo T, Yasue S, et al. Clinical significance of a minor population of paroxysmal nocturnal hemoglobinuria-type cells in bone marrow failure syndrome. Blood. 2002;100:3897-3902.
8. Jin JY, Tooze JA, Marsh JC, Matthey F, Gordon-Smith EC. Myelodysplasia following aplastic anaemia-paroxysmal nocturnal haemoglobinuria syndrome after treatment with immunosuppression and G-CSF: evidence for the emergence of a separate clone. Br J Haematol. 1996;94:510-512.
9. Ishihara S, Nakakuma H, Kawaguchi T, et al. Two cases showing clonal progression with full evolution from aplastic anemia-paroxysmal nocturnal hemoglobinuria syndrome to myelodysplastic syndromes and leukemia. Int J Hematol. 2000;72: 206-209.
10. van Kamp H, Smit JW, van den Berg E, Ruud Halie M, Vellenga E. Myelodysplasia following paroxysmal nocturnal haemoglobinuria: evidence for the emergence of a separate clone. Br J Haematol. 1994;87:399-400.
11. Maciejewski JP, Rivera C, Kook H, Dunn D, Young NS. Relationship between bone marrow failure syndromes and the presence of glycophosphatidyl inositol-anchored protein-deficient clones. Br J Haematol. 2001;115:1015-1022.
12. Raghavachar A, Janssen JW, Schrezenmeier H, et al. Clonal hematopoiesis as defined by polymorphic X-linked loci occurs infrequently in aplastic anemia. Blood. 1995;86:2938-2947.
13. Josten KM, Tooze JA, Borthwick-Clarke C, Gordon-Smith EC, Rutherford TR. Acquired aplastic anemia and paroxysmal nocturnal hemoglobinuria: studies on clonality. Blood. 1991;78:3162-3167.
14. van Kamp H, Landegent JE, Jansen RP, Willemze R, Fibbe WE. Clonal hematopoiesis in patients with acquired aplastic anemia. Blood. 1991; 78:3209-3214.
15. Mortazavi Y, Chopra R, Gordon-Smith EC, Rutherford TR. Clonal patterns of X-chromosome inactivation in female patients with aplastic anaemia studies using a novel reverse transcription polymerase chain reaction method. Eur J Haematol. 2000;64:385-395.
16. van Kamp H, Fibbe WE, Jansen RP, et al. Clonal involvement of granulocytes and monocytes, but not of T and B lymphocytes and natural killer cells in patients with myelodysplastia: analysis by X-linked restriction fragment length polymorphisms and polymerase chain reaction of the phosphoglycerate kinase gene. Blood. 1992;80:1774-1780.
17. Champion KM, Gilbert JG, Asimakopoulos FA, Hinshelwood S, Green AR. Clonal haemopoiesis in normal elderly women: implications for the myeloproliferative disorders and myelodysplastic syndromes. Br J Haematol. 1997;97:920-926.
18. Guan Y, Ralph S, Hogge DE. Polyclonal normal hematopoietic progenitors in patients with acute myeloid leukemia. Exp Hematol. 2002;30:721-728.
19. Gale RE, Mein CA, Linch DC. Quantification of X-chromosome inactivation patterns in haematological samples using the DNA PCR-based HUMARA assay. Leukemia. 1996;10:362-367.
20. Abkowitz JL, Linenberger ML, Newton MA, Shelton GH, Ott RL, Guttorp P. Evidence for the maintenance of hematopoiesis in a large animal by the sequential activation of stem-cell clones. Proc Natl Acad Sci. 1990;87:9062-9066.
21. Gale RE, Wheadon H, Linch DC. X-chromosome inactivation patterns using HPRT and PGK polymorphisms in haematologically normal and post-chemotherapy females. Br J Haematol. 1991;79: 193-197.
22. Nakao S, Yamaguchi M, Takamatsu H, Shiobara S, Matsuda T. Clonal hematopoiesis detected in an allogeneic marrow transplant recipient with poor engraftment. Transplantation. 1994;57: 1266-1268.
23. Mortazavi Y, Tooze JA, Gordon-Smith EC, Rutherford TR. N-RAS gene mutation in patients with aplastic anemia and aplastic anemia/paroxysmal nocturnal hemoglobinuria during evolution to clonal disease. Blood. 2000;95:646-650.
24. Murai K, Numaoka H. Frequent transformation to overt myelodysplastic syndrome in patients with aplastic anemia showing hematopoietic monoclonality at onset. Jpn J Clin Hematol. 2000;41: 391-396.
25. Karasawa M, Tsukamoto N, Yamane A, et al. Analysis of the distribution of CAG repeats and X-chromosome inactivation status of HUMARA gene in healthy female subjects using improved fluorescence-based assay. Int J Hematol. 2001; 74:281-286.
26. Ma W, Ikeda H, Yoshimoto T. Clinicopathologic study of 123 cases of prolactin-secreting pituitary adenomas with special reference to multihormone production and clonality of the adenomas. Cancer. 2002;95:258-266.
27. D'Adda T, Candidus S, Denk H, Bordi C, Hofler H. Gastric neuroendocrine neoplasms: tumour clonality and malignancy-associated large X-chromosomal deletions. J Pathol. 1999;189: 394-401.
28. Lucas DR, Shroyer KR, McCarthy PJ, Markham NE, Fujita M, Enomoto TE. Desmoid tumor is a clonal cellular proliferation: PCR amplification of HUMARA for analysis of patterns of X-chromosome inactivation. Am J Surg Pathol. 1997;21: 306-311.
29. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ. Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med. 1985;313:776-783.
30. Busque L, Mio R, Mattioli J, et al. Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. Blood. 1996;88:59-65.
31. Gale RE, Fielding AK, Harrison CN, Linch DC. Acquired skewing of X-chromosome inactivation patterns in myeloid cells of the elderly suggests stochastic clonal loss with age. Br J Haematol. 1997;98:512-519.
32. Tonon L, Bergamaschi G, Dellavecchia C, et al. Unbalanced X-chromosome inactivation in haemopoietic cells from normal women. Br J Haematol. 1998;102:996-1003.
33. van Dijk JP, Heuver L, Stevens-Linders E, et al. Acquired skewing of lyonization remains stable for a prolonged period in healthy blood donors. Leukemia. 2002;16:362-367.
34. Asimakopoulos FA, Gilbert JG, Aldred MA, Pearson TC, Green AR. Interstitial deletion constitutes the major mechanism for loss of heterozygosity on chromosome 20q in polycythemia vera. Blood. 1996;88:2690-2698.
35. Sashida G, Ohyashiki JH, Ito Y, Ohyashiki K. Monoclonal constitution of neutrophils detected by PCR-based human androgen receptor gene assay in a subset of idiopathic thrombocytopenic purpura patients. Leuk Res. 2002;26:825-830.
36. Shih LY, Lin TL, Lai CL, et al. Predictive values of X-chromosome inactivation patterns and clinicohematologic parameters for vascular complications in female patients with essential thrombocythemia. Blood. 2002;100:1596-1601.
37. Mutter GL, Boynton KA. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acids Res. 1995;23:1411-1418.
38. Yamaguchi M, Nakao S, Takamatsu H, Chuhjo T, Shiobara S, Matsuda T. Quality of hematologic recovery in patients with aplastic anemia following cyclosporine therapy. Exp Hematol. 1995;23: 341-346.
39. Nakahara Y, Suzuki H, Ohashi H, et al. Clonality analysis of granulocytes and T lymphocytes in healthy females by the PCR-based HUMARA method. Int J Hematol. 1999;69:237-243.
40. Anan K, Ito M, Misawa M, et al. Clonal analysis of peripheral blood and haemopoietic colonies in patients with aplastic anaemia and refractory anaemia using the polymorphic short tandem repeat on the human androgen-receptor (HUMARA) gene. Br J Haematol. 1995;89:838-844.
41. Tsuge I, Kojima S, Matsuoka H, et al. Clonal haematopoiesis in children with acquired aplastic anaemia. Br J Haematol. 1993;84:137-143.
42. Vulliamy T, Marrone A, Dokal I, Mason PJ. Association between aplastic anaemia and mutations in telomerase RNA. Lancet. 2002;359:2168-2170.
43. Young NS. The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. Blood. 1992;79:1385-1392.
44. Aivado M, Rong A, Stadler M, et al. Favourable response to antithymocyte or antilymphocyte globulin in low-risk myelodysplastic syndrome patients with a 'non-clonal' pattern of X-chromosome inactivation in bone marrow cells. Eur J Haematol. 2002;68:210-216.

Bone Marrow Transplantation (2006) 38, 577–579

www.nature.com/bmt

LETTER TO THE EDITOR

Oral beclomethasone dipropionate as an initial treatment of gastrointestinal acute graft-versus-host disease after reduced-intensity cord blood transplantation

Bone Marrow Transplantation (2006) **38,** 577–579. doi:10.1038/sj.bmt.1705489; published online 4 September 2006

Beclomethasone dipropionate (BDP) is a potent topically active corticosteroid. BDP is hydrolyzed to an active metabolite, beclomethasone 17-monopropionate, by esterase enzymes in the intestinal and bronchial mucosa. Systemic adverse effects are limited by incomplete mucosal absorption and rapid metabolism in the liver.[1] BDP had been developed as inhalant corticosteroid for the treatment of asthma and allergic rhinitis, then the efficacy of topical BDP was shown in the treatment of Crohn's or inflammatory bowel disease.[2,3] Oral BDP (6–10 mg/body/day) or BDP enema (3–5 mg/body/day) was used in these studies.

The pathological and clinical findings of gastrointestinal (GI) graft-versus-host disease (GVHD) are similar to those of inflammatory bowel disease. Three previous studies had shown the safety and efficacy of the topical BDP for GI GVHD. A phase I trial from the Fred Hutchinson Cancer Research Center (FHCRC) showed that 23 patients with mild to moderate GI acute GVHD were given oral BDP at a daily dose of 8 mg/body as an initial therapy. The response rate was 77%, and no patient presented with severe infection or adrenal insufficiency.[4] A randomized placebo-controlled trial from FHCRC demonstrated that response rates were significantly higher in patients given oral BDP and prednisone than those given prednisone alone as initial therapy for GI acute GVHD (71 versus 41%).[5] A phase II trial by Iyer *et al.*[6] showed that long-term use of oral BDP for the treatment of GI GVHD, which included chronic GVHD mainly, was safe and effective.

Infection is a significant problem, contributing to a majority of transplant-related mortality following cord blood transplantation (CBT). Systemic use of corticosteroid is a standard treatment of acute GVHD; however, it is associated with a high incidence of infection, impairing patients' survival.

The optimal treatment for immune reactions after CBT has not been established.[7] We have been undertaking clinical trials on CBT using reduced-intensity preparative regimen (reduced-intensity (RI)-CBT).[8] Since March 2004, we have used oral BDP as initial treatment of GI acute GVHD following RI-CBT instead of systemic corticosteroid. We will review medical, pathological and laboratory records of five patients with GI GVHD who were treated with oral BDP.

Between March 2003 and December 2004, 38 patients with hematological diseases or solid tumors underwent RI-CBT at Toranomon Hospital. Details of transplantation procedures and supportive cares were reported previously.[8] The preparative regimen mainly comprised fludarabine 125 mg/m^2, melphalan 80 mg/m^2 and 4 Gy total body irradiation. GVHD prophylaxis was cyclosporine 3 mg/kg or tacrolimus 0.03 mg/kg. CMV pp65 antigenemia was monitored weekly after engraftment. If CMV antigenemia exceeded 10/50 000, patients preemptively received ganciclovir 5 mg/kg or foscarnet 30 mg/kg intravenously twice a day. When patients developed pathologically proven GI acute GVHD with or without stage 1 and 2 skin involvement, patients received one enteric-coated cellulose capsule and an aqueous suspension by mouth every 6 h for a total daily dose of 8 mg. Both enteric-coated cellulose capsule and aqueous suspension containing 1 mg BDP powder (Wako Pure Chemical Industries, Ltd, Osaka, Japan) were prepared in our hospital. BDP in the form of aqueous suspension is activated in the stomach and the upper intestine. The enteric-coated capsule dissolves in the small intestine, delivering active drug into the small intestine and the colon. The duration of BDP was at the discretion of the primary physicians. All patients provided written informed consent in accordance with the requirements of the institutional review board. The other patients with grade II–IV acute GVHD were treated with systemic methylprednisolone 0.5–2.0 mg/kg. Response to BDP was determined according to the study by Martin *et al.*[9]

A total of 17 (44.7%) patients developed grade II–IV acute GVHD, and five of them were treated with oral BDP. Their clinical characteristics and outcomes were shown in Table 1. Responses to BDP included complete response (CR) ($n=3$) and progression ($n=2$). Acute GVHD had not recurred in the three patients (Cases 1–3) who achieved CR. None of these three patients developed either bacterial or fungal infection, whereas cytomegalovirus enterocolitis was diagnosed in a patient (Case 1). The remaining two patients (Cases 4 and 5) who had not responded to oral BDP were given methylpresonisolone 1–2 mg/kg as a second treatment, and all of them achieved CR. These two patients were complicated with cytomegalovirus enterocolitis during administration of methylprednisolone, and were successfully treated with ganciclovir or foscarnet. As of July 2006, one of the five patients (Case 1) is alive without disease progression. The other four patients died of recurrence (Cases 2, 3 and 4) and chronic GVHD (Case 5).

The present study showed that oral BDP is a useful agent for the treatment of GI acute GVHD following RI-CBT.

Table 1 Characteristics of BDP

	Sex	*Age (years)*	*Primary disease*	*Onset of gut GVHD*	*HLA match*	*GVHD stage and grade at diagnosis (skin/gut)*	*Duration of BDP (days)*	*Diarrhea at the end of BDP*	*Response*	*CMV disease*	*CMV antigenemia*	*BSI*	*Other infection*	*Survival (causes)*
Case 1	Female	32	Hodgkin's lymphoma	Day 45	4	(2/2), grade III	35	0	CR	Enterocolitis	Yes	No	No	Alive
Case 2	Male	68	ATL	Day 27	5	(1/3), grade III	60	0	CR	No	No	No	No	Dead (relapse)
Case 3	Male	45	AML	Day 29	5	(2/3), grade III	39	0	CR	No	No	No	No	Dead (relapse)
Case 4	Male	40	Malignant lymphoma	Day 53	4	(2/3), grade III	9	1196 ml/day	Progression	Enterocolitis	Yes	No	No	Dead (relapse)
Case 5	Male	59	AML	Day 26	4	(0/3), grade III	7	10 times/day	Progression	Enterocolitis	Yes	No	No	Dead (chronic GVHD)

AML = acute myeloid leukemia; ATL = adult T-cell leukemia; BDP = beclomethasone dipropionate; BSI = bloodstream infection; CMV = cytomegalovirus; CR = complete response; GVHD = graft-versus-host disease; HLA = human leukocyte antigen; RI-CBT = reduced-intensity cord blood transplantation.

When patients with acute GVHD are given systemic corticosteroid, it involves a high risk of bloodstream infection (BSI). We previously reported that 32% of RI-CBT recipients developed BSI within 100 days of transplantation, and that it was associated with systemic use of corticosteroid (relative risk, 43.1).[10] However, none of the five patients developed BSI during and after administration of oral BDP in the present study. Oral BDP causes limited immunosuppression to the systemic organs other than the gut,[4,6] and the risk of BSI might not be elevated in patients given oral BDP for the treatment of GI acute GVHD.

Considering that three of the five patients achieved CR with oral BDP, it is reasonable to assume that systemic administration of corticosteroid is not requisite for GI acute GVHD, and that oral BDP is occasionally sufficient for its control. However, it should be noted that the remaining two patients failed to achieve CR, and that one of them finally died of chronic GVHD. The two patients with treatment failures (Cases 4 and 5) might have responded to short-course prednisone along with oral BDP, followed by a rapid prednisone taper as reported by McDonald *et al.*[5] These findings suggest that systemic corticosteroid might have been required for these two patients. Alternatively, oral BDP at a dose of 8 mg/day might have been suboptimal in these patients. Further studies are warranted to clarify the eligibility of oral BDP, and its optimal dose for the treatment of GI acute GVHD.

In conclusion, the present study demonstrated the feasibility of oral BDP in the treatment of GI acute GVHD following RI-CBT. It suppresses allogeneic immune responses in the gut without causing significant immunosuppression. However, this study is too small to draw a definite conclusion on oral BDP, and we are now planning a large-scale prospective study.

Y Miura[1], H Narimatsu[1], M Kami[2], E Kusumi[1], T Matsumura[1], K Yuji[1], A Wake[1], S Miyakoshi[1] and S Taniguchi[1]

[1]*Department of Hematology, Toranomon Hospital, Tokyo, Japan and*

[2]*Division of Exploratory Research, Institute of Medical Science, University of Tokyo, Tokyo, Japan*

E-mail: ug-miu@umin.ac.jp

References

1 Martin LE, Tanner RJ, Clark TJ, Cochrane GM. Absorption and metabolism of orally administered beclomethasone dipropionate. *Clin Pharmacol Ther* 1974; **15**: 267–275.

2 Tursi A, Giorgetti GM, Brandimarte G, Elisei W, Aiello F. Beclomethasone dipropionate for the treatment of mild-to-moderate Crohn's disease: an open-label, budesonide-controlled, randomized study. *Med Sci Monit* 2006; **12**: PI29–PI32.

3 Rizzello F, Gionchetti P, Galeazzi R, Novelli G, Valpiani D, D'Arienzo A *et al.* Oral beclomethasone dipropionate in patients with mild to moderate ulcerative colitis: a dose-finding study. *Adv Ther* 2001; **18**: 261–271.

4 Baehr PH, Levine DS, Bouvier ME, Hockenbery DM, Gooley TA, Stern JG *et al.* Oral beclomethasone dipropionate for treatment of human intestinal graft-versus-host disease. *Transplantation* 1995; **60**: 1231–1238.

5 McDonald GB, Bouvier M, Hockenbery DM, Stern JM, Gooley T, Farrand A *et al.* Oral beclomethasone dipropionate for treatment of intestinal graft-versus-host disease: a randomized, controlled trial. *Gastroenterology* 1998; **115**: 28–35.
6 Iyer RV, Hahn T, Roy HN, Battiwalla M, Cooper M, Anderson B *et al.* Long-term use of oral beclomethasone dipropionate for the treatment of gastrointestinal graft-versus-host disease. *Biol Blood Marrow Transplant* 2005; **11**: 587–592.
7 Kishi Y, Kami M, Miyakoshi S, Kanda Y, Murashige N, Teshima T *et al.* Early immune reaction after reduced-intensity cord-blood transplantation for adult patients. *Transplantation* 2005; **80**: 34–40.
8 Miyakoshi S, Yuji K, Kami M, Kusumi E, Kishi Y, Kobayashi K *et al.* Successful engraftment after reduced-intensity umbilical cord blood transplantation for adult patients with advanced hematological diseases. *Clin Cancer Res* 2004; **10**: 3586–3592.
9 Martin PJ, Schoch G, Fisher L, Byers V, Anasetti C, Appelbaum FR *et al.* A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. *Blood* 1990; **76**: 1464–1472.
10 Narimatsu H, Matsumura T, Kami M, Miyakoshi S, Kusumi E, Takagi S *et al.* Bloodstream infection after umbilical cord blood transplantation using reduced-intensity stem cell transplantation for adult patients. *Biol Blood Marrow Transplant* 2005; **11**: 429–436.