Table 1. Hematologic parameters, fetal hemoglobin (HbF) and endothelin-3 (ET-3) concentrations in 19 patients with sickle cell disease before and during hydroxyurea treatment.

	Before treatment (mean±SD)	*1st month (mean±SD)*	*5th month (mean±SD)*	*Controls (mean±SD)*	*Mann-Whitney (p)*	*Wilcoxon (p)*
Hb (mmol/L)	1.10±0.28	1.18±0.19	1.32±0.28			NS
WBC ($\times 10^9$/L)	12.2±2.7	11.9±2.4	11.1±3.8			NS
Reticulocytes ($\times 10^9$/L)	335±155	280±135	210±180			NS
HbF (%)	7.6±3.8	8.1±2.9	15.8±7.2			< 0.05
ET-3 (pmol/L)	5.66±2.23	2.16±1.21	1.05±0.46	0.99±0.58	< 0.005	< 0.005

Hb: hemoglobin; WBC: white blood cell count; NS: non significant.

with red blood cells. ET-3 could play a role in the cycle of ischemia and inflammation by deregulating vascular tone, and by increasing ET-1 and IL-6 production. The early decrease of ET-3 levels, at a time when HbF elevation was not evident in most patients, is a particularly interesting finding of this study. It indicates that increased HbF is not the only mechanism by which hydroxyurea can affect SCD and this is in concert with previous studies, as it has been shown that the beneficial clinical effect of HU precedes any significant increase in HbF and is not correlated with the achieved level of HbF.[6] At a molecular level, HU reduces the expression of adhesion molecules on the surface of red blood cells, lymphocytes, monocytes and neutrophils before HbF increases.[7,8,9] In addition, it downregulates ET-1 gene expression on endothelial cells.[10]

In summary, the elevated steady state plasma ET-3 levels and their early decrease during HU treatment, indicate that ET-3 may have a role in endothelial deregulation and inflammation in SCD patients. More studies are needed to determine ET-3 levels and their correlation with the extent of vascular damage and the severity of SCD.

Alexandros C. Makis, Eleftheria C. Hatzimichael,* George Kolios,° Konstantinos L. Bourantas**

**Hematology Clinic and °Laboratory of Biological Chemistry, Medical School, University of Ioannina, Ioannina, Greece*

Key words: sickle cell disease, endothelin-3, hydroxyurea.

Correspondence: Konstantinos L. Bourantas, Professor of Internal Medicine/Hematology, P.O. Box 37, GR-45002 Ioannina, Greece. Phone: international +30.2651024794. Fax: international +30 2651039356. E-mail: kbouran@cc.uoi.gr

References

1. Serjeant GR. The emerging understanding of sickle cell disease. Br J Haematol 2001;112:3-18.
2. Makis AC, Hatzimichael EC, Bourantas KL. The role of cytokines in sickle cell disease. Ann Hematol 2000;79:407-13.
3. Rybicki AC, Benjamin LJ. Increased levels of endothelin-1 in plasma of sickle cell anemia patients. Blood 1998;92:2594-6.
4. Yokokawa K, Kohno M, Yasunari K, Murakawa K, Takeda T. Endothelin-3 regulates endothelin-1 production in cultured human endothelial cells. Hypertension 1991;18:304-15.
5. Xin X, Cai Y, Matsumoto K, Agui T. Endothelin-induced interleukin-6 production by rat aortic endothelial cells. Endocrinology 1995; 136:132-7.
6. Maier-Redelsperger M, Labie D, Elion J. Long-term hydroxyurea treatment in young sickle cell patients. Curr Opin Hematol 1999; 6:115-20
7. Styles LA, Lubin B, Vichinsky E, Lawrence S, Hua M, Test S, et al. Decrease of very late activation antigen-4 and CD36 on reticulocytes in sickle cell patients treated with hydroxyurea. Blood 1997; 89:2554-9.
8. Okpala I, Daniel Y, Haynes R, Odoemene D, Goldman J. Relationship between the clinical manifestations of sickle cell disease and the expression of adhesion molecules on white blood cells. Eur J Haematol 2002;69:135-44.
9. Benkerrou M, Delarche C, Brahimi L, Fay M, Vilmer E, Elion J, et al. Hydroxyurea corrects the dysregulated L-selectin expression and increased H(2)O(2) production of polymorphonuclear neutrophils from patients with sickle cell anemia. Blood 2002;99:2297-303.
10. Brun M, Bourdoulous S, Couraud PO, Elion J, Krishnamoorthy R, Lapoumeroulie C. Hydroxyurea downregulates endothelin-1 gene expression and upregulates ICAM-1 gene expression in cultured human endothelial cells. Pharmacogenomics J 2003;3:215-26.

Phagocytes

High incidence of neutropenia in patients treated with rituximab after autologous stem cell transplantation

We report a high incidence of neutropenia in patients treated with rituximab prior to and following autologous stem cell transplantation (ASCT). Fourteen patients with follicular or mantle–cell lymphoma were treated with high dose (HD) therapy followed by an *in vivo*–purged autologous graft. Ten of these patients received two additional courses of rituximab after the transplant. Seven experienced severe neutropenia after the second administration. Our data suggest that early administration of rituximab following a transplant may favor the onset of neutropenia.

haematologica 2004; 89:361-362
(*http://www.haematologica.org/journal/2004/3/361*)

Rituximab is a chimeric IgG-1 monoclonal antibody which binds to the CD20 antigen with high affinity.[1] When used in the pretransplant regimen, it can be considered an *in-vivo purging* agent that can remove CD20+ lymphoma cells from the graft.[2] The occurrence of neutropenia has rarely been reported in patients exposed to rituximab,[3-6] although this complication seems more frequent in the setting of autologous stem cell transplantation (ASCT).[7] From 1999 to 2003, 14 consecutive patients with confirmed histologic diagnosis of follicular (n=9) or mantle cell (n=5) lymphomas were referred to our institution and treated with a protocol integrating chemotherapy, rituximab and ASCT[8] (Table 1). After a phase of debulking with VACOP-B, the patients received rituximab, vincristine and cyclophosphamide and underwent mobilization and harvest of CD34+ cells after high dose cytarabine and rituximab. The final phase of the protocol consisted of ASCT, using BEAM as the conditioning regimen, followed by two courses of rituximab (375 mg/m^2).

All 14 patients underwent ASCT using peripheral blood as the source of stem cells. In 13 cases the apheresis products

Table 1. Patients' characteristics and outcomes.

No. of patients	*14*
Male:Female	9:5
Median age (range)	48 years (32-60)
Disease characteristics	
Follicular lymphoma grade 1-2	9
Mantle cell lymphoma	5
Status at protocol enrollment	
relapse/progression	10
at diagnosis	4
Patients with bone marrow involvement at diagnosis	11
Patients with bone marrow involvement at transplant (histologic)	0
Patients with bone marrow involvement at transplant (molecular)	0
Post-transplant rituximab	
Patients receiving rituximab after transplant	10
Median neutrophil count at first rituximab administration (range)	2.35×10^9/L (1.2-4.2)
Median time from transplantation to first rituximab administration (range)	52 days (34-63)
Post-transplant neutropenia	
Patients developing neutropenia	7 (70%)
Median time from 2nd rituximab course to neutrophil $<1.0\times10^9$/L (range)	38 days (14-84)
Median neutrophil count nadir (range)	0.38×10^9/L (0.1-0.8)
Median duration of neutrophil count $<1.0\times10^9$/L (range)	12 days (7-145)
Episodes of fever during neutropenia	None
Findings on bone marrow biopsy at time of neutropenia (all patients studied)	Hypocellular
Follow-up	
Median time from ASCT (range)	15 months (4-40)
Patients in continuous complete remission	14

were negative for Bcl-2/IgH and Bcl-1/IgH rearrangements. The median number of $CD34^+$ cells infused was 17.15 $\times10^6$/kg (range 6.21-32). Engraftment was rapid in all patients. The median time to a neutrophil count $>0.5\times10^9$/L was 9 days (range 8-18) and a self-sustained platelet count $>20\times10^9$/L was reached after 9 days (range 0-25). Ten out of 14 patients (71%) received rituximab at a median of 52 days (range 34-63) post-transplant while the median neutrophil count was 2.35×10^9/L (range 1.2-4.2). A second dose of rituximab was administered two weeks later. Seven out of the 10 patients who received post-transplant rituximab developed severe neutropenia at a median of 38 days (range 14-84) after the second administration. The median neutrophil nadir was 0.38×10^9/L (range 0.1-0.8) while the median duration of the neutropenic phase was 12 days (range 7-145). Two patients experienced multiple episodes of neutropenia; one of them responded to granulocyte colony-stimulating factor given after 14 days with a neutrophil count $<0.5\times10^9$/L. The neutrophil count recovered spontaneously in the other 6 patients. There were no episodes of infections during the neutropenia. An evaluation of bone marrow in the neutropenic period showed hypocellular marrow with normal trilineage hematopoiesis consistent with the recent transplant. Cytogenetic analysis demonstrated a normal karyotype in all cases. In 5 cases the bone marrow was evaluated by flow cytometry and a predominance of cells reactive to CD3, CD4, CD8, CD16-56 was not found. An infectious etiology of the neutropenia was excluded in all cases. In particular cytomegalovirus antigenemia and Parvovirus B19 tests were negative.

Published reports of agranulocytosis possibly related to rituximab use are scanty. In a study of an *in vivo* purging with rituximab, Flinn *et al.*, observed 6 cases of transient neutropenia occurring at a median of 99.5 days after ASCT in patients with indolent non-Hodgkin's disease. All patients had received rituximab early after their transplant. Unfortunately, a systematic evaluation of these cases was not reported.[9] Papadaki *et al.* reported on 34 NHL patients of whom 15 developed profound neutropenia following rituximab, often associated with T-cell large granular lymphocyte (T-LGL) proliferation. The authors postulated a rituximab-induced T-LGL proliferation that secretes large amounts of Fas and Fas-ligand leading to apoptosis of mature neutrophils.[10] Voog *et al.* described 8 cases of neutropenia in patients previously treated with rituximab. An IgG-type antibody, bound to the neutrophil surface, was detected in two patients. The authors speculated that rituximab-induced B-cell depletion may favor autoantibody production in the context of a new immune repertoire.[11] Horwitz *et al.* reported that 54% of patients with aggressive NHL developed severe neutropenia following ASCT. Patients were scheduled to receive four weekly infusions of rituximab starting on day +40; a second 4-week course of rituximab was repeated 6 months after transplant. Serious infections were not observed despite delayed B-cell recovery in all patients.[7] In our study we found an incidence of 70% of severe neutropenia after ASCT in patients treated with rituximab prior to and following transplantation. All episodes either resolved spontaneously or responded to a few days of treatment with granulocyte colony-stimulating factor. No severe infections occurred during the neutropenia. In all cases bone marrow biopsies at the time of neutropenia were hypocellular with myeloid precursors present; no LGL infiltration was noted in bone marrow specimens or by flow cytometry. In conclusion our experience suggests that early administration of rituximab during the post-transplant period may favor the onset of severe neutropenia. Further studies are warranted to define the best dose and schedule for rituximab administration after transplantation.

Roberto Cairoli, Giovanni Grillo, Alessandra Tedeschi, Giovanna D'Avanzo, Paola Marenco, Enrica Morra

Division of Hematology, Ospedale Niguarda, Milan, Italy

Key words: rituximab, neutropenia, autologous transplant.

Correspondence: Roberto Cairoli, Division of Hematology, Ospedale Niguarda, piazza Ospedale Maggiore 3, 20162 Milan, Italy. Phone: international +39.02.64443187. E-mail: ematologia@ospedaleniguarda.it

References

1. Reff ME, Carnel K, Chambers KS, Chinn PC, Leonard JE, Raab R, et al. Depletion of B cells in vivo by chimeric mouse human monoclonal antibody to CD20. Blood 1994;83:435-45.
2. Blum KA, Bartlett NL. Antibodies for the treatment of diffuse large cell lymphoma. Semin Oncol 2003;30:448-56.
3. Davis TA, Grillo-Lopez A, White CA, McLaughlin P, Czuczman MS, Link BK, et al. Rituximab anti-CD20 monoclonal antibody therapy

in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. J Clin Oncol 2000;18:3135-43.
4. Maloney DG, Grillo-Lopez A, White CA, Bodkin D, Schilder RJ, Neidhart JA, et al. IDEC-C2B8 (rituximab) anti CD-20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. Blood 1992;6:2188-95.
5. Maloney DG, Liles TM, Czerwinski DK, Waldichuk C, Rosenberg J, Grillo-Lopez A, et al. Phase I clinical trial using escalating single dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. Blood 1994; 8:2457-66.
6. McLaughlin P, Grillo-Lopez A, Link KB, Levy R, Czuczman MS, Williams ME, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a 4-dose treatment program. J Clin Oncol 1998;16: 2825-33.
7. Horwitz SM, Negrin RS, Blume KJ, Breslin S, Stuart MJ, Stockerl-Goldstein KE, et al. Rituximab as adjuvant to high-dose therapy and autologous hematopoietic cell transplantation for aggressive non-Hodgkin's lymphoma. Blood 2004;103:777-83.
8. Lazzarino M, Arcaini L, Bernasconi P, Alessandrino EP, Gargantini L, Cairoli R, et al. A sequence of immuno-chemotherapy with rituximab, mobilization of in vivo purged stem cells, high dose chemotherapy and autotransplantation is an effective and non-toxic treatment for advance follicular and mantle cell lymphoma. Br J Haematol 2002;116:229-35.
9. Flinn IW, O' Donnell PV, Goodrich A, Vogelsang G, Abrams R, Noga S, et al. Immunotherapy with rituximab during peripheral blood stem cell transplantation for non-Hodgkin's lymphoma. Biol Bone Marrow Transplant 2000;6:628-32.
10. Papadaki T, Stamatopoulos K, Anagnostopoulos A, Fassas A. Rituximab-associated immune myelopathy. Blood 2003;102:1557-8.
11. Voog E, Morschhauser F, Solal-Céligny P. Neutropenia in patients treated with rituximab. N Engl J Med 2003;348:2691-4.

Acute Myeloid Leukemia

Differences in the expression pattern of apoptosis-related molecules between childhood and adult *de novo* acute myeloid leukemia

Distinct expression patterns of pro- and anti-apoptotic proteins may contribute to different prognoses and therapy outcomes in adult versus childhood acute myeloid leukemia (AML). Therefore, we investigated whether expression levels of apoptosis-related proteins CD95, Bcl-2, Bax, Bcl-xL, procaspase-3, XIAP, cIAP-1, and survivin differ between children and adults with *de novo* AML.

haematologica 2004; 89:363-364
(*http://www.haematologica.org/journal/2004/3/363*)

Prognosis in acute myeloid leukemia (AML) is age-related, with childhood AML having a better treatment outcome than adult AML. Within adults, patients < 60 years old have a better prognosis than patients ≥60 years old. For the current chemotherapy regimens, these prognostic differences are valid for response to induction chemotherapy, event-free survival (EFS) as well as overall survival (OS).[1-4]

It has been speculated that differences in the expression patterns of anti- and pro-apoptotic molecules between childhood and adult acute leukemia might contribute to the different treatment outcomes of age-stratified leukemia groups.[5] However, systematic investigations on possible expression differences of apoptosis-related molecules in children and adults with acute leukemia are rare. To evaluate this hypothesis, we examined consecutively collected leukemic cell samples from children (n=45) and adults (n=92; < 60 years: n=44, ≥ 60 years: n=48) with *de novo* AML for the expression levels of several apoptosis-related molecules (CD95, Bcl-2, Bax, Bcl-xL, caspase-3, XIAP, cIAP-1, survivin). All samples contained more than 80% leukemic cells based on morphologic criteria.

Surface CD95 expression and intracellular expression of Bcl-2 and Bax were determined by flow cytometry, as described previously, using the PE-conjugated anti-CD95 monoclonal antibody DX2, the FITC-conjugated anti-Bcl-2 monoclonal antibody 124, and the polyclonal rabbit-anti-human antibody I-19 raised against Bax-specific peptide sequences.[6] Antigen expression distribution in individual cell samples was quantified as relative fluorescence intensity (RFI), determined by the ratio of mean fluorescence intensity of cells stained for the respective antigen to mean fluorescence intensity of the corresponding negative control. Expression levels of Bcl-xL, caspase-3, XIAP, cIAP-1 and survivin were determined by Western blotting using monoclonal antibodies specific for XIAP (Transduction Laboratories, Lexington, KY, USA), cIAP-1, survivin (R&D Systems, Minneapolis, MN, USA), procaspase-3 (Pharmingen, San Diego, CA, USA), and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Data on X-ray films were quantified by scanning-densitometry using the NIH Image analysis system. To normalize for variation in antibody concentration or time of exposure, the protein signal from the respective patient was normalized against the protein signal of the control cell line BJAB (human Burkitt-like lymphoma cell line). Western blot results are expressed in terms of this ratio (relative optical density, ROD).[7] Differences in the expression levels of apoptosis-related molecules between childhood and adult AML were evaluated using the Mann Whitney-test.

As outlined in Table 1, expression of Bax, procaspase-3, XIAP and c-IAP1 differed between the age groups. The most striking finding in our study was the much higher expression of cIAP-1 in childhood AML than in adult AML. In contrast, all other observed statistically significant age-related expression differences (Bax, procaspase-3, XIAP) were for higher protein levels among the adults than among the children. However, neither the higher expression of cIAP-1 nor the lower expression levels of Bax and procaspase-3 in childhood AML observed in this series fits with the general expectation that expression levels of anti-apoptotic proteins (e.g. cIAP-1) would be lower and expression levels of pro-apoptotic proteins (e.g. Bax, Procaspase-3) would be higher in the prognostically more favorable pediatric AML group.[5]

Only the higher expression of the anti-apoptotic molecule XIAP in the prognostically more unfavorable adult AML matches this expectation.[7] Possible explanations for these rather unexpected findings may include: (i): the intracellular location of these molecules influences their apoptotic activity. For example, to be pro-apoptotic, Bax must translocate from the cytoplasm to mitochondria, where it triggers cytochrome c release; (ii) post-translational modifications (e.g. phosphorylation) of Bcl-2 family members might be of importance for their functional activity; (iii) measurement of the active form of caspase-3 rather than the inactive proform might be more informative as a potential prognostic marker; (iv) expression analysis of recently characterized molecules (e.g. Diablo/Smac) counteracting the anti-apoptotic activity of IAP molecules might be helpful to understand the observed expression pattern of apoptosis-related molecules within this study; (v) protein families other than apoptosis-regu-