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and the Italian Society of Experimental Hematology

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# Haematologica

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2. Burgess AW, Begley CG, Johnson GR, et al. Purification and properties of bacterially synthesized human granulocyte-macrophage colony stimulating factor. *Blood* 1987; 69:43-51.
3. The Royal Marsden Hospital Bone-Marrow Transplantation Team. Failure of syngeneic bone-marrow graft without preconditioning in post-hepatitis marrow aplasia. *Lancet* 1977; 2:242-4.
4. Anonymous. Red cell aplasia (Editorial). *Lancet* 1982; 1:546-7.
5. Karlsson S, Humphries RK, Gluzman Y, Nienhuis AW. Transfer of genes into hemopoietic cells using recombinant DNA viruses (Abstract). *Blood* 1984; 64(Suppl 1):58a.

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6. Ferrata A, Storti E. *Le malattie del sangue*. 2nd ed. Milano: Vallardi, 1958.
7. Hillman RS, Finch CA. *Red cell manual*. 5th ed. Philadelphia: FA Davis, 1985.

8. Bottomley SS. Sideroblastic anaemia. In: Jacobs A, Worwood M, eds. *Iron in biochemistry and medicine*, II. London: Academic Press, 1980:363-92.
9. DuPont B. Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. *Proceedings of the third annual meeting of the International Society for Experimental Hematology*. Houston: International Society for Experimental Hematology, 1974:44-6.
10. Bieber MM, Kaplan HS. T-cell inhibitor in the sera of untreated patients with Hodgkin's disease (Abstract). Paper presented at the International Conference on Malignant Lymphoma Current Status and Prospects, Lugano, 1981:15.
11. Worwood M. Serum ferritin. In: Cook JD, ed. *Iron*. New York: Churchill Livingstone, 1980:59-89. (Chanarin I, Beutler E, Brown EB, Jacobs A, eds. *Methods in hematology*; vol 1).
12. Ranofsky AL. *Surgical operation in short-stay hospitals: United States-1975*. Hyattsville, Maryland: National Center for Health Statistics, 1978; DHEW publication no. (PHS) 78-1785, (Vital and health statistics; series 13; no. 34).

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### *Selected Abstracts*

The Scientific Committee of the 35<sup>th</sup> Congress of the Italian Society of Hematology has received 703 abstracts. All of them, without the names of the Authors and Institutions, have been examined by three Referees, chosen by the Board of the Society. Each of the abstracts has received a score (maximum 36) based on methodology, exposition and interpretation of the results, originality and interest of the study. Due to these criteria 651 abstracts have been accepted as oral presentations or as posters. Almost two thirds of abstracts have been received in English to be published in *Haematologica*, but only those with a score above 21 will be published in the Journal. The rigorous criteria for selection warranty the good quality of the abstracts published.

*Prof. Carlo Bernasconi*  
*Chairman of the Scientific Committee*

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001

**SIGNALLING OF G-CSF THROUGH SHC AND GRB2 IS MEDIATED BY G-CSF-R CYTOPLASMIC DISTAL REGION**

Santini V, de Koning J, Dong F, Rossi Ferrini P, Löwenberg B, Touw I

*Divisione e Cattedra di Ematologia, Università di Firenze and Dept. of Hematology, Dr. Dan den Hoed Cancer Center and Institute of Hematology, Erasmus University, Rotterdam, The Netherlands*

Human G-CSF receptor (G-CSF-R) is a single polypeptide of 813 AA able to transduce growth signals through its membrane proximal region and maturative signals through its cytoplasmic distal region. Although G-CSF-R lacks a tyrosine kinase consensus sequence, G-CSF stimulation induces the tyrosine phosphorylation of several proteins in G-CSF responsive cells. We analyzed whether Shc proteins were involved in this signalling and we compared the activity of wild type G-CSF-R, several mutants (deletion and tyrosine mutants) and a natural splice variant in 32d myeloid cell transfectants. G-CSF induces the transient tyrosine phosphorylation of Shc, especially the p46 isoform of these proteins. This tyrosine phosphorylation is time dependent, peaking around 7 min after stimulation. G-CSF stimulation of 32d mutant M1 ( $\Delta 685$ ), 32d/DA ( $\Delta 715$ ), 32d/M4 ( $\Delta 746$ ) and 32d/M4 ( $\Delta 756$ ) transfectants did not induce phosphorylation of Shc. Binding of Shc to grb2 was demonstrated by anti-grb2 immunoblots of Shc immunoprecipitants of 32d/WT cell lysates. We deduced that G-CSF-R activates the ras-raf-1 pathway, most probably through Shc proteins and grb2 adaptor protein, similarly to what is observed for EPO-R and GM-CSF-R. From our observations, the G-CSF-R cytoplasmic region which regulates Shc phosphorylation is located distal to box 2 and includes the box 3 subdomain. Thus, Shc phosphorylation and grb2 protein binding could be involved in granulocytic maturation signal transduction in myeloid cells.

002

**SINGLE OR MULTI-STEP ORIGIN OF HEMOPOIETIC TUMORS: THE CONTRIBUTION OF CLONALITY STUDIES**

Guerrasio A,\* Rosso C,\* Panetta P,<sup>o</sup> Cilloni D,\* Saglio G,\* Lo Coco F<sup>o</sup>

*\*Dip. di Scienze Biomediche e Oncologia Umana e CNR-CIOS, Università di Torino, Osp. San Luigi Gonzaga, Orbassano-Torino, Italy; <sup>o</sup>Dip. di Biopatologia Umana, Sezione di Ematologia, Università "La Sapienza", Roma, Italy*

Several types of human cancer, particularly solid tumors, arise as a consequence of a multistep process. The situation is more puzzling for hematopoietic tumors. Clonality assays based on study of X chromosome inactivation patterns provide a helpful approach to understanding the problem of a single or multistep pathogenesis for hematologic malignancies. Apparent clonal hematopoiesis in a portion (25%) of AML patients during morphological remission induced by chemotherapy was previously inter-

preted as evidence of a multistep origin of AML. We used highly informative X-linked polymorphisms in conjunction with a recently developed PCR assay to assess the X chromosome methylation pattern and stringent criteria in order to identify apparently clonal patterns due to constitutionally skewed methylation, we studied a series of patients affected by several types of acute and chronic primary leukemias. This series was selected on the basis of the presence of a tumor-specific and pathogenetically relevant marker (i.e. PML/RAR- $\alpha$ , BCR/ABL, AML1/ETO, CBF $\beta$ /MYH11 rearrangements), and only patients who achieved suppression of their respective leukemia-associated marker through various treatments were included in the study. We were not able to detect clonal hematopoiesis during remission in 51 patients with APL, CML, AMLs and Ph<sup>+</sup>-ALL. We conclude that the persistence of clonal hematopoiesis must be a rare event in *primary* leukemias once the major burden of the leukemic clone is suppressed by treatment, as is reliably documented by the disappearance of specific rearrangements. Although not conclusive for several reasons, these observations do not support the notion of a clonal expansion preceding the leukemic process, at least not in the majority of primary leukemias. Furthermore, clonality studies have been documented to be helpful in identifying cases in which overt leukemia represents the evolution of a previously unrecognized myelodysplastic syndrome and in which the leukemic phenotype is likely to be due to an accumulation of multiple *minor* genetic lesions rather than to a single strong leukemogenic event.

003

**THE PROTEIN-TYROSINE KINASE INHIBITOR GENISTEIN HAS A STRONG ANTIPROLIFERATIVE EFFECT ON NORMAL AND LEUKEMIC HEMATOPOIETIC PROGENITORS BUT SPARES THE MORE PRIMITIVE LTC-IC**

Regazzi E, Carlo-Stella C, Mangoni L, Garau D, Almici C, Rizzoli V

*Bone Marrow Transplantation Unit, University of Parma, Italy*

Protein-tyrosine kinases (PTKs) mediate critical aspects of cellular signalling associated with cell proliferation. It was the aim of the present study to evaluate the effect of the natural PTK inhibitor genistein (Sigma) on hematopoietic progenitors from consenting normal donors (n=5) and chronic myelogenous leukemia (CML, n=5) patients. Three cell fractions were used in this study: light-density mononuclear cells (MNCs), soybean agglutinin-negative (SBA-neg) cells and CD34<sup>+</sup>CD45RA<sup>-</sup> cells obtained by flow sorting (FACSsort, Becton-Dickinson). The effect of genistein was investigated at the level of multilineage (CFU-Mix), erythroid (BFU-E) and granulopoietic (CFU-GM) progenitors. In addition, the antiproliferative action of genistein on the primitive progenitors capable of initiating hematopoiesis in long-term culture (LTC-IC) was also investigated by evaluating the relative frequency (per 2 $\times$ 10<sup>6</sup>) of LTC-IC generated by untreated and genistein-treated cell fractions cultured for

5 weeks over irradiated M2-10B4 cells. Increasing doses (1-100  $\mu\text{M}$ ) of genistein induced statistically significant ( $p \leq 0.05$ ), dose-dependent suppression of colony formation from normal CFU-Mix, BFU-E, and CFU-GM generated by MNCs, SBA-neg cells and CD34<sup>+</sup>CD45RA<sup>-</sup> cells. For normal progenitors, genistein concentrations inducing 50% inhibition ( $\text{ID}_{50}$ ) of colony formation ranged from 18 to 53  $\mu\text{M}$ . Similarly, genistein (1-100  $\mu\text{M}$ ) suppressed in a dose-dependent manner CFU-Mix, BFU-E, and CFU-GM ( $\text{ID}_{50}$  values: 20, 28, and 40  $\mu\text{M}$ , respectively) generated by CML samples. The growth inhibitory values for CML progenitors were not significantly different from those calculated for normal progenitors. Preincubation experiments revealed that a one-to-two-hour exposure of either normal or CML-derived MNCs to genistein (200  $\mu\text{M}$ ) followed by repeated washings induced a significant suppression of CFU-Mix, BFU-E and CFU-GM progenitors (range: 30 to 80%). The relative frequency of LTC-IC was not affected by genistein concentrations (50-200  $\mu\text{M}$ , 1-2 hrs) capable of inducing a complete suppression of CFU-Mix, BFU-E, and CFU-GM. To detect significant suppression of LTC-IC an 18-hr incubation with genistein (200  $\mu\text{M}$ ) was required. In conclusion, the present data demonstrate that: (i) continuous exposure to genistein induces a significant growth inhibition of normal and leukemic CFU-Mix, BFU-E, CFU-GM; (ii) the antiproliferative effect of genistein following transient drug exposure suggests an irreversible effect of genistein on cell proliferation; (iii) LTC-IC are spared at genistein concentrations that induce complete inhibition of multipotent and lineage-restricted progenitors. The therapeutic potential of PTK inhibitors will require further investigation.

004

#### **AM580, A STABLE BENZOIC DERIVATIVE OF ALL-TRANS RETINOIC ACID, HAS POWERFUL AND SELECTIVE CYTO-DIFFERENTIATING EFFECTS ON APL CELLS**

Gianni M, Li Calzi M, Terao M, Guiso G, Caccia S, Barbui T, Rambaldi A, Garattini E  
*Istituto di Ricerche Farmacologiche "Mario Negri", Milano e  
 Divisione di Ematologia, Ospedali Riuniti, Bergamo, Italy*

All-trans retinoic acid (ATRA) is used successfully in the cyto-differentiating treatment of acute promyelocytic leukemia (APL). APL cells express PML-RAR, an aberrant form of the retinoic acid receptor derived from the leukemia-specific t(15;17) chromosomal translocation. Here, we demonstrate that AM580, a stable synthetic derivative of retinoic acid, is a powerful inducer of granulocytic maturation in NB4, an APL-derived cell line, and in freshly isolated APL blasts. Following treatment of APL cells with AM580 either alone or in combination with two other differentiation agents, i.e. G-CSF and dibutyryl cAMP, the compound induces granulocytic maturation, as assessed by determining the levels of LAP, CD11b, CD33 and G-CSF receptor mRNA, at concentrations that are 10-100 times lower than those of all-trans retinoic acid (ATRA) necessary to produce similar effects. By contrast, ATRA is more so than AM580 in modulating the expression of these differentiation markers in the HL-60 cell line. In addition, the natural retinoid is as effective or

more effective than the synthetic congener in regulating the expression of LAP in freshly isolated granulocytes obtained from the peripheral blood of patients during the stable phase of the disease. These results demonstrate that AM580 is more powerful than ATRA in modulating the expression of differentiation antigens only in cells where PML-RAR is present. Binding experiments, using transiently transfected PML-RAR and the normal retinoic acid receptor type  $\alpha$  (RAR $\alpha$ ), demonstrate that AM580 has a lower affinity than ATRA for both receptors. However, in the presence of PML-RAR, the synthetic retinoid is a much better transactivator of retinoic-acid-responsive-element-containing promoters than the natural retinoid, whereas AM580 and ATRA have similar activity in the presence of RAR $\alpha$ . This may explain the strong cyto-differentiating activity of AM580 in PML-RAR-containing leukemic cells.

005

#### **QUANTIFICATION OF HUMAN EARLY HEMOPOIETIC PROGENITOR CELLS (pre-CFU) IN LIQUID CULTURE: CORRELATION WITH PREVIOUS CHEMOTHERAPY**

Bruno B, Ferrero D, Cherasco C, Gallo E, Pileri A  
*Dip. di Medicina ed Oncologia Sperimentale, Div. Universitaria di Ematologia, Azienda Ospedaliera S. Giovanni, Torino, Italy*

Quantification of normal early hemopoietic progenitor cells (pre-CFU) is commonly evaluated by complex methods, such as long-term culture initiating cell assay, that cannot be routinely performed to determine marrow repopulating capacity after intensive chemotherapy. We recently devised a simple technique to quantify, by liquid culture only without the need for stromal layers, a pre-CFU population, the growth of which is inversely correlated with previous chemotherapy. Bone marrow samples from 9 normal subjects and 9 patients treated with different chemotherapeutic protocols were enriched in hemopoietic progenitors by removal of mature myelo-monocytic cells on a density gradient after phagocytosis of opsonized yeast particles. Baseline CFU-GM formation was evaluated, while the remaining cells were treated with two monoclonal antibodies, CD33 and CD38, plus rabbit complement complex that killed over 99% of CFU-GM, BFU-E and CFU-GEMM. Cells were then cultured in liquid medium (IMDM+20%FBS) supplemented with stem cell factor (20 ng/mL) and 10% conditioned medium from the 5637 cell line (5637-CM) in microwells at different concentrations ( $10^4$ - $10^3$ /well). Evaluation of the wells as positive or negative for cell growth at day 21 allowed determination of clonogenic cells. Further immunological characterization indicated that more than 90% of these cells expressed the HLA-DR antigen, indicating a population that lies somewhere between stem cells and CFU. Pre-CFU concentration was  $6.8-88.9/10^5$  cells (non phagocytosing), median 25, and  $0.27-7.2$ , median 0.55, in normal controls and in previously treated samples, respectively ( $p$  0.013). Slightly significant differences were noticed in terms of baseline CFU-GM: 145-590 (median 285) in normal controls and 27-280 (median 185) in treated samples. Our method allows quantification after 3

weeks of culture a pre-CFU population that may be a better indicator of post-chemotherapy marrow repopulating capacity than CFU-GM evaluation.

006

#### INTERLEUKIN-6 (IL-6) AS AUTOCRINE GROWTH AND ANTI-APOPTOTIC FACTOR IN PRIMARY HUMAN ACUTE MONOBLASTIC LEUKEMIA

D'Ippolito G, Santini V, Bernabei P, Rossi Ferrini P.  
Dept. of Hematology, University of Florence, and Azienda Ospedaliera di Careggi, Florence, Italy

The leukemic cell line FLG 29.1, derived from a patient with acute monoblastic leukemia, has been demonstrated to differentiate towards macrophages and osteoclasts. Since this cell line grows continuously in culture without the addition of exogenous factors, we evaluated whether this autonomous growth was regulated by autocrine factors. FLG 29.1 cells were maintained in continuous culture in RPMI 1640, 10% FCS or, alternatively, in serum free medium at 37°C, 5% CO<sub>2</sub>. We employed ELISA assays to investigate the presence of several growth factors and cytokines in cell culture supernatants and we could demonstrate the production of minimal amounts of IL-6. Stimulation of FLG 29.1 cells with 8Br-cAMP 500 µM for 18 hours, both in serum-free and in serum-containing medium, led to a significant increase in the production of IL-6, as demonstrated by ELISA assay, Northern blot and RT-PCR. Other compounds, such as all-trans retinoic acid (ATRA) and TNFα, were not able to induce any increment. FLG 29.1 autonomous proliferation was inhibited in the presence of anti-IL-6 blocking antibody, up to a 43% decrease in tritiated thymidine uptake with respect to control cells. The addition of anti-IL-6-receptor blocking antibody to cell cultures also yielded 61% growth inhibition, clearly indicating that IL-6 is a determinant autocrine factor in FLG 29.1 proliferation. FLG 29.1 cells could be induced to undergo apoptotic cell death by TGFβ or daunorubicin incubation. We demonstrated that contemporary treatment of cells with IL-6 1000 U/mL or 8Br-cAMP protecting against apoptosis, as shown by the decrease in the G<sub>0</sub> peak at FACScan analysis of cells incubated with propidium iodide or by quantitation of DNA digestion products in agarose gel electrophoresis.

These observations demonstrate an important role for autocrine IL-6 in determining human acute monoblastic leukemia cell growth and survival, and suggest that exogenous stimuli may modulate autocrine properties of AMLs.

007

#### EFFECTS OF ALL-TRANS RETINOIC ACID ON SURVIVAL, PROLIFERATION AND DIFFERENTIATION OF HUMAN FETAL PROGENITOR CELLS

Visani G, Zauli G,\* Tosi P, Manfroi S, Ottaviani E, Remiddi C, Capitani S,\* Tura S  
Institute of Hematology "L. e A. Seràgnoli", University of Bologna; \*Institute of Human Anatomy, University of Ferrara, Italy

To evaluate the effects of all-trans retinoic acid (RA) on fetal hematopoiesis, we performed serum-free liquid and semisolid cultures using CD34<sup>+</sup> cells purified from human fetal blood samples. RA, both at physiological (10<sup>-12</sup>M) and pharmacological (10<sup>-7</sup>M) concentrations, significantly (p<0.01) promoted the survival of fetal CD34<sup>+</sup> cells in liquid cultures from day 3 onwards by suppressing apoptosis induced by serum and growth factor deprivation. On the other hand, RA alone had no significant effect on the proliferation or differentiation of fetal hematopoietic progenitors. In the presence of optimal concentrations of recombinant interleukin-3 (IL-3), stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin (Epo), low and high doses of RA induced striking differential effects on CD34<sup>+</sup> cell proliferation in liquid cultures and colony formation in semisolid assays. In fact, 10<sup>-11</sup>M, 10<sup>-12</sup>M RA were able to: 1) significantly (p<0.05) increase [3H]thymidine uptake by fetal CD34<sup>+</sup> cells in liquid cultures; 2) variably promote the growth of pluripotent (CFU-GEMM, p<0.05), early (BFU-meg) and late (CFU-meg, p<0.01) megakaryocyte, granulocyte-macrophage (CFU-GM, p<0.01) and erythroid (BFU-E) progenitors in semisolid cultures.

On the contrary, 10<sup>-6</sup>, 10<sup>-7</sup>M RA induced: 1) an overall inhibition (p<0.01) of CD34<sup>+</sup> cell growth in liquid cultures; 2) a marked suppression of BFU-E colony formation (p<0.01) at all Epo concentrations examined (0.002-0.004 IU/mL); 3) a significant (p<0.01) stimulation of CFU-GM with a shift from mixed granulocyte-macrophage to pure granulocyte colonies, while it had little effect on the growth of CFU-GEMM, BFU-meg and CFU-meg. As a whole, our data demonstrate that RA has direct complex effects on the survival, growth and clonal expansion of fetal hematopoietic progenitor cells, that depend mainly on the presence of recombinant cytokines, the type of progenitor and the RA concentration.

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008

#### TARGETED INTEGRATION OF HHV-6 GENOME NOT ONLY IN LYMPHOID BUT ALSO IN MYELOID LINEAGES

Torelli G, Barozzi P, Marasca R, Morselli M, Ferrari MG, Bocchi A, Luppi M  
Department of Medical Sciences, Section of Hematology, University of Modena, Italy

During a survey for the presence of the human Herpes virus-6 (HHV-6) genome both in lymphoproliferative and immune disorders we selected 8 patients: 3 Hodgkin's diseases (HD), 2 non-Hodgkin's lymphoma (NHL) and 3 multiple sclerosis (MS) cases, which represented a previously unreported kind of HHV-6 latency and/or persistence, characterized by an unexpectedly high number of viral sequences either in peripheral blood mononuclear cells (PBMCs) or in the neoplastic lymph nodes and easily detectable by Southern blot analysis. Using *pulsed field gel electrophoresis* (PFGE) we demonstrated the integration of the entire viral genome in the PBMCs of 3 patients (1 HD, 1 NHL and 1 MS case) as well as in the diagnostic lymph node of 1 NHL patient. Finally, we used *fluorescence in situ hybridization* (FISH) to visualize the region of HHV-6

integration, which proved to be identical in the PBMCs of all 3 patients and was represented by the most distal portion of the p arm of chromosome 17 (17p13). We are currently performing the molecular cloning of the genomic sequences flanking the viral integration site in the PBMCs of one lymphoma patient, hoping to obtain new insights into the biologic meaning of this unexpected phenomenon. Furthermore, we showed by PFGE in the same 3 patients that the *integration pattern* of HHV-6 genome is the same not only in the mononuclear cell fraction, but also in the peripheral blood granulocytes. Thus, HHV-6 might have latently infected a bone marrow precursor cell with the ability to give rise to cells of both myeloid and lymphoid origin. However, we cannot rule out the possibility of an intrauterine viral infection in such cases and we are now also looking for possible integration of the HHV-6 genome in non hematopoietic tissues.

009

#### MDR GENE EXPRESSION IN AVIAN LYMPHOCYTIC DEVELOPMENT

Galimberti S,\* Bernardini N,<sup>o</sup> Bianchi F,<sup>o</sup> Dolfi A,<sup>o</sup> Lupetti M,<sup>o</sup> Petrini M\*

\*U.O. Ematologia, Clinica Medica I and <sup>o</sup>Istituto di Anatomia Umana, Normale Università di Pisa, Italy

Multidrug resistance (MDR) is still the main cause of chemotherapy failure in cancer, and *mdr* gene expression has been reported in bone marrow CD34<sup>+</sup>/CD33<sup>-</sup> cells, in mature T-lymphocytes and in CD5/CD19<sup>+</sup> B-lymphocytes. In a previous report we evaluated the expression of *mdr*-mRNA in the thymus and in the bursa of Fabricius in chicken embryos from day 12 to day 21 in order to monitor mRNA appearance/disappearance during T- and B-cell ontogenesis. PCR assays detected the *mdr*-gene expression between day 14 and 17 in the bursa and from day 12 to birth in the thymus. In order to clarify which bursal population was actually positive, *in situ hybridization* assays employing the specific digoxigenin-labelled probe pCHP1 were performed; *mdr* positivity was detected in the follicula at day 15, whereas samples were as negative at days 13 and 20. These results were also confirmed by *in situ*-PCR assays. Moreover, we detected P-170 protein with two monoclonal antibodies: MM4:17 and JSB1. Immunohistochemistry revealed clear positivity at day 15 in the follicula, without glycoprotein expression at day 13, and strongly reduced perifollicular positivity at day 20. A subsequent purification of lymphocytes demonstrated their exclusive positivity at day 15. These data could confirm the hypothesis of modulation of *mdr* expression during avian lymphocyte ontogenesis.

010

#### EFFECTS OF ERYTHROPOIETIN (EPO) AND DIFFERENTIATING AGENTS ON THE EXPRESSION OF EPO RECEPTOR AND THE SYNTHESIS OF FETAL HEMOGLOBIN IN A LEUKEMIC CELL LINE EXPRESSING ERYTHROID AND MEGAKARYOCYTIC PHENOTYPE AND FUNCTIONAL CHARACTERISTICS

Grossi A, Bagnara G, Bonsi L, Vannucchi AM, Strippoli P, Visconti G, Ieri A, Rossi Ferrini P

Divisione di Ematologia, Università degli Studi, Azienda Ospedaliera Careggi, Firenze; Istituto di Istologia, Università degli Studi, Bologna, Italy

A human megakaryocyte cell line has been established from bone marrow cells obtained from a patient with acute myelogenous leukemia. Cells are CD34<sup>+</sup>, CD33<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>+</sup>, and express immunophenotypic markers of megakaryocyte lineage, such as CD41, (glycoprotein IIb/IIIa) and von Willebrand factor, although they are CD42 (glycoprotein Ib) negative. The megakaryocytic nature of the cell line is further demonstrated by the incorporation of 5-hydroxytryptamine, the release of platelet-like particles in the culture medium, as well as the expression of mRNA for the thrombopoietin receptor (c-MPL). These cells also express an erythroid phenotype; in fact, they are glycophorin A positive and 10% of unstimulated cells stain with an anti-globin  $\gamma$  chain MoAb. Moreover, they express Epo receptor.

Cell proliferation is not dependent on the presence of growth factors, including thrombopoietin, in the culture medium, and cells grow in the presence of 5% of a pool of normal human serum; cytokines are undetectable in the culture medium and cells do not express the corresponding mRNAs. Therefore cell proliferation is not based on autocrine production of growth factors.

Modulation of the erythroid phenotype is observed in an Epo containing medium since cytofluorimetric and mRNA expression of Epo receptor are increased, but not that of the globin  $\gamma$  chain. Only in the presence of sodium butyrate, hemin and hydroxyurea is cells the synthesis of  $\gamma$  chain increased. On the contrary, neither growth factors (MGDF, IL-6, Epo) nor PMA modulate the megakaryocytic phenotype; in particular expression of CD42, a later marker of differentiation with respect to CD41, is not induced.

The biological characteristics of this new cell line lend further support to the hypothesis that megakaryocytes and erythrocytes share a common precursor cell. Moreover, these cells represent a novel model for studying the late differentiation events that drive this bipotential precursor toward a megakaryocytic or an erythropoietic lineage.

011

#### CONSTITUTIVE EXPRESSION OF INTERFERON- $\gamma$ IN STROMAL HUMAN MARROW CULTURES MEDIATES HEMATOPOIETIC SUPPRESSION AND APOPTOSIS OF CD34<sup>+</sup> CELLS

Selleri C,\*<sup>s</sup> Maciejewski JP,\* Sato T,\* Anderson S,\* Young NS\*

\*Hematology Branch, National Heart, Lung, and Blood Institute, Bethesda, MD, Usa; <sup>s</sup>Hematology Division, Federico II University of Naples, Italy

Several lines of evidence suggest that IFN- $\gamma$  plays a pathophysiologic role in aplastic anemia (AA). We used long-term bone marrow cultures (LTBMC) to investigate the pathophysiologic role of IFN- $\gamma$  in BM failure disease. IFN- $\gamma$  added to LTBMC showed a potent inhibitory effect

on the generation of long-term culture-initiating cells (LTC-IC) from CD34<sup>+</sup> cells cultured on preformed, irradiated allogeneic stroma; 1×10<sup>3</sup> CD34<sup>+</sup> plated in LTBMC exposed weekly to 1000 U/mL of IFN-γ did not give rise to any LTC-IC, compared to 6±2 LTC-IC/10<sup>3</sup> CD34<sup>+</sup> in untreated LTBMC. To study the effects of locally secreted IFN-γ on the hematopoietic microenvironment as a model for IFN-γ overproduction *in vivo*, human stromal cells were engineered by retroviral-mediated gene transfer to express a transduced IFN-γ gene. Production of IFN-γ by stroma was detected by RT-PCR and measured by ELISA. Untransduced stromal cultures and those transduced with control virus did not produce IFN-γ, but stromal layers transduced with the IFN-γ virus secreted IFN-γ at a concentration of about 1000 pg/mL (20 U/mL). IFN-γ-transduced stroma strongly inhibited the generation of LTC-IC. Control stromal layers and those transduced with Neo virus showed comparable function in support of LTC-IC generation. Addition of IFN-γ at 20 U/mL to LTBMC showed only a marginal effect on the number of LTC-IC in culture. To determine whether the decreased generation and survival of LTC-IC on stromal layers expressing IFN-γ was related to decreased survival or cell cycle inhibition of CD34<sup>+</sup> cells, we examined the DNA content of CD34<sup>+</sup> cells by flow cytometry. Compared to control cultures, a decreased percentage of CD34<sup>+</sup> cells cultured on IFN-γ-expressing stromal layers was in the G1 and S phases of the cell cycle. A distinct hypodiploid peak, characteristic of apoptosis, indicated that a portion of cells were undergoing programmed cell death in these cultures. LTBMC with genetically altered stromal cells offers an *in vitro* model of immune suppression of hematopoiesis in AA and may be helpful in testing certain therapeutic modalities. Our results strongly suggest that local IFN-γ action in BM tissue can result in hematopoietic suppression and permanent depletion of hematopoietic progenitor and stem cells.

012

#### FLT3 LIGAND IS REQUIRED FOR THE MAINTENANCE OF LTC-IC DURING *EX VIVO* AMPLIFICATION OF G-CSF-MOBILIZED CD34<sup>+</sup>CD45RA<sup>-</sup> HEMATOPOIETIC PROGENITORS

Garau D, Carlo-Stella C, Mangoni L, Regazzi E, Almici C, Rizzoli V  
Bone Marrow Transplantation Unit, University of Parma, Italy

A ligand for the human flt3 tyrosine kinase receptor has recently been cloned. CD34<sup>+</sup>CD45RA<sup>-</sup> cells represent a primitive subset of hematopoietic cells containing CFU-Mix, BFU-E and CFU-GM, as well as long-term culture-initiating cells (LTC-IC). It was the aim of the present study to investigate the role of the flt3 ligand (FL) in the *ex vivo* amplification of CD34<sup>+</sup>CD45RA<sup>-</sup> cells isolated from the peripheral blood of non-Hodgkin's lymphoma patients mobilized with G-CSF. After depletion of soybean agglutinin (SBA)-positive cells (AIS, MicroCELLector™), the SBA-neg fraction was labelled with 8G12-PE (CD34, Becton-Dickinson) and L48-FITC (CD45RA, B-D). Flow sorted (FACSsort, B-D) CD34<sup>+</sup>CD45RA<sup>-</sup> cells were pure up to 95%±2% and negative for CD19 (95%±3%), CD7 (97%±1%), and CD33 (75%±8%). Short-term culture of

sorted cells with SCF, IL-3, GM-CSF, G-CSF and EPO revealed the presence of CFU-Mix, BFU-E and CFU-GM, with a plating efficiency ranging from 15% to 26%. FL (50 ng/mL) was additive with (IL-3+EPO) or (IL-3+EPO+SCF) on CFU-Mix, BFU-E and CFU-GM growth. The relative frequency (per 2×10<sup>6</sup>) of LTC-IC supported by M2<sup>-10</sup>B4 cells ranged from 3,375 to 22,000. For *ex vivo* amplification, sorted cells (1,000/mL) were cultured in suspension in 24-well plates for 7-28 days in a stromal cell-free, serum-containing system supplemented with optimal concentrations of growth factors. Cells were assayed at weekly intervals in short-term and long-term culture. FL synergistically augmented the ability of (SCF+IL-3+IL-6) to promote CFU-GM expansion (18-, 34-, 36- and 30-fold on day 7, 14, 21, and 28, respectively). FL also synergized with (SCF+IL-3+IL-6+G-CSF) and amplified CFU-GM up to 60-fold (Day 21). CFU-Mix were maintained up to day 14 but subsequently disappeared. Using the combination (FL+SCF+IL-3+IL-6+EPO), a 2- to 8-fold increase in BFU-E was observed. Although significantly decreased, LTC-IC were still detectable after 14-21 days of amplification. Only the combination (FL+IL-3+IL-6), associated with a 30-fold increase in CFU-GM, was able to maintain of LTC-IC (70% of day 0 input). In conclusion, our data demonstrate that: (a) FL synergistically interacts with a number of cytokines in the expansion of CD34<sup>+</sup>CD45RA<sup>-</sup>-derived progenitors; (b) *in vitro* amplification in our stroma-free system requires FL for maintenance of LTC-IC.

013

#### CLONALITY AND ELEVATED SERUM LEVELS OF SOLUBLE(s) CD30: NEW INSIGHTS INTO THE PATHOGENESIS OF HYPEREOSINOPHILIC SYNDROME (HES)

Morselli M, Luppi M, Marasca R, Barozzi P, Morabito F,<sup>§</sup> Masini L,\* Pizzolo G,<sup>°</sup> Torelli G  
Department of Medical Sciences, Section of Hematology, University of Modena; \*Division of Hematology, Arcispedale S. Maria Nuova, Reggio Emilia; <sup>§</sup>Division of Hematology, Ospedali Riuniti, Reggio Calabria; <sup>°</sup>Division of Hematology, University of Verona; Italy

A similarity between HES and other myeloproliferative syndromes has been suggested, although the evidence for clonal proliferation of eosinophils has been sparse and based only on the detection of chromosomal abnormalities in the bone marrow of patients with the clinically aggressive variant of HES, otherwise known as eosinophilic leukemia. This prompted us to perform a clonal analysis on DNA extracted from the granulocytes of six female patients affected with HES in order to look for the possible presence of a clonal population, as shown by the methylation status of the X-linked phosphoglycerate kinase (PGK) gene.

CD30 is preferentially expressed on and released by human CD4<sup>+</sup> cells producing type 2 helper T cell (Th2) cytokines, and elevated serum levels of sCD30 have been found in conditions in which a pathogenic role for Th2 cells has been suggested. Since the pathologic features of HES appear to be linked to the production of Th2-type cytokines, we judged it appropriate to measure sCD30 levels on serum samples from these same six patients.

In two patients molecular analysis showed the clonal nature of the disease; it is of interest that the methylation pattern of the PGK gene, which was consistent with clonal hematopoiesis, was also found at recurrence of the disease. Thus, blood hypereosinophilia may be sustained by a myeloproliferative process that is clonal, although probably not fully malignant, as testified by the favorable clinical course.

Serum levels of sCD30 (range of values in normal controls: 0-20 U/mL) was high in three patients (range: 53-119 U/mL), including one of the two patients with a circulating clonal granulocyte population. Of interest, the clinical and hematologic features in these three patients were unequivocally consistent with an active phase of the disease. In contrast, serum levels of sCD30 were found to be low in other two patients, both of whom suffered from the disease in a *plateau phase*: one untreated and the other after steroid treatment. sCD30 may thus be proposed as a new indicator of disease activity in HES, and a possible correlation between serum levels of sCD30 and the clonal nature (not only myeloid but also lymphoid) of the disease merits further investigation.

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014

#### EXPRESSION OF p64/ $\gamma_c$ AND COGNATE CYTOKINE RECEPTOR SUBUNITS IN HUMAN MYELOID CELLS

Cuomo C,<sup>\*o</sup> Agosti V,<sup>#</sup> Bond HM,<sup>#</sup> Carandente Giarrusso P,<sup>#</sup> Petrella A,<sup>o</sup> Notaro R,<sup>s</sup> Camera A,<sup>s</sup> Biondi A,<sup>@</sup> Rambaldi A,<sup>‡</sup> Rotoli B,<sup>s</sup> Morrone G,<sup>\*o</sup> Venuta S<sup>\*o</sup>

<sup>\*</sup>Dept. of Experimental and Clinical Medicine, Catanzaro;

<sup>#</sup>Dept. of Biochemistry and Medical Biotechnology and

<sup>s</sup>Division of Clinical Hematology, Federico II University,

Naples; <sup>o</sup>CEINGE, Genetic Engineering Centre, Naples;

<sup>@</sup>Dept. of Pediatrics, Univ. of Milan; <sup>‡</sup>Dept. of Hematology,

Ospedali Riuniti and Istituto Mario Negri, Bergamo, Italy

The  $\gamma$  common chain (p64/ $\gamma_c$ ) is a shared subunit of multiple hemopoietin receptor complexes (IL2R, IL4R, IL7R, IL9R, IL15R), where its presence is required for the generation of high-affinity binding sites and for signal transduction. So far, p64 has been studied mainly in cells of lymphoid origin. We sought to investigate the expression and possible role of this molecule and of its cognate cytokine receptor chains in human normal and malignant myeloid cells.

$\gamma_c$  mRNA expression is ubiquitous in all human myeloid cells. CD34<sup>+</sup> hematopoietic progenitors conserve relatively high levels of this transcript throughout their granulo-monocytic as well as erythroid differentiation. A strong increase in the expression of p64 mRNA and protein occurs during the megakaryocytic, but not the erythroid, maturation of K-562 cells. Cell-surface IL-2 binding and transcripts for p64-related chains are scarcely detectable in these cells, whereas the number of GM-CSF binding sites is up-regulated by TPA in a fashion resembling that of p64, suggesting that, at least in some cell types, functional interactions or cross-talk may occur between  $\gamma_c$  and GM-CSFR, and/or additional receptor complexes.

IL-7, one of the cytokines acting through p64 and chiefly known as a growth factor for lymphoid progeni-

tors, has recently been demonstrated to stimulate human granulo-monopoiesis in cooperation with other hemopoietins. We analyzed IL7R expression in erythroid cells and found high levels of the mRNAs for both the IL7Ra chain and  $\gamma_c$  in cord blood erythroblasts. A significant up-regulation of IL7Ra mRNA occurred as a late event during the erythroid differentiation of CD34<sup>+</sup> cells. In *in vitro* colony assays, IL-7 enhanced consistently, although to a modest extent, the erythropoietin-dependent generation of erythroid colonies, thereby supporting the notion that IL-7 may act as a co-factor in the regulation of erythropoiesis.

Acute myelogenous leukemia (AML) cells were also investigated and found to express consistently p64 mRNA and protein. The majority of the cases studied also expressed mRNAs for other IL2R, IL4R and IL7R chains, indicating that AML blasts are likely to possess functional receptor complexes for, and therefore be potentially responsive to, these cytokines *in vivo*. In preliminary experiments IL-7 has indeed proven to be capable of modulating the proliferation of the majority of AMLs tested. Study of the signal transduction molecules associated with p64 will help clarify the role of this and other related cytokine receptor chains in myeloid leukemic cells.

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014bis

#### FLOW CYTOMETRY QUANTITATION OF GM-CSF RECEPTORS IN ACUTE LEUKEMIA AND NORMAL HEMOPOIETIC CELLS

Lanza F, Rigolin GM, Moretti S, Latorraca A, Bardi A, Castagnari B, Ferrari L, Spanedda R, Scapoli GL, Castoldi GL

*Institute of Hematology, University of Ferrara, Italy*

Cell reactivity for the GM-CSF/R was evaluated by flow cytometry in different disease categories: AML (n. 35), ALL (n. 9), MDS (n. 10), and healthy volunteers (n. 10), using three different GM-CSF/R monoclonal antibodies (MoAbs) (HGM-CSFR, M5D12, 4B5F5). Flow cytometry data were expressed in the form of molecular equivalents of soluble fluorochrome (MESF) based on FITC expression by quantitative microbead calibration standards. Through assessment of the protein-fluorochrome ratio for the various antibodies, we also calculated the antibody-binding capacities (ABC) per cell. In healthy subjects GM-CSF/R was detectable on blood monocytes, neutrophils and bone marrow myeloid precursors, including a subset of CD34<sup>+</sup> progenitors committed to the myelo-monocytic pathway. Among AML samples, M5D12 McAb was positive in 33%, 4B5F5 Mc Ab in 90%, H-GMCSF/R Mc Ab in 80% of the cases examined (range of ABC: 1,000-66,000). The highest ABC values were within the M4 and M5 FAB subvarieties, while ALL samples were negative except in two cases (My+ALL: 1; pro-B ALL: 1). In patients with FAB M0-M1, GM-CSF/R<sup>+</sup> blasts co-expressed CD34<sup>low</sup> HLA-DR<sup>high</sup>, CD33, CD38 antigens, and had little capacity to form CFU-GM colonies. Taking the data altogether, we found the HGM-CSF/R MoAb correlated positively with CD13 (p < 0.017), CD14 (p < 0.0001), and CD33 (p < 0.012). In conclusion, correct application of flow cytome-

try technology together with calibration microbeads allows precise and reliable quantitation of MESF and ABC values, permitting comparability of data over time and between laboratories. The determination of GM-CSF receptors also offers the possibility of selecting patients suitable for GM-CSF therapy following intensive myeloablative chemotherapy regimens, thus avoiding the risk of stimulating the proliferation of the leukemic clone. Moreover, in AML patients expressing the receptor for GM-CSF, the possibility of using GM-CSF is proposed with the aim of recruiting blast cells into the active phase of the cell cycle prior to chemotherapy. It is conceivable that this last technique could increase the cell killing rate and possibly the remission rate in patients with GM-CSF/R<sup>+</sup> leukemic cells.

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015

#### INTERFERON REGULATORY FACTOR-1 PARTIALLY MEDIATES INTERFERON- $\gamma$ HEMATOPOIETIC SUPPRESSION

Selleri C,<sup>\*</sup> Sato T,<sup>\*</sup> Anderson S,<sup>\*</sup> Young NS,<sup>\*</sup> Maciejewski JP<sup>\*</sup>

<sup>\*</sup>Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, MD, USA; <sup>†</sup>Hematology Division, Federico II University of Naples, Italy

Interferon (IFN) regulatory factor-1 (IRF-1) is a member of a transcription factor family that also includes IRF-2 and IFN consensus sequence binding protein. IRF-1 was originally implicated in the IFN- $\alpha$  signal transduction pathway but subsequently was also found to be induced by IFN- $\gamma$ , tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, leukemia inhibitory factor (LIF) and prolactin. IRF binding sequences (IFN-stimulated responsive element) have been localized in the promoter regions of several genes, including p53, inducible nitric oxide synthase (iNOS), ornithine decarboxylase, intercellular adhesion molecule-1 (ICAM-1), FAS ligand and IRF-2. Both IFN- $\gamma$  and TNF- $\alpha$  exert inhibitory activity on hematopoiesis *in vitro*, and they may play a pathophysiologic role in BM failure syndromes. We investigated whether the inhibitory effects of these cytokines were intracellularly mediated through the expression of IRF-1 or IRF-2 in target cells. Northern blot analysis showed that both IFN- $\gamma$  and TNF- $\alpha$  enhanced constitutive levels of IRF-1 expression in total BM cells; IFN- $\gamma$  demonstrated the stronger effect. In contrast, IRF-2 expression levels were not affected by IFN- $\gamma$  or TNF- $\alpha$ . Using reverse-transcriptase polymerase chain reaction (RT-PCR) we demonstrated very low levels of IRF-1 expression in highly purified fresh or unstimulated CD34<sup>+</sup> cells; after 18 hours of exposure to IFN- $\gamma$  and TNF- $\alpha$ , amplified IRF-1 mRNA showed a much stronger signal than controls. To exclude the possibility that IRF-1 mRNA expression was related to the differentiation of the CD34<sup>+</sup> population to more mature cells and not to the induction of IRF-1 in immature CD34<sup>+</sup> cells, BM CD34<sup>+</sup> cells were enriched, stimulated with IFN- $\gamma$  or TNF- $\alpha$ , and subsequently resorted based on the presence of the CD34 antigen in order to exclude cells which differentiated dur-

ing the culture period. PCR analysis revealed that IRF-1 mRNA expression is induced in CD34<sup>+</sup> cells and that this effect is not related to differentiation. Since IFN- $\gamma$  and TNF- $\alpha$  both suppress colony formation by CD34<sup>+</sup> cells in methylcellulose cultures, we tested whether the effects of these cytokines were mediated through IRF-1. IRF-1 antisense oligonucleotides that targeted the translational initiation site partially reversed the suppressive effects on CD34<sup>+</sup> cell-derived colony formation by IFN- $\gamma$ , but not by TNF- $\alpha$ . These results suggest that IRF-1 is involved in the activation of cellular genes responsible for IFN- $\gamma$  suppressive effects on hematopoiesis.

016

#### DEFICIENCY OF GLYCOSYL-PHOSPHATIDYL-INOSITOL (GPI)-LINKED MOLECULES IN CYTOPENIC PATIENTS: A PHENOTYPIC AND GENOTYPIC STUDY

Musto P, Cascavilla N, D'Arena G, Scalzulli P, Savoia A,<sup>\*</sup> Ianzano L,<sup>\*</sup> Matera R, Carotenuto M

<sup>\*</sup>Hematology and <sup>†</sup>Genetics, IRCCS "Casa Sollievo della Sofferenza", S.Giovanni Rotondo, Italy

We employed peripheral blood flow cytometry to analyze surface expression of 3 GPI-linked molecules (CD66b on neutrophils, CD14 on monocytes, CD59 on erythrocytes) in 36 cytopenic patients with heterogeneous clinical and laboratory features all negative for qualitative Ham's test, and in 4 subjects with classic paroxysmal nocturnal hemoglobinuria (PNH). Six patients with a partial or total deficiency of at least one of the antigens tested were identified. Previous diagnoses in these subjects were: thrombosis of hepatic veins (1 case), bone marrow hypoplasia (1 case), myelodysplasia (4 cases). In 2 patients with initially normal expression of GPI-linked molecules, a PNH phenotype appeared several months after diagnosis of a myelodysplastic syndrome and it was associated with symptoms and hematological signs typical of PNH, but with persistent negativity for Ham's test. The deficiency of GPI-linked molecules observed in the patient with bone marrow hypoplasia was transient. All patients with classic PNH showed a marked deficiency (>90%) of the antigens tested. A mutation of the PIG-A gene, identified by means of gene amplification followed by RNA-SSCP, was found in 3/4 patients with *overt* PNH and in 2/3 of those with a PNH phenotype *associated* with myelodysplasia. Our study suggests that deficiency of GPI-linked molecules is not strictly associated with positivity for Ham's test and demonstrated the clinico-biological heterogeneity of this phenomenon.

016bis

#### MOLECULAR HETEROGENEITY OF G6PD DEFICIENCY IN CAM-PANIA

Alfinito F, Filosa F,<sup>\*</sup> Cimmino A, Pandolfi G, Martini G,<sup>\*</sup> Rotoli B

<sup>\*</sup>Cattedra di Ematologia, Università Federico II and <sup>†</sup>IIGB, CNR, Naples, Italy

G6PD deficiency is the enzymopathy occurring most frequently in the world. Nearly 300 variants have been identified so far and biochemical characterization shows that they are produced by allelic mutations of the G6PD gene. Molecular analysis has only partially confirmed this heterogeneity, defining 50 different point mutations. G6PD Mediterranean is the most common variant found in Italy, although a certain biochemical heterogeneity has been reported among the various regions of the country. Here we report on the molecular characterization of G6PD mutations in 36 G6PD-deficient unrelated, male patients. Thirty-four were from Campania, one from Calabria and one from Puglia; 33 patients showed a severe (G6PD activity < 5%) and 3 a moderate (>10%) defect. Investigation was carried out using the PCR technique for known mutations. Fourteen out of the 33 patients with a severe defect (42.4%) demonstrated the Mediterranean mutation (563 C→T), 7 (21.2%) the Seattle mutation (844 G→C); 4 (12.1%) were A and the mutation that produces this deficiency is still under study. Two patients presented the Cassano mutation (1347 G→C), one the Maewo (1360 C→T) and one the Cosenza (1376 G→T); 2 out of the 3 patients with a moderate defect exhibited the Seattle mutation. We also looked for other mutations such as Coimbra (592 C→T), Montalbano (854 A→G), Sibari (634 A→G), S. Antioco (1342 A→G). All the samples with an unidentified mutation (5 with severe and 1 with moderate defect) were amplified for all exons and processed by the SSCP technique. In two samples a shift in the fragment amplified from exons XI-XIII was found. We are now sequencing these exons. Our data suggest there is molecular heterogeneity in the G6PD defect in Campania and that the incidence of the Mediterranean mutation here differs from that found in other Italian regions.

017

#### **HYPOREGENERATIVE ANEMIA DUE TO PARVOVIRUS B19 INFECTION: DIFFERENT BEHAVIOR IN ONE OF TWO BROTHERS WITH HEREDITARY SPHEROCYTOSIS CAUSED BY IMMUNE RESPONSE DEFICIT**

Salmaso F, Silvestri F, Barillari G, Infanti L, Zaja F, Zorzenon M,\* Squecco D,\* Botta GA,\* Zerbinì M,<sup>o</sup> Bacarani M

*Division of Hematology, \*Chair of Microbiology, Udine University Hospital, Udine and <sup>o</sup>Chair of Microbiology, University of Bologna, Italy*

Parvovirus B19, the agent of the so-called *fifth disease*, is well recognized as a cause of hyporegenerative anemia in patients affected by hereditary spherocytosis. A congenital or acquired immunodeficiency has been indicated as the mechanism responsible for the persistence of the virus in some affected patients, resulting in the continuation of anemia. We report herein the cases of two brothers affected by hereditary spherocytosis with hyporegenerative anemia due to B19, in whom resolution of the condition was dependent on the immune response, which was different in the two patients. In June 1994 two brothers PM and PA (25 and 30 years, respectively) affected by hereditary spherocytosis came to our attention for severe anemia (Hb 6.1 g/dL and 4.0 g/dL, respectively). This consultation had

been recommended by the family doctor because the two had been febrile and very tired for several days. Physical examination revealed subicterus, tachycardia and splenomegaly. Among the laboratory tests, the Coombs' test was negative, total bilirubin was less than 2 mg% and reticulocytosis was absent. Bone marrow aspirates, perfectly superimposable in the two, showed a hypercellular picture with hyperplastic erythropoiesis represented only by giant E1 cells. The suspicion of B19 infection was confirmed by the finding of specific IgM positivity (IIF). The two patients were started on therapy with steroids and folic acid; however, the clinical behavior was very different. After ten days of therapy, PM's hemoglobin rose from 6.1 to 9.7 g/dL, the steroids were tapered and, in a subsequent test, the hemoglobin returned to baseline levels. PA's hemoglobin levels followed a very different course, with severe anemia persisting for 45 days and moderate anemia for another 60 days. During the course of the disease the viral genome was serially detected in both serum and marrow. It was sought in the serum with dot-blot hybridization and polymerase-chain-reaction (PCR) while *in situ hybridization* was used to search for the viral genome in marrow erythroid precursors. While it was possible to document clearance of the virus in PM one month after diagnosis, the same result was obtained in PA only four months later. At the same time the IgG assay, using the Ideia-Dako kit, showed a great antibody response (4+) that was already evident by the tenth day in PM, while in PA the specific IgG was stable (1+) over the entire time. The cases reported confirm that: 1) the B19 virus infects marrow erythroid precursors, producing a cytolytic effect; 2) the virus is detectable in the patient's serum, and with sensitive methods such as PCR it is possible to follow its clearance; 3) the antibody response is responsible for the clearance of the virus and, consequently, for the duration of the anemia. Our results also indirectly indicate that treatment with hyperimmune specific immunoglobulins is probably the most correct strategy for obtaining quick clearance of the virus.

018

#### **ALTERED N-LINKED CARBOHYDRATE BIOSYNTHESIS IN CONGENITAL ANEMIA HEMPAS**

Izzo P,\* Zingoni A,<sup>o</sup> Forbici F,<sup>o</sup> Baschieri S,<sup>o</sup> Bendiak B,<sup>#</sup> D'Agostaro G<sup>o</sup>

*\*Dipartimento di Scienze Biomediche ed Oncologia Umana, Università di Bari; <sup>o</sup>Dipartimento Ambiente, ENEA, Rome; Italy; <sup>#</sup>Biomembrane Institute and University of Washington, Seattle, Usa*

Congenital dyserythropoietic anemia type II or HEMPAS (*Hereditary Erythroblastic Multinuclearity with a positive acidified serum lysis test*) is a rare anemia in humans, inherited as an autosomal recessive trait. Biochemical and structural studies have indicated that HEMPAS anemia is caused by defects in the biosynthesis of N-linked complex carbohydrates (1). A marked defect of GnT II activity has been observed in two HEMPAS patients, while a defect of  $\alpha$ -Man II activity has also been reported in another patient (1). These two Golgi-localized enzymes act sequentially in the biosynthetic pathway leading from hybrid to complex N-linked carbohydrates.

cDNA and genomic clones for human GnT II were isolated using rat GnT II cDNA (2) as a probe. The human GnT II gene, which contains a single exon uninterrupted by introns, was mapped to the long arm of chromosome 14 (14q21) by fluorescent *in situ* hybridization. Transcription of the GnT II gene starts at ~450 bp upstream of the translation initiation codon and generates two mature transcripts of 2.8 and 2.0 kb, which terminate downstream of two distinct poly A addition signals. Sequence analysis indicates that the 2.0 kb GnT II transcript is devoid of structural motifs that might affect mRNA stability and/or translational proficiency. Northern Blot analysis showed that the abundance of the 2.0 kb GnT II transcript is reduced in four HEMPAS patients out transcripts of 4.6 and 4.2 kb, which presumably result from a differential utilization of distinct polyadenylation signals, was also observed in the HEMPAS patients. It is intriguing to speculate that HEMPAS anemia may be the result of genetic mutation(s) which affect(s) the molecular mechanisms controlling the maturation of the primary transcripts of the GnT II and  $\alpha$ -Man II genes. Structural and genetic linkage studies are in progress.

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019

#### A NOVEL DELETION, WITH A 3' BREAKPOINT IN AN hsRTVL-H ELEMENT, ASSOCIATED WITH $\beta^0$ THALASSEMIA AND HFPFH

Carestia C, Lacerra G, Sabato V, De Angioletti M, De Mattia D, Schettini F

IIGB, CNR, Napoli, Dipartimento di Biomedicina e dell'Età Evolutiva, Università degli Studi di Bari, Italy

$\beta$ -thalassemia is extremely heterogeneous at the molecular level; in the majority of cases this is due to point mutations and only rarely to deletions.

We report here a novel deletion detected in a family from Southern Italy. Hematological data were obtained by standard methods and globin chains were analyzed by HPLC and biosynthesis *in vitro*; the structure of the globin gene clusters was analyzed by restriction mapping, the deletion breakpoints were identified by inverse-PCR and PCR-sequencing and screening of point mutations was carried out by allele-specific-PCR and PCR-sequencing.

The propositus, a 13-year-old boy, suffered from a mild thalassemia intermedia syndrome. Hb (10.6 g/dL), MCV (60 fL) and MCH (19 pg) were low; Hb F was 95%, Hb A2 5%, bilirubin 1.3 mg/dL, serum iron 167 mg/dL.

The parents presents hematological alterations of the  $\beta$ -thalassemia heterozygous type; in addition, the father possessed Hb F 9%. Globin chain HPLC in the propositus showed that  $\beta$ -chains were completely absent and the G $\gamma$ :A $\gamma$  ratio was 43:57; the G $\gamma$ +A $\gamma$ / $\alpha$  chain biosynthesis ratio was 0.47. DNA analysis revealed that the patient had inherited the  $\beta$ -thalassemia codon 44 -C mutation from the mother and a long deletion from the father. This deletion completely removed the  $\beta$ -globin gene. At 5' it starts 2,134 bp 3' to the polyA of the  $\delta$ -globin gene and is about 67 kb long; the 3' is inside an hsRTVL-H element located about 60 kb 3' to the  $\beta$ -globin gene. This element, about 6

kb long, belongs to a human DNA repetitive family and includes terminal direct repeats of 415 bp with features of long terminal repeats (LTRs) of retroviruses. In our case the LTR at the 5' extremity is absent. The deletion blocks the  $\gamma$ -globin gene switch-off and allows  $\gamma$ -globin gene expression to continue into adult age, but Hb F synthesis in the propositus is less than Hb A synthesis in the  $\beta$ -thalassemia heterozygotes and this causes a thalassemia intermedia phenotype. Several factors may play a role in blocking the switch-off of the  $\gamma$ -globin genes: the absence of the  $\beta$ -globin gene and its 5' sequences (3,632 bp 5' from the cap site); the remotion of the LTR element; the shortening of the distance between the  $\gamma$ -globin genes and enhancer elements possibly present at 3' of the deletion.

020

#### ANEMIA IN LIVER CIRRHOSIS: NEW INSIGHTS INTO THE PATHOGENETIC MECHANISMS

Ricerca BM, Siciliano M,\* Milani A,\* Storti S, Tomasello D,\* Vigevani F, Mezzabotta M, Rossi L\*

Istituto di Semeiotica Medica; \*Istituto di Patologia Medica. Università Cattolica del Sacro Cuore, Rome, Italy

Chronic anemia is a common feature in liver cirrhosis patients. Several factors are involved in the pathogenetic mechanism, such as chronic gastrointestinal bleeding, the resulting iron deficiency, folate and cobalamin deficiencies. It is well known that increase in erythropoietin (EPO) secretion is a major physiologic response to anemia, but only scant literature data are available about serum EPO in liver cirrhosis anemia.

We measured serum EPO in 48 cirrhotic patients with different etiologies, without obvious gastrointestinal bleeding in the two months before the investigation and with normal renal function. According to the Child-Pough classification 13 patients belonged to class A, 14 to class B and 21 to class C; 38 of them were anemic. Eleven normal subjects and 23 patients with uncomplicated iron deficiency anemia were studied as controls. EPO was measured with an immune-enzyme assay (EPO-EIA, bioMerieux). In non-anemic cirrhotic patients the serum EPO level was similar to that of healthy controls (mean $\pm$ SE: 15.66 $\pm$ 2.48 U/L vs 12.46 $\pm$ 1.02 U/L). Regression analysis between EPO and Hb showed a significant inverse exponential correlation both in the cirrhotic patients ( $r=-0.55$ ) and in the controls ( $r=-0.92$ ). The slopes of the two correlation curves were significantly different (cirrhotic =  $-0.07$ ; controls =  $-0.19$ ,  $p<0.005$ ), with lower EPO levels in the liver patients for the same degree of anemia. Furthermore, covariance analysis showed lower Hb-adjusted EPO levels in Child class C patients than in classes A and B. Our data indicate that an EPO response to anemia is present in liver cirrhosis, but that it is blunted in correlation with the severity of the disease.

We retain that impaired EPO response could play a role in the pathogenesis of liver cirrhosis anemia. Several hypotheses can be formulated about the causes of the reduced hormone levels; among them, the intervention of cytokines with an inhibitory effect on EPO secretion, as is observed in the anemia of chronic disorders (ACD), could be involved.

021

**THALASSEMIA INTERMEDIA IN PATIENTS FROM EAST SICILY: MOLECULAR BASIS AND PHENOTYPES**

De Angioletti M, Lacerra G, Alessi M, Sortino G, Crisafulli S, Cacciola E, Carestia C  
 IIGB, CNR, Napoli. *Cattedra di Ematologia, Università degli Studi di Catania, Italy*

*Thalassemia intermedia* is a very heterogeneous  $\beta$ -thalassemic syndrome characterized by anemia that does not require regular blood-transfusion therapy.

We studied genotypes and phenotypes in 28 patients from East Sicily.

We analyzed hematological parameters by standard methods, the structure of the  $\alpha$ - and  $\beta$ -globin gene clusters by restriction mapping, point mutations by allele-specific PCR and PCR direct sequencing. The patients ranged in from 6 to 78 years; 22 of them had never or rarely been transfused, 6 were transfusion dependent for various periods of time ranging from 5 to 53 yrs. Patients were divided into five principal groups on the basis of molecular data regarding  $\beta$ -thalassemia mutations, Hb variants and  $\alpha$ -globin genotype. Six were  $\beta$ -IVS-I-6 T $\rightarrow$ C homozygotes; seven were compound heterozygotes for -87 C $\rightarrow$ G and  $\beta$ -IVS-I-6 (5) or  $\beta$ -IVS-I-110 (1) or  $\beta$ -39 (1); four were double heterozygotes for triplication of the  $\alpha$ -globin genes and  $\beta$ -IVS-I-1 (1) or  $\beta$ -IVS-I-110 (1) or  $\beta$ -IVS-I-110/ $\beta$ -101 (2); seven were compound heterozygotes for Hb variants and  $\beta$ -thalassemia: Hb S/ $\beta$ -39 (2), Hb S/ $\beta$ -IVS-I-110 (1), Hb S/ $\beta$ -IVS-I-1 (1), Hb C/ $\beta$ -39 (2), Hb O-Arab/ $\beta$ -39 (1); four presented interaction of mild thalassemia with  $\beta^0$  or  $\beta^+$  mutations:  $\beta$ -IVS-I-110/ $\beta$ -IVS-I-6 (1),  $\beta$ -39/ $\beta$ -101 (1),  $\beta$ -IVS-I-110/ $\beta$ -101 (1), Hb Lepore/ $\beta$ -IVS-II-745 (1).

Analysis of phenotypes in each group led to some conclusions which appear to be relevant to genetic counselling and prognosis. First, the molecular basis of thalassemia intermedia in East Sicily is extremely heterogeneous considering that 16 different genotypes were detected in 28 patients. Second, the majority (4/7) of the compound heterozygotes for -87 and another  $\beta$ -thal mutation became transfusion dependent in adult age, and this was not correlated with the type of  $\beta$ -thalassemia mutations in trans to the -87; of the remaining 21 patients, only 2 (genotypes:  $\beta$ -IVS-I-6 homozygote;  $\beta$ -IVS-II-745/Hb Lepore) became transfusion dependent. Third, there was a heterogeneity in the extent of the disturbance, in the age at diagnosis and in the need for blood transfusions in all five groups of patients, and this was detected even in patients belonging to the same family and carrying the same genotype.

022

**WIDE-SCALE RE-EVALUATION OF THE INCIDENCE OF  $\beta$ -THALASSEMIA IN SARDINIA**

Longinotti M, Dore F, Pardini S, Pistidda P,\* Guiso L,\* Frogheri L,\* Bonfigli S, Nieddu RM, Oggiano L, Inzaina A,<sup>o</sup> Mulas P<sup>o</sup>

*Istituto di Ematologia, Università di Sassari; \*Istituto CNR per lo Studio delle Patologie del Sangue e degli Organi Emopoietici, Sassari; <sup>o</sup>Lab. Analisi USL 3, Tempio; <sup>o</sup>Centro Trasfusionale USL 4, Olbia; Italy*

During scholastic screening for the prevention of thalassemia carried out in north Sardinia, 11,527 youngsters from the provinces of Sassari and Oristano were examined. The screening, which was accompanied by conferences, debates and informative lessons, was directed at two age groups: 1) pupils in their last year of obligatory education in order to examine and inform the maximum number of subjects; 2) youngsters in their last year of higher education in order to identify carriers of  $\beta$ -thalassemic trait and to enable them to benefit from eugenic information in the near future.

The scholastic screening allowed us to obtain information about the frequency of thalassaemic syndromes and about hemoglobinopathies in north Sardinia; 10,527 subjects from the chief town and from 28 communities in the province of Sassari and 1,101 subjects from 7 villages in the province of Oristano were examined. The overall frequency of  $\beta$ -thalassaemic carriers was 12.2% but there were differences between the two provinces; the rate was 10.4% among the inhabitants in the province of Sassari and 13.9% among those in the towns around Oristano. The frequency of  $\beta$ -thalassaemic trait was not homogeneous but varied from 6.5% in some hilly hinterland areas to 18.8% in some of the villages situated in former marshy areas in the province of Oristano.

Moreover, it can be seen that there is a great difference even within the same territorial area, but that there exists a correspondence between the high frequency of thalassaemic traits and former malaria areas is not always evident. The non  $\beta$ -thalassaemic microcytoses (MCV  $\leq$  77 fL), which include  $\alpha$ -thalassaemia traits and rare cases of iron deficiency anemia, are particularly frequent in the province of Oristano (26%), with a variable incidence that ranges from 21 to 42% and represents 11.2% of all subjects examined. There were, however, towns in which 60% of the subjects examined showed a reduction of red blood cell parameters. During the screening no case of  $\delta\beta$ -thalassaemia was found, whereas HPPH carriers with Hb F above 2% represented 0.3% of the cases. Hemoglobin  $\alpha$ -chain variants (J Sardinia and G Philadelphia) showed an overall incidence of 0.4% but were endemic in some villages in the province of Sassari.

023

**DEFECTIVE GLYCOSYLATION OF BAND 3 AND GLYCOPHORIN A IN CONGENITAL DYSERYTHROPOIETIC ANEMIA TYPE II OR HEMPAS REVEALED BY SDS-PAGE: A PATHOGNOMONIC MARKER?**

Qualtieri A, Bria M, Bisconte MG, Pasqua A,\* Le Pera M,\* Ruggiero S,<sup>o</sup> Brancati C\*

*Centro Studi della Microcitemia, Cosenza; \*CNR IMSEB, Cosenza; <sup>o</sup>Div. Medicina Ospedale Civile, Cosenza, Italy*

Congenital dyserythropoietic anemia type II (CDA II or HEMPAS) is a genetic anemia caused by a membrane abnormality in RBCs. Previous studies indicate that in HEMPAS erythrocyte band 3 and band 4.5 are not glycosylated by polylactosaminoglycans. Fukuda *et al.* demonstrated that a defect due to lowered N-acetylglucosaminyltransferase II activity is present in HEMPAS.

We report the hematological and biochemical character-

ization of CDA II both in the heterozygous and the homozygous state in a Calabrian family (Southern Italy). The proband was a 67-year-old woman submitted to our attention by the Hospital of Cosenza.

We examined the proband for hemoglobinopathies and enzymopathies. Normochromic and normocytic hemolytic anemia was present (Hb = 7.7 g/dL; MCV = 86 fL; MCH = 29 pg, reticulocytes = 5%, orthochromatic erythroblasts = 3%). HbA<sub>2</sub>, HbF, serum Fe, vitamin B12 and folate levels were normal. No abnormal hemoglobins (charged or neutral) were found and the  $\alpha$ /non $\alpha$  globin synthesis ratio was in the normal range. Serum ferritin, total and indirect bilirubin and osmotic fragility increased, while autohemolysis, both with and without glucose, was in the normal range. The Coombs' test was negative. The erythrocytic enzymes G6PD, PK, AK, hexokinase were in the normal range. These preliminary results led us to suspect an erythrocyte membrane defect.

To evaluate this hypothesis we examined the RBC membrane proteins by SDS-PAGE using 3.5-17% acrylamide exponential gradient Fairbanks slab gel and SDS-PAGE 10% acrylamide homogeneous Laemli slab gel. The Fairbanks gel stained with blue coomassie showed a faster migration of band 3 than that of normal RBCs and seemed more homogeneous. The Laemli gel stained for glycoproteins (PAS method) revealed a faster migration of glyophorin A (monomer and dimer). These observations, indicative of altered glycosylation, suggested a CDA type II. To confirm this hypothesis we determined the RBC anti-titer, the positivity of the acidified serum lysis test, bone marrow erythroblastic multinuclearity and ultrastructural erythrocyte double membranes. The results were compatible with a CDA type II. In a proband sister we were able to demonstrate on SDS-PAGE the same characteristic anomalies found in the proband; in another sister and her child we found intermediate characteristics indicating a heterozygous state.

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024

#### EARLY DETECTION OF NEPHROTOXICITY CAUSED BY DEFERRIOXAMINE IN THALASSEMIC PATIENTS

Cianciulli P, Sorrentino F, Forte L, Morino L, Amadori S  
*Centro Microcitemie, Ospedale S. Eugenio, Cattedra di Ematologia, Università Tor Vergata, Rome, Italy*

Some authors (1,2) have described renal damage caused by desferrioxamine (DFX). Nineteen transfusion-dependent thalassemia major patients were examined. Six of these received DFX (50 mg/kg b.w./12 hr) by subcutaneous infusion, thirteen patients were treated intravenously (six with 50 mg/kg body wt/6 hr and seven with 100 mg/kg body wt/24hr).

The following parameters were evaluated: BUN, creatinine, creatinine clearance,  $\beta_2$ -microglobulin, urinary  $\beta_2$ -microglobulin (u- $\beta_2$ m) and urinary growth hormone (u-GH) excretion.

Thirteen patients showed evident tubular damage by an increase of u- $\beta_2$ m; of these, eleven (85%) showed more serious tubular damage demonstrated by a contemporary increment of the u-GH excretion. We found a positive correlation between the increased excretion of u-GH and

that of u- $\beta_2$ m ( $p < 0.05$ ). It is therefore recommended that attention be paid to possible tubular damage (often sub-clinical) in patients receiving subcutaneous or intravenous iron chelation therapy through the simple determination of u- $\beta_2$ m excretion.

A reduction in the dosage of DFX is suggested in the presence of increased u- $\beta_2$ m levels, or a temporary suspension if there is an associated progressive reduction of the creatinine clearance. High levels of u-GH indicate more serious tubular damage. Therefore u-GH determination to evaluate hypothalamo-pituitary function is not admissible in patients with subclinical renal damage, above all in those undergoing iron chelation therapy.

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025

#### BUTYRATE DERIVATIVES IN HOMOZYGOUS B-THALASSEMIA, A SUMMARY OF THE TARANTO EXPERIENCE

Rizzo C, Masi C, Lazzari G, Amurri B, Peluso A, Cervellera M, Maggi A, Mazza P  
*Servizio di Ematologia, Taranto, Italy*

It is well known that fetal globin chains can substitute for absent or decreased  $\beta$ -globin in thalassemia. Butyric acid derivatives can stimulate the fetal globin gene promoter and increase fetal globin production.

We evaluated the effect on fetal globin of isobutyramide in a group of 6 pts with homozygous  $\beta$ -thalassemia; one pt with thalassemia intermedia received arginine butyrate. The protocol consisted of isobutyramide given orally at 60 mg/kg over 5 days/week, arginine butyrate was given i.v. as an 8-hour infusion at 1000 mg/kg.

Patient characteristics were as follow: 3 pts had circulating irregular antibodies with a positive Coombs' test which led to many difficulties in finding compatible blood; their Hb levels ranged from 5.6 to 6.8 g/dL. Three patients had high HbF levels (ranging from 8 to 15% of total hemoglobin). The patient who received arginine butyrate presented perimalleolar ulcers and 90% fetal hemoglobin. Laboratory analyses showed that the non  $\alpha/\alpha$  ratio moved over a few weeks from 0.53 (range 0.46-0.65) to 0.87 (range 0.68-1.05), with a variable increase ranging from 12 to 47%. F reticulocytes also increased variably. One patient stopped his transfusion program due to spontaneous maintenance of Hb level at 8.3 g/dL, and another required fewer transfusions to maintain Hb level at 8.0 g/dL. The patient with perimalleolar ulcers recovered from this problem and experienced a transient elevation of his Hb level from 10.2 to 12.4 g/dL. The other patients showed non clinical improvement during 1 year of therapy. We conclude that butyrate in thalassemia could be of benefit in a few selected patients with high fetal hemoglobin production. No serious side effects were recorded.

026

### SERUM TRANSFERRIN RECEPTOR ASSAY: A USEFUL TEST FOR DIFFERENTIATING IRON DEFICIENCY ANEMIA FROM THE ANEMIA OF CHRONIC DISORDERS?

Ricerca BM, Storti S, Vigevani F, Rumi C, Mezzabotta M, Zoli A,\* Altomonte L,\* Santacesaria G\*  
*Istituto di Semeiotica Medica and \*Istituto di Clinica Medica, Università Cattolica del Sacro Cuore, Rome, Italy*

The soluble form of transferrin receptors (sTfR) can be assayed using an immune-enzyme method. This is considered a parameter that is closely linked to erythropoiesis. In fact, the erythroid bone marrow is the main source of sTfR and its value is influenced by the iron balance. Our aim was to verify whether the sTfR assay can differentiate a true iron deficiency anemia from the anemia of chronic disorders (ACD). We examined 23 rheumatoid arthritis (RA) patients (3 males and 20 females), 16 healthy subjects and 14 patients with a simple iron deficiency anemia not secondary to other diseases. We performed the following tests for each subject: complete blood count, iron balance (serum iron, transferrin and ferritin), sTfR (Quantikine-Amgen), peripheral blood lymphocyte immunophenotyping with evaluation of the percentage of lymphocytes positive for TfR (CD71<sup>+</sup>). sTfR and CD71<sup>+</sup>(%) were higher in all RA patients than in the normal group (sTfR: 520±499 ug/dL versus 235.6±69.3 ug/dL; p=0.013; CD71<sup>+</sup>: 15.7±13% vs 9±2.8% p=0.02). When the RA patients were compared with the iron deficiency group, only the CD71<sup>+</sup>(%) was significantly higher in the first group (p=0.04). Moreover, the RA patients were divided into two subgroups, with comparable hemoglobin levels, according to iron balance (iron deficiency anemia, ACD). The sTfR and CD71<sup>+</sup>(%) were similar in these two subgroups. Thus different iron balance status did not influence the sTfR level in RA.

These data suggest two possible hypotheses: a) sTFR are equally sensitive to actual iron depletion and to *functional* iron deficiency, as the ACD model can be interpreted; b) in inflammatory diseases other sources of TfR can contribute to determining sTfR levels. The increase of CD71<sup>+</sup>(%) observed in our RA patients supports this interpretation.

027

### FAS-ANTIGEN EXPRESSION ON NORMAL AND APLASTIC BONE MARROW CD34<sup>+</sup> CELLS

Selleri C,\*<sup>§</sup>, Maciejewski JP,\* Sato T,\* Anderson S,\* Young NS\*

*\*Hematology Branch, National Heart, Lung and Blood Institute, National Institute of Health, Bethesda, MD, USA;*

*§Hematology Division, Federico II University of Naples, Italy*

Fas antigen (CD95), a receptor molecule mediating signals for apoptosis, is involved in T-cell-mediated killing of malignant, virus-infected or allogenic target cells. In aplastic anemia (AA), T-cells secrete IFN- $\gamma$  and TNF- $\alpha$  *in vitro*; activated cytotoxic lymphocytes infiltrate bone marrow (BM), and enhanced IFN- $\gamma$  and TNF- $\beta$  mRNA is

detected in BM from most patients. Using two-color fluorescent staining with FITC-conjugated anti-CD95 mAb and PE-conjugated anti-CD34 mAb, we detected an average of 8.3% CD34<sup>+</sup> cells expressing Fas antigen in 30 normal BM samples. Similar results were also obtained when highly purified CD34<sup>+</sup> from normal BM were used for analysis. Peripheral blood CD34<sup>+</sup> cells showed FAS antigen expression comparable to CD34<sup>+</sup> cells derived from BM (mean 7.3% FAS cells). Utilizing anti-Fas mAb, which mimics the biologic activity of Fas ligand on Fas receptor-expressing cells, we found that normal CD34<sup>+</sup> cells showed low sensitivity to Fas-mediated inhibition of colony formation. The level of FAS antigen expression on CD34 cells correlates with the sensitivity of these cells to FAS-mediated hematopoietic colony formation. CD34<sup>+</sup> cells stimulated by IFN- $\gamma$  and TNF- $\alpha$  to express Fas antigen were much more susceptible to suppression by anti-Fas antibody. Although BM from AA patients contains decreased percentages of cells expressing CD34 antigen, we found significantly increased expression of Fas antigen on CD34<sup>+</sup> cells in 30 patients with severe to moderate AA. In 15 patients who showed hematologic improvement in response to immunosuppressive therapy, the expression of Fas antigen decreased but remained higher than in normal controls; similarly, some patients with myelodysplastic syndrome showed increased FAS receptor expression on cells derived from BM. This finding correlated with the increased sensitivity of AA marrow cells to anti-Fas antibody-mediated inhibition of colony formation. Similar findings were obtained when fractionated CD34<sup>+</sup> cells were cultured in progenitor assays in the presence of anti-FAS mAb: 55% and 63% inhibition in two AA patients compared to 15% and 11% inhibition in two normal controls. Thus, it is possible that AA CD34<sup>+</sup> cells, including hematopoietic progenitor cells, express higher levels of Fas receptor due to *in vivo* exposure to IFN- $\gamma$  and TNF- $\alpha$  and are suitable targets for Fas ligand mediated cytotoxicity.

028

### DNA ANALYSIS IN PATIENTS WITH PYRUVATE KINASE DEFICIENCY

Pastore L, Della Morte R, Frisso G, Vitale D, Alfinito F\*, Salvatore F, Rotoli B\*

*Dipartimento di Biochimica e Biotecnologie Mediche e \*Cattedra di Ematologia, Università Federico II; CEINGE-Biotecnologie Avanzate, Naples, Italy*

Pyruvate kinase is a glycolytic enzyme that in humans has four isoenzymes. The R-type (R-PK) is expressed in erythrocytes and a deficiency of it leads to a defect of the glycolytic pathway (over 300 cases reported). The disease is heterogeneous in both clinical presentation and biochemical abnormalities. In a search for the molecular alteration, we examined nine PK-deficient patients from Southern Italy for a new Sty restriction site created by the 1529A mutation (R510Q), believed to be the most frequent in the European population, as well as for the presence of other restriction sites altered by other mutations (BsmAII for 1456T, AluI for 1179A). We found one patient with the 1529A mutation and another bearing the 1456T (R486W), both in the homozygous state. We

screened several exons in the other 7 patients by solid phase automated sequencing and so far we have found two undescribed mutations: a 1523 G transversion that leads to a stop codon instead of a Leu (L508Z) in exon 10, and a 1010 C transversion that leads to a Pro instead of an Arg (R337P) in exon 8, both in the heterozygous state. This last mutation changes the R located in an  $\alpha$ -helix to a P, thus drastically modifying the protein structure at that site. The 1523G mutation was confirmed by ASO-dot blot assay and was not found in any of the other patients analyzed. A screening of normal subjects never revealed the 1010 C mutation.

This preliminary study suggests that the mutation spectrum causing PK deficiency in Italian patients is different from that of the other European populations studied so far.

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029

#### ARE $\beta$ +45 G $\rightarrow$ C AND B-IVS-II-478 CCA NOVEL SILENT $\beta$ -THALASSEMIA MUTATIONS?

Carestia C, Lacerra G, De Angioletti M, de Bonis C, Palamone G, Sabato V, De Mattia D, Schettini F  
IIGB, CNR, Napoli, Dpt di Biomedicina dell'Età Evolutiva, Università degli Studi di Bari; Ospedale di Scafati (NA), Italy

Silent  $\beta$ -thalassemia mutations do not cause hematological alterations in carriers, but lead to mild thalassemia intermedia in patients who are compound heterozygotes for silent and severe  $\beta$ -thalassemia mutations. During a screening of unusual  $\beta$ -thalassemia genes we detected in families originating from Southern Italy two single-base substitutions in the  $\beta$ -globin gene,  $\beta$ +45 G $\rightarrow$ C and  $\beta$ -IVS-II-478 C $\rightarrow$ A, which could represent novel silent  $\beta$ -thalassemia mutations. Hematological data were obtained by standard methods. Globin patterns were analyzed by HPLC and *in vitro* biosynthesis;  $\beta$ - and  $\alpha$ -globin genes were examined by PCR-sequencing and restriction mapping. In the first family (parents, three daughters and two sons) we detected the novel  $\beta$ +45 G $\rightarrow$ C substitution, the  $\beta$ -thalassemia mutation IVS-II-654 C $\rightarrow$ T, and the  $\alpha\alpha$ -3.7 allele (triplication of the  $\alpha$ -globin genes). The proposita (16 years old) inherited all three alleles and was affected with thalassemia intermedia characterized by consistent chronic hemolysis; her Hb was 9 g/dL and indirect-reacting bilirubin 4.6 mg/dL. The mother presented the same genotype but without the novel  $\beta$ +45 G $\rightarrow$ C substitution and showed a  $\beta$ -thalassemia heterozygous phenotype. The other two daughters (19 and 17 years old) inherited both the novel and the thalassaemic  $\beta$ -globin alleles; they showed only slightly more severe hematological alterations than  $\beta$ -thalassemia heterozygotes and their Hb ranged from 10 to 11 g/dL. In the second family (parents and one son) we detected the novel  $\beta$ -IVS-II-478 C $\rightarrow$ A and the  $\beta$ -thalassemia mutation cod 39 C $\rightarrow$ T. The

propositus (13 years old) showed a reduction in Hb (9.7 g/dL), MCV (58 fL) and MCH (18 pg), an increase in Hb A<sub>2</sub> (5.2%) and normal Hb F; iron parameters were normal. The father presented typical alterations of  $\beta$ -thalassemia heterozygosity and was  $\beta$ -thalassemia cod 39 C $\rightarrow$ T heterozygous; the mother demonstrated no hematological alterations and was heterozygous for the novel substitution  $\beta$ -IVS-II-478 C $\rightarrow$ A. Globin chain biosynthesis was carried out in the propositus and in the mother, but an imbalance in globin chain biosynthesis due to the  $\beta$ -IVS-II-478 C $\rightarrow$ A substitution was not detected. These data indicate that the novel substitutions could play a role only in trans to  $\beta^0$  or  $\beta^+$  thalassemia globin genes.

030

#### INCREASED MEMBRANE PROTEIN PHOSPHORYLATION AND ANION TRANSPORT FUNCTION IN CHOREOACANTHOCYTOSIS

Olivieri O,\* De Franceschi L,\* Clari G,<sup>o</sup> Fenzi F,<sup>#</sup> Manfredi M,<sup>§</sup> Corrocher R\*

\*Institute of Medical Pathology, Chair of Internal Medicine, University of Verona; <sup>o</sup>Department of Biological Chemistry, University of Padova; <sup>#</sup>Department of Neurology, University of Verona, Italy

Choreoacanthocytosis is a rare disorder characterized by central and peripheral neuronal degeneration and the presence of acanthocytosis on blood smears. Structural and functional changes in membrane proteins, especially on the ubiquitous anion transporter (band 3), have been suspected to be involved in the pathophysiology of this disorder. In a 43-year-old woman presenting typical neurological symptoms and peripheral acanthocytosis, we studied: a) RBC membrane proteins by SDS-PAGE electrophoresis; b) RBC anion transport function by  $\text{SO}_4^{35}$  influx measurement; c) RBC membrane protein phosphorylation on  $\text{P}^{32}$ -labelled erythrocytes; d) RBC membrane casein kinase and poly(Glu,tyr) kinase activities on membrane ghosts.

**Results:** a) a normal membrane protein pattern was obtained on SDS-PAGE electrophoresis; b)  $V_{\text{max}}$  of  $\text{SO}_4^{35}$  influx was increased by 60-65% compared to normal controls ( $123 \pm 24.5$  mmol/L cells/h,  $n=6$ ); c) there was a remarkable (40%) increase in tyr-phosphorylation of membrane band 3 on ATP-depleted  $\text{P}^{32}$ -labelled erythrocytes with respect to normal controls; d) similarly, membrane-associated casein-kinase and poly(Glu,tyr) kinase activities were also increased by 22% and 7%, respectively.

**Conclusions:** in choreoacanthocytosis, tyr-phosphorylation of RBC membrane band 3 is increased and is associated with a remarkable stimulation of anion transporter function.

031

#### ABNORMALITIES IN ANION TRANSPORT AND IN K/CL COTRANSPORT ACTIVITY IN HEREDITARY SPHEROCYTOSIS (HS) ASSOCIATED WITH DIFFERENT MEMBRANE PROTEIN DEFECTS

De Franceschi L,\* Olivieri O,\* Miraglia del Giudice E,<sup>o</sup> Perrotta S,<sup>o</sup> Iolascon A,<sup>§</sup> Corrocher R\*

\*Cattedra di Medicina Interna, Università di Verona, °Dip. di Pediatria, II Università di Napoli; §Dip. di Biomedicina dell'Età Evolutiva, Università di Bari, Italy

Hereditary spherocytosis (HS) is heterogeneous in terms of inheritance, red cell morphology, clinical severity and underlying molecular defects. The most frequent defect is a spectrin (Sp) deficiency or combined deficiency of spectrin (Sp) and ankirin (Ank), but the primary molecular lesion could also involve band 3 or protein 4.2. There are no systematic studies for HS on the effects of different membrane protein defects on the activity of the anion transport (band 3). The two ions involved in anion transport system are  $\text{HCO}_3^-$  and  $\text{Cl}^-$ . K/Cl cotransport (cot) is responsible for the transport of K and Cl in the red cells, which in HS are characterized by a loss of K. We studied K/Cl cot activity in order to evaluate the role of this mechanism in promoting K loss in HS.

**Patients.** We studied 5 subjects with HS due to a band 3 deficiency (23-25%), 4 subjects with a combined Sp (22%) and Ank (25%) deficiency and 3 patients with HS associated with a band 4.2 deficiency. Normal subjects without HS or other hematological diseases were used as control; their reticulocyte values were similar to those observed in HS patients.

**Methods.** Anion transport was studied as  $\text{SO}_4^{35}$  influx in the presence of DIDS, using the Schofield AE method (*Nature* 1992; 355:836-8). K/Cl cot was measured as chloride-volume dependent K efflux (Brugnara C, *Am J Physiol* 1989; 256:C993-C1004).

**Results.** In the HS group characterized by a band 3 deficiency, we observed a 42% decrease in anion transport activity (Vmax), as well as a decrease in the inhibitory concentration of DIDS with respect to normal subjects. In HS due to a deficiency of Sp+Ank we did not observe any differences in anion transport activity compared to the control group. In HS associated with a 4.2 deficiency we observed a 35-40% increase in the anion transport activity (Vmax) and an increase in the inhibitory concentration of DIDS as compared to normal subjects. K/Cl cot activity was decreased in all HS cases studied with respect to normal controls, and was unaffected by the various protein membrane defects.

**Conclusions.** 1)  $\text{SO}_4^{35}$  influx may discriminate between HS types due to different membrane protein defects; band 3 deficiency: reduction of  $\text{SO}_4^{35}$  influx, Sp+Ank deficiency: no changes in anion transport activity, band 4.2 deficiency: increase in  $\text{SO}_4^{35}$  influx.

2) K/Cl cot activity was decreased with respect to normal subjects in all the HS studied. The K loss observed in HS is not related to K/Cl cot activity.

032

#### ANALYSIS OF RETICULOCYTE AND RED BLOOD CELL INDICES IN HETEROZYGOTES FOR A SILENT $\beta$ -THALASSEMIA MUTATION (THE C $\rightarrow$ T SUBSTITUTION AT POSITION -92)

Danise P,\* Amendola G,° Clemente L,† Ingenito M,® Rolando P,° Spagnoletti G\*

Department of \*Clinical Pathology and °Pediatrics, St. Leonardo Hospital, Castellammare di Stabia; Department of Clinical Pathology, †Pagani Hospital and ®Nocera Inferiore Hospital, Italy

Two sisters, aged 4 years and 16 months, respectively, with thalassemia intermedia were compound heterozygotes for the  $\beta^+$  -92 C $\rightarrow$ T mutation and severe  $\beta^+$  thalassemia ( $\beta^+$  IVS II - nt 745). Among the relatives of these patients, DGGE (denaturing gradient gel electrophoresis) and sequencing analysis led to the detection of 6 heterozygotes (4 adults and 2 children) for the C $\rightarrow$ T mutation at position -92. The hematologic phenotype of all these subjects was fully investigated and characterized. Hematologic analysis included: complete blood cell count and measurement of reticulocyte and red blood cell indices (performed on a Technicon H\*3 Bayer), erythrocyte morphological studies (on dried smears stained with May-Grunwald-Giemsa), hemoglobin electrophoreses, HbA2 levels (by microchromatography) and HbF levels (by alkaline denaturation), osmotic fragility test, measurement of bilirubin, haptoglobin, serum iron, transferrin and serum ferritin concentrations.

**Results:** i) erythrocyte morphology was normal, as were bilirubin, haptoglobin, iron balance and osmotic fragility; ii) HbF was always lower than 1%; iii) HbA2 levels showed a borderline increase (3.6-3.7%) in all subjects but one, whose HbA2 level was within the  $\beta$ -thalassemia range (4.2%).

Analysis of red blood cell and reticulocyte indices revealed:

- MCV and MCH were only mildly reduced (MCV 79.8-81.7 fL in adults, 71.8-74.5 in children; MCH 26.3-27.5 pg in adults and 25.2-25.7 in children);
- RDW, HDW, microcytic (<60 fL) and hypochromic (<28 pg) red cell populations were in the normal range;
- reticulocyte counts were normal;
- the values of two reticulocyte indices (MCVr and Chr) were only mildly reduced in comparison to the reference range, but moderately elevated if compared to the  $\beta$ -thalassemia trait.

In conclusion, the results of this study show that heterozygotes for the -92 C $\rightarrow$ T mutation have a nearly normal hematologic phenotype; on the other hand, compound heterozygotes for this mutation and severe  $\beta$ -thalassemia show a clinical phenotype of thalassemia intermedia. Therefore, for the purposes of genetic counseling and prenatal diagnosis, it is necessary to carry out globin chain synthesis analysis or gene analysis in the case of a marriage between a typical heterozygote for  $\beta$ -thalassemia and a person with even minimally altered red cell indices or borderline HbA2.

033

#### MOLECULAR ORGANIZATION OF TRANSLOCATION (15;17) AND ANALYSIS OF THE JUNCTIONAL SEQUENCE OF ITS FUSION PRODUCTS

Zangrilli D,\* Alcalay M,° Migone N,† Talamo G,° Papa G,\* Pelicci PG°

\*Cattedra di Ematologia, Dipartimento di Medicina Interna, University Tor Vergata of Rome; °Clinica Medica I, Policlinico Monteluce, University of Perugia; †Dipartimento Genetica, Biologia e Chimica Medica, CII/CIOS, Torino, Italy

In order to investigate the existence of signals specific for the fusion product involved in translocation t(15;17), we studied the mole-

cular organization of fusion genes PML/RAR $\alpha$  and RAR $\alpha$ /PML and of the corresponding region of the parent genes RAR $\alpha$  and PML. We performed the sequence analysis of a region spanning about 1 Kb that contained the genomic junctions in three acute promyelocytic leukemia (APL) cases representative of the three regions where the breakpoints on chromosome 15 (bcr1, bcr2, bcr3) are located. In two cases with breakpoints in the sixth intron (bcr1) and exon VI (bcr2) of PML, respectively, we could not detect any specific sequence in the regions flanking the breakpoints. In the third case, with the breakpoint located within the third intron of PML (bcr3), heptamer-like sequences originating from the parent genes were found flanking the RAR $\alpha$ /PML junction. We evaluated possible involvement of the recombinase system in causing the translocation by screening 12 more cases of APL for the presence of immunoglobulin (Ig) or T-cell receptor (TCR) rearrangement. Only the case with heptamer-like sequences flanking the junction showed rearrangement of the TCR $\delta$  locus. The demonstration of recombinase system activation in this case seems to confirm its involvement in the translocation. Our data suggest that in most cases t(15;17) is likely to be a random event and that APL-specific proteins give leukemic cells a biological advantage.

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034

#### MOLECULAR CLONING OF TWO DIFFERENT ISOFORMS OF TRANSCRIPTION FACTOR ZFM1 IN A GM-CSF DEPENDENT ACUTE MYELOID LEUKEMIA CELL LINE

Caslini C, Cazzaniga G, Golay J, Spinelli O, Amaru R, Attuati V, Barbui T, Introna M, Biondi A, Rambaldi A  
*Istituto di Ricerche Farmacologiche Mario Negri, Clinica Pediatrica dell'Università di Milano, Ospedale San Gerardo Monza e Div. di Ematologia Ospedali Riuniti Bergamo, Italy*

the aim of this study was to identify new genes specifically regulated by the hematopoietic growth factor GM-CSF. Using differential screening of a cDNA library obtained in  $\lambda$ -ZAPII vector from polyA<sup>+</sup> RNA isolated from a GM-CSF dependent human myeloid leukemia cell line (GF-D8), we identified two new isoforms of the recently described transcription factor ZFM1, which is apparently linked to multiple endocrine neoplasia type 1 (MEN1) (Toda et al. *Hum Mol Gen* 1994; 3:465-70). The transcription pattern of this gene is highly complex; it is characterized by four different transcripts of 3.9, 3.7, 3.2 and 2.9 Kb, respectively. Although constitutively present in basal conditions, the expression of the four transcripts increases in GF-D8 after GM-CSF stimulation as well as in T cells after incubation with mitogens. Among the several cDNA clones isolated, B74b3 is 2847 bp long and presents an open reading frame (ORF) of 1713 bp coding for 571 amino acids. The B74b3 amino acid sequence analyzed for functional protein domains (PROSITE) identified the presence of the eight amino acids (G-X-X-X-X-G-K-S) that determine the consensus sequence of the A motif of the ATP/GTP binding site characteristic of a protein family

including protein kinases, proteins involved in active transport and GTP binding elongation factors, etc. The cDNA clone B74b4 is 3484 bp long and presents an ORF of 1833 bp coding for 661 amino acids; it differs from B74b3 for the insertion at position 1925 of 636 additional nucleotides that determine different terminal sequences for the two isoforms. Northern blot analysis using a B74b4-specific sequence as a probe demonstrated that this cDNA clone is only representative of the high molecular weight transcripts and identifies one of the various isoforms of this gene. The role of B74 gene expression in response to GM-CSF stimulation of myeloid progenitor cells will be further elucidated by the stable transfection in GF-D8 cells of full length cDNA clones representative of the different isoforms of this gene.

035

#### FUNCTIONAL CHARACTERIZATION OF HUMAN C-SEA ISOFORMS

Catalano G,\* Zangrilli D,\* Chan AML<sup>o</sup>

\*Cattedra di Ematologia, Dip. di Medicina Interna, Tor Vergata University of Rome, Italy; <sup>o</sup>LCMB, NCI, NIH, Bethesda, USA

The viral SEA oncogene of avian erythroblastosis virus induces erythroleukemia in chicken and transformed rodent fibroblasts in cell cultures at high frequency. Molecular cloning experiments have shown that the avian SEA protooncogene encodes a molecule with sequence similarity to transmembrane receptor tyrosine kinases. To study the role of SEA gene products in regulating cellular events in human cells, we isolated overlapping cDNA clones from a human mammary epithelial cell line for the construction of the full-length form of human SEA messenger RNA. Several variant cDNA clones that represent isoforms of the SEA gene potentially generated by alternative splicing mechanisms were also identified. All of the isoforms were subcloned in a eucaryotic expression vector and transfected in NIH3T3 and 32D cell lines. One isoform that lacked the extracellular growth-factor-binding portion, being constitutively phosphorylated in tyrosine, caused transformation when overexpressed in monolayer-growing NIH3T3 cells. SEA expression seems to be restricted to the hemopoietic system of the chicken, and this is true for its homologues STK in the mouse and, according with our data, SEA/RON in man. All these data suggest the involvement of SEA in regulating bone marrow erythropoiesis.

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036

#### COMBINED USE OF CYCLOSPORIN A, VERAPAMIL AND THE DIHYDROPYRIDINE DERIVATIVE GR66234A IN MODULATING MULTIDRUG RESISTANCE IN HUMAN LEUKEMIA CELLS

Gancitano RA, Tolomeo M, Barbusca E, Abbadessa V, Perricone R, Cajozzo A

Chair of Hematology, University of Palermo, Italy

Kinetic analysis showed that the inhibitory effect of vinblastine, verapamil and cyclosporin A (CsA) on azidopine binding to the plasma membranes of multidrug resistant (MDR) cells is non competitive, suggesting that azidopine binds to P-glycoprotein (Pgp) at a binding site different from that of these drugs. These data indicate that there are at least two different drug-binding sites on Pgp and that they are kinetically distinguishable: one for vinca alkaloids, verapamil and cyclosporin A, and one for azidopine, a dihydropyridine calcium channel blocker. Recent observations suggest that the binding site for dihydropyridine compounds is located on an intracellular domain of Pgp. Therefore an attractive approach would be the simultaneous use of two MDR modulators that interact with the different Pgp binding sites. We evaluated on MDR cells K562/R and HL60/R the daunorubicin (DNR) and vincristine (VCR) resistance reversing activity of R-teludipine (GR66234A), a new dihydropyridine calcium channel blocker, in combination with CsA. The effects of this combination were evaluated by cytotoxicity test and by cell drug uptake studies. The resistance reversing activity of verapamil (VER)-CsA and GR66234A-VER combinations was also evaluated. VCR and DNR were used at IC10, GR66234A and CsA at concentrations between 0.2 and 2  $\mu\text{M}$ , VER at concentrations between 0.6 and 5  $\mu\text{M}$ . The combination of these drugs resulted mostly in an additive effect. Synergistic effects were observed only when the revertants of each combination were used at low concentrations. Moreover, the effects were different in relation to the antitumoral drug used. The GR66234A-CsA combination showed synergistic effects only for the modulation of VCR resistance, while a synergistic effect was observed with the VER-CsA combination for DNR resistance reversal. Data from cell uptake of the antitumoral drugs used confirmed the results obtained with the cytotoxicity test.

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037

#### ACTIVITY OF IDARUBICIN AND IDARUBICINOL IN COMBINATION WITH CYCLOSPORIN A IN MDR LEUKEMIAS.

Tolomeo M, Gancitano RA, Barbusca E, Abbadessa V, Perricone R, Cajozzo A  
Chair of Hematology, University of Palermo, Italy

Idarubicin (IDA) is a 4-demethoxydaunorubicin analog with greater *in vitro* cytotoxicity against tumor cell lines than daunorubicin (DNR). It shows a low degree of cross-resistance when tested *in vitro* against doxorubicin-resistant and multidrug resistant (MDR) cell lines; however, many clinical studies have shown no important increase in the complete response rate after the administration of IDA to patients with malignancies resistant to DNR. The discrepancy between *in vitro* and *in vivo* data may be related to the pharmacokinetic properties of IDA. IDA is converted to idarubicinol (IDAol) in the liver and in this form it seems to exert its antitumoral activity *in vivo*. We compared the *in vitro* activity of IDA and IDAol in MDR cell lines K562/R, FLCR, and in their sensitive parent cell

lines. IDA and IDAol showed the same cytotoxic activity in sensitive cells, which was about 5 times higher than that showed by daunorubicin (DNR). After one-hour exposure to each drug the cellular uptake of IDA and IDAol was also similar. In resistant cells IDAol was 3-4 times less active than IDA and its cytotoxic activity was intermediate between that showed by IDA and DNR. Intracellular uptake of IDAol was lower than that of IDA and this could be correlated with the capacity of P-glycoprotein to expel more IDAol than IDA. The IDA resistance index was 5 and 8.57 for K562/R and FLCR cells, while the IDAol resistance index was 20 and 36 for K562/R and FLCR cells, respectively. We observed that 2  $\mu\text{M}$  cyclosporin A (CsA) was able to reverse IDA and IDAol resistance completely in FLCR and K562/R cells. The cytotoxicity and cell drug uptake data obtained with these anthracyclines in combination with 2  $\mu\text{M}$  CsA in resistant cells were analogous to those obtained with these anthracyclines alone in sensitive cells. Since IDA shows good activity in resistant cells, the combination with CsA determined an increase in cytotoxic activity of only 5-8 times. On the other hand, a 20-36 fold increase in IDAol cytotoxic activity was observed when this drug was associated with CsA. In conclusion, the discrepancy between *in vitro* and *in vivo* data obtained with IDA may be related to the metabolism of IDA into IDAol, which is less active than IDA in MDR cells. Since the cytotoxic activity of IDA and IDAol in resistant cells becomes similar when these anthracyclines are associated with CsA, our data suggest the utility of combining IDA with a MDR reversing agent in hematological malignancies displaying the MDR phenotype.

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038

#### PCR AMPLIFICATION OF REARRANGED IMMUNOGLOBULIN HEAVY-CHAIN (IgH) GENES FOR MINIMAL RESIDUAL DISEASE DETECTION IN B-CELL MALIGNANCIES

Astolfi M, Corradini P, Ladetto M, Campana S, Comoglio F, Boccadoro M, Pileri A

Dip. di Medicina ed Oncologia Sperimentale, Div. Universitaria di Ematologia, Az. Ospedaliera S. Giovanni, Torino, Italy

We developed a novel PCR-based approach for amplifying rearranged variable regions (VDJ) of IgH genes for minimal residual disease (MRD) detection in B-cell malignancies. VDJ regions contain four framework (FR) regions and three complementarity-determining regions (CDR). CDR regions are unique to each B-cell clone and have been used to generate tumor-specific primers and probes. VDJ genes were amplified from tumor DNA or RNA using sense primers derived from the leader, FR1 and FR3 regions, and a consensus antisense primer from the six joining (JH) regions. Amplified VDJs were then sequenced and the CDR2 and/or CDR3 identified. MRD was assessed by PCR using CDR2 and JH primers. PCR products were then hybridized to a CDR3 probe. When

VDJs were amplified with and FR3 primer, only a CDR3 probe was available. CDR2 and CDR3 with JH primers were also used to develop a nested-PCR approach which showed high sensitivity ( $10^{-6}$ ) and specificity. A panel of mature and immature B-cell malignancies was analyzed to compare the efficiency of leader, FR1 and FR3 primers.

Diag.	Leader	FR1	FR3	Total
ALL	8/10	5/10	7/8	9/10 (90%)
CLL	5/8	6/9	4/8	6/9 (66%)
MM	13/22	16/22	16/22	20/22 (90%)
NHL	8/22	15/22	7/22	16/22 (72%)
Total	34/62 (55%)	42/63 (66%)	34/60 (56%)	51/63 (81%)

The combined use of leader, FR1, and FR3 primers allowed amplification of tumor VDJ in 51 of 63 cases (81%).

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039

#### ROLE OF FLUORESCENT *IN SITU* HYBRIDIZATION FOR THE DETECTION AND MONITORING OF TRISOMY 8 IN ACUTE MYELOID LEUKEMIA

Bigoni R, Cuneo A, Roberti MG, Bardi A, Balsamo R, Rigolin GM, Piva N, Castoldi GL  
Istituto di Ematologia, Università di Ferrara, Italy

In order to better define the role of fluorescent *in situ* hybridization (FISH) for the detection and monitoring of trisomy 8 in acute myeloid leukemia (AML), we analyzed data obtained by conventional cytogenetic analysis (CCA) and by FISH using a chromosome-8-specific pericentromeric probe in 30 newly diagnosed patients.

Normal karyotypes were found in 9, while 8 cases showed +8 in metaphase cells, with complex karyotypes present in two; 10 patients, 4 of whom demonstrated complex karyotypes, showed clonal chromosome abnormalities without +8. In 3 cases no analyzable metaphases were obtained by CCA.

FISH detected 10-71% of interphase nuclei with three fluorescent signals in all cases of +8 detected by CCA, with a fairly good correspondence between the % of trisomic cells detected by CCA and by FISH.

In addition, +8 was seen by FISH in 14-22% of interphase cells in 6 cases: 2 with no mitosis, 2 with normal karyotypes and 2 with complex aberrations without +8, giving a 46.6% overall incidence of trisomy 8 in this series.

Complete remission was achieved in 11/22 patients treated with myeloablative chemotherapy; however, only 1/10 cases with trisomy 8 detected by FISH obtained complete remission, as compared with 10/12 patients without +8.

Five percent of interphase cells with three signals were seen by FISH in 1 patient with trisomy 8 in cytologic remission, and a reduction in the size of the trisomic clone was detected in a patient who achieved partial remission.

We conclude that: a) FISH may be more sensitive than CCA for the detection of trisomy 8 in AML in those patients with no analyzable mitosis, in those with normal karyotypes and in those with complex karyotypes without

+8 by CCA; b) FISH may provide information on variations in the size of the trisomic clone during clinical follow-up, and c) the study of more cases is warranted in order to confirm the relationship between trisomy 8 and the low CR rate observed in this series.

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040

#### G6PD DEFICIENCY: STUDY OF A COHORT OF FEMALES

Pietrapertosa A, D'Agostino C, Ranieri P, Rizzo A, Tannoia N

*Chair of Hematology II, University of Bari, Italy*

Biochemical diagnosis of G6PD deficiency in males is not difficult since the defect is clearly demonstrable, but in heterozygous females it poses problems. Due to X chromosome inactivation such patients have two RBC populations: one consists of normal RBCs and the other of RBCs with the enzyme deficiency. For this reason enzyme activity reflects the ratio of the two RBC populations so that females with a severe deficiency have primarily defective cells, while those with mostly normal cells will be asymptomatic.

Forty-five female members of 25 families were studied. After assaying enzyme activity it was possible to divide them into 4 groups: 12 females with enzyme activity >6.8 I.U./g Hb, 28 with enzyme activity >2<6.8 I.U./g Hb, 4 with enzyme activity <2 I.U./g Hb and 1 female with activity of enzyme >9.8 I.U./g Hb who also presented a  $\beta$  thalassemia trait. Such great phenotype heterogeneity required molecular typing in order to evidence a possible correspondence between genotype and phenotype.

Thirty females were found to be heterozygous for the Mediterranean variant, 10 heterozygous for the Seattle variant and 5 are still being studied. Comparing genotype with phenotype, 4 females with a severe deficiency who reported hemolytic crises presented the Mediterranean variant; the other 36 indifferently showed one of the two variants.

In conclusion, the enzyme assay activity is not always diagnostic in female heterozygotes for a G6PD deficiency. The only sure method for identifying heterozygotes is a molecular study, and this is necessary in heterozygotes with normal activity as well as in those who also present associated hemolytic anemia (thalassemia). In Apulia the Mediterranean variant is the most frequent. At present a correspondence genotype/phenotype is not possible, but the severe phenotypes seem to be those with the Mediterranean variant.

041

#### MOLECULAR HETEROGENEITY OF *MLL*<sup>+</sup> ACUTE LEUKEMIAS: ASSOCIATION OF P53 MUTATIONS WITH MYELOID/MONOBlastic FEATURES

Lanza C,\*# Gaidano G,\*° Cimino G,§ Pastore C,\* Nomdedeu J,\* Volpe G,\* Vivenza C,# Mazza U,® Basso G,# Madon E,# Lo Coco F,§ Saglio G\*°

\*Laboratorio di Medicina e Oncologia Molecolare, Dip. di Scienze Biomediche e Oncologia Umana, Ospedale San Luigi Gonzaga; °CNR-CIOS; °Istituto di Discipline Pediatriche, and °Dipartimento di Scienze Cliniche e Biologiche, Università di Torino; °Sezione di Ematologia, Dipartimento di Biopatologia Umana, Università La Sapienza, Rome, Italy

Acute leukemias carrying MLL rearrangements are characterized by a high degree of clinical and immunologic heterogeneity, as demonstrated by immunophenotypic variability consistent with lymphoid or myeloid/monoblastic derivation and occurrence in distinct age groups, ranging from infancy to adulthood. In order to clarify whether distinct patterns of genetic lesions could contribute to the heterogeneity of MLL-positive (MLL<sup>+</sup>) acute leukemias, we tested the involvement of the p53 tumor suppressor gene in 29 patients displaying lymphoid (13 cases) or myeloid/monoblastic (16 cases) features and belonging to different age groups. p53 mutations were detected in 6/16 myeloid/monoblastic cases and in 2/13 lymphoid cases. Among the myeloid/monoblastic leukemias, p53 mutations occurred in the majority of infant cases (3/4), whereas they were restricted to a quarter (3/12) of the cases belonging to other age groups. Overall, our data suggest that 1) the heterogeneity of MLL<sup>+</sup> acute leukemias may be partially explained by the presence of distinct ongoing molecular pathways in different clinical subgroups of the disease; 2) at least two genetic lesions have accumulated in the short time (a few weeks after childbirth or, alternatively, conception) corresponding to the development of acute leukemia in these infants.

042

#### SENSITIVE DETECTION BY RT-PCR OF MOBILIZED TUMOR CELLS IN THE PERIPHERAL BLOOD OF PATIENTS WITH BREAST CANCER

Luppi M, Morselli M, Marasca R, Bandieri E, Sabbatini R, Federico M, Barozzi P, Ferrari MG, Torelli G  
Department of Medical Sciences, Section of Hematology and Oncology, University of Modena, Italy

Sensitive detection of occult carcinoma in the bone marrow and peripheral blood of patients with breast cancer may have important therapeutic and prognostic implications. We evaluated the RNA transcript encoding for *maspin* as a marker of mammary carcinoma cells. *Maspin* is a recently identified protein related to the serpin family of protease inhibitors, proposed to be a candidate tumor suppressor gene in human breast cancer (Zou et al. *Science* 1994; 263:526). Using a reverse-transcriptase polymerase chain reaction assay (RT-PCR), it was possible to amplify *maspin* mRNA in a large series of both primary and metastatic breast cancer specimens. The 14 peripheral blood as well as the 4 bone marrow samples obtained from healthy donors were found to be negative with the same technique. Thus, detection of a *maspin* transcript in the peripheral blood or marrow of a patient with known breast cancer should indicate the presence of mammary carcinoma cells. RT-PCR for *maspin* generated a single 443 band, identifiable after hybridization with a specific internal oligonucleotide probe, and detectable in 1 to 10 ng of total

RNA from the MCF 7 mammary tumor cell line. Neither the 7 patients with stage II nor the 3 with stage III breast cancer showed a detectable *maspin* transcript in their peripheral blood. Of interest, 3 of 8 pts with stage IV breast cancer receiving systemic therapy at the time of sample collection did have a detectable *maspin* transcript in their peripheral blood. In addition, 1 of 11 pts with stage IV breast cancer not receiving therapy also showed a positive *maspin* RT-PCR assay. Moreover, 3 bone marrow biopsies showing very few tumor cells at standard histologic examination resulted clearly positive with this RT-PCR assay, and we are currently testing whether this assay is also powerful in identifying occult mammary carcinoma cells in histologically negative marrows. Concomitant tumor cell recruitment upon mobilization of peripheral blood progenitor cells in stage IV breast cancer patients was recently documented by immunocytochemical methods (Brugger et al. *Blood* 1994; 83:636). We propose the *maspin* RT-PCR assay as a new specific, sensitive and rapid tool, alone or in combination with immunocytochemical assays, for the detection of mobilized tumor cells in the peripheral blood of patients with breast cancer.

043

#### FREQUENCY AND PHENOTYPE OF $\delta^{+27}$ THALASSEMIA IN SARDINIA

Guiso L, Froggheri L, Pistidda P, Angioni L, Bonfigli S, Lonignotti M, Dore F  
Istituto di Ematologia, Università di Sassari, Istituto CNR per lo studio della Patologia del Sangue, Sassari, Italy

Different  $\delta$ -thalassemia mutations have been reported in the Mediterranean area and, among them, the G→T substitution at the first nucleotide of codon 27 of the  $\delta$  globin gene ( $\delta^{+27}$ ) is the most frequent mutation found in Sardinian people. The frequency of this thalassemic mutation in Sardinia has only been hypothesized by a reduction of HbA2 levels.

In the present study, 750 subjects drawn from preventive scholastic screening for  $\beta$ -thalassemia were examined consecutively to verify the precise frequency of  $\delta^{+27}$  thalassemia. Polymerase chain reaction (PCR) and successive hybridization with an allele-specific oligonucleotide (ASO) probe for the  $\delta^{+27}$  mutation was performed in all samples.

Nine subjects (one of them was also a carrier of  $\beta$ -thalassemic trait) were found to be positive for this molecular mutation, and the frequency of  $\delta^{+27}$  thalassemia in the Sardinian population was estimated at 1.2%. For a better characterization of the hematological phenotype of  $\delta^{+27}$  thalassemia and of double heterozygotes for  $\delta^{+27}$ / $\beta$ -thal, we included another 38 previously studied subjects in our investigation (29  $\delta^{+27}$  and 9  $\delta^{+27}$ / $\beta$ -thal carriers).

The  $\delta^{+27}$  thalassemia carriers showed normal hematological parameters and a mean HbA2 percentage of  $1.58 \pm 0.31$  (range 0.74-2.2), which was significantly lower ( $p < 0.0001$ ) than that found in normal subjects of the same population ( $2.45 \pm 0.26$ ). The 10 subjects with  $\delta^{+27}$  thal/ $\beta$ thal presented mean values of MCV ( $60.3 \pm 4.2$ ), MCH ( $19.4 \pm 0.8$ ) and  $\alpha/\beta$  chain ratio ( $2.1 \pm 0.4$ ) similar to those reported in  $\beta$ -thalassemic trait. On the contrary, the mean HbA2 level ( $3.07 \pm 0.38$ ), which is significantly lower

( $p < 0.0001$ ) than that observed in the typical  $\beta$ -thal trait, suggested the presence of a *silent*  $\beta$ -thalassemic trait.

Since the frequencies of  $\beta$ -thalassemic and  $\delta^+27$  thalassemic traits in the Sardinian population are 11% and 1.2%, respectively, the possibility of finding a  $\delta/\beta$  silent carrier is 0.0003.

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044

**THE *A-myb* GENE IS HIGHLY EXPRESSED IN BURKITT'S LYMPHOMA AND (sIg<sup>+</sup>) ACUTE LYMPHOBLASTIC LEUKEMIA**

Golay J, Luppi M, Songia S, Biondi A, Barbui T, Rambaldi A, Introna M

*Istituto di Ricerche Farmacologiche Mario Negri, Milano, Clinica Pediatrica dell'Università di Milano, Ospedale San Gerardo, Monza e Divisione di Ematologia, Ospedali Riuniti Bergamo, Italy*

The *A-myb* gene is a transcription factor that is related both functionally and structurally to the *v-myb* oncogene. Following our observations that *A-myb* is expressed in a restricted subset of normal activated human B lymphocytes, we investigated the pattern of *A-myb* expression in neoplastic B-cells representing the whole spectrum of B-cell differentiation. In a panel of 32 B-cell lines, *A-myb* was very strongly expressed in most Burkitt's lymphoma (BL) cell lines, but negative in 2 pre-B acute lymphoblastic leukemia (ALL), 4 non Hodgkin lymphoma, 6 EBV-LCL and 6 myeloma lines. We also investigated *A-myb* expression in 49 fresh cases of B leukemias. Among 24 ALL, 6 were of the null and 11 of the common type and all of these were negative for *A-myb* expression; on the other hand, all 7 B-ALL cases (sIg<sup>+</sup>) as well as one fresh BL case with bone marrow infiltration strongly expressed *A-myb*. *A-myb* was undetectable in four prolymphocytic leukemias but was strongly expressed in 5 out of 20 (25%) chronic lymphocytic leukemias (CLL). In this last group, *A-myb* did not correlate with phenotype or clinical stage. Thus high levels of *A-myb* RNA and protein are expressed in a restricted subset of B-cell neoplasias (BL and sIg<sup>+</sup> ALL) representative of a specific stage of B-cell differentiation. This expression may in part reflect the expression of *A-myb* by the normal germinal center B cells, which are the normal counterpart of these transformed B cells. The data presented strongly support a role for this transcription factor in B-cell differentiation and perhaps in B-cell transformation in some neoplasias.

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045

**RED CELL PYRUVATE KINASE (PK) DEFICIENCY IN ITALY: MOLECULAR STUDY**

Bianchi P, Terragna C, Bredi E, Zappa M, Vercellati C, Pelissero G, Alfinito G,\* Zanella A

*Divisione di Ematologia e Centro TrASFusionale e di Immunologia dei Trapianti, Ospedale Maggiore Policlinico, Milan; \*Università Federico II, Naples, Italy*

PK deficiency is the most common glycolytic enzyme defect and it is associated with chronic non-spherocytic hemolytic anemia. The disease is very heterogeneous in

terms of clinical presentation and biochemical abnormalities. The gene encoding for PK (*LR-Pk*) has been cloned and sequenced. So far, molecular studies have led to the description of 16 different mutations in the Japanese and Chinese populations, and 29 in Caucasians, most of which are point mutations. A<sup>1529</sup> is considered to be the most common mutation in the white race.

The aim of this paper was to study the molecular defect in 18 PK-deficient patients: 9 from Northern, 1 from Central and 7 from Southern Italy. Parental consanguinity was documented in one family only. A screening for most of the known mutations was performed for each patient by enzymatic digestion on amplified DNA. Moreover, the entire coding region of the *L-Pk* gene and the flanking intronic regions were analyzed by SSCP and direct sequencing.

The following mutations were identified: A<sup>994</sup> (1/39 alleles), C<sup>1269</sup> (1/39), T<sup>1456</sup> (7/39), A<sup>1529</sup> (2/39), T<sup>1594</sup> (1/39). Furthermore, a c to t nucleotide substitution at nt +50 in intron 5 and a C/T polymorphism at nt 1738, never before described, were found. Mutation C<sup>1269</sup> (GCC to GCC) does not lead to an amino acid substitution, but results in abnormal splicing in exon 8.

In conclusion, a new mutation (C<sup>1269</sup>) was found. Moreover, the most frequent mutation in Italy appears to be T<sup>1456</sup>, contrary to what is reported in Northern American and Northern European populations where A<sup>1529</sup> is the most common.

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046

**ROLE OF INTERLEUKIN-3 (IL-3) PLUS INTERLEUKIN-6 (IL-6) IN THE CYTOGENETIC ANALYSIS OF MALIGNANT PLASMA CELLS IN MULTIPLE MYELOMA**

Balsamo R, Cuneo A, Roberti MG, Bardi A, Balboni M, Bigoni R, Piva N, Rigolin GM, Castoldi GL

*Istituto di Ematologia, Università di Ferrara, Italy*

In order to define better the role of interleukin-3 (IL-3) plus interleukin-6 (IL-6) in the cytogenetic analysis of multiple myeloma, we studied 34 patients with the following protocol based on cytologic and cytogenetic techniques:

1. preparation of two cultures for each patient containing  $6 \times 10^6$  bone marrow cells/mL in RPMI1640 + 30% fetal calf serum with and without IL-3+IL-6;
2. evaluation of the number of mitotic plasma cells at 24, 48, 72, 96 hours after incubation of 1 mL aliquots of each culture with colcemide for two hours. Cytologic study was performed on cytocentrifuge samples colored with panoptical stain and through a cytoimmunological method (APAAP) with monoclonal antibody directed against the specific Ig light chain;
3. evaluation of karyotype at 72-96 hours by conventional cytogenetic analysis.

An increased number of mitotic plasma cells was found in the IL-3+IL-6 stimulated cultures with respect to the unstimulated ones in 15/34 cases. Of the remaining 19, an increased number of mitotic erythroblasts and granulocyte precursors was detected in the stimulated cultures in 15, whereas no difference was observed in 4 cases.

Conventional cytogenetic analysis from stimulated culture yielded analyzable metaphases in 29/34 cases. In five

cases without analyzable metaphases less than  $0.7 \times 10^6$  cells/mL were obtained at BM aspiration, compared to a median of  $3 \times 10^6$  cells/mL cells in the patients with analyzable metaphases.

Clonal karyotype abnormalities were seen in 25/29 cases. It was concluded that:

- a) IL-3 plus IL-6 may significantly increase the number of mitotic plasma cells with respect to traditional culture media in approximately half of the cases;
- b) IL-3 plus IL-6 may disclose clonal abnormalities in the majority of patients with MM;
- c) success in obtaining mitoses is largely dependent on the number of cultured cells.

Supported by MURST, fondi 40% and 60%.

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047

**AML PRESENTING A VARIANT PH CR. (V-PH) WITH P190 EXPRESSION, DUP 3Q AND -7, FOLLOWING TREATMENT OF NON-HODGKIN LYMPHOMA (NHL) WITH ALKYLATING AGENTS AND TOPOISOMERASE II INHIBITORS**

Cedrone M, Nanni M, De Cuia MR, Lo Coco F, De Sanctis V, Cimino G, Mancini M, Alimena G  
Hematology, Department of Human Biopathology, University "La Sapienza" Rome, Italy

Two different subtypes of acute leukemia (s-AL) following prior chemotherapy have recently been recognized: one related to alkylating agents and the other secondary to chemotherapy with topoisomerase II inhibitors. S-ALs of the latter type present with distinctive biological and clinical aspects: 1) mostly M4-M5 cytology; 2) absence of a dysplastic phase; 3) balanced chromosome aberrations, including those specific for *de novo* AL; 4) a less unfavorable prognosis.

We describe a case of s-AL, FAB-M5, not preceded by a dysplastic phase in a patient heavily pre-treated with alkylating agents and topoisomerase II inhibitors for a NHL. Cytogenetics displayed the presence of a v-Ph originating from a complex  $t(2;9;22)(q37;q34;q11)$ , transcribing for an abnormal p190 protein, and associated with a  $dup(3q)(q21q26)$  and  $-7$ . The Ph chromosome, which is the hallmark of CML, is also present in 5-25% of *de novo* AL; it is only found exceptionally in s-AL, where it has never been described in a variant form. At the molecular level, p190 is almost exclusively found in *de novo* AL.  $Dup(3q)$  and  $-7$  are *specific* aberrations often observed in AL secondary to alkylating agents. Ours is the first observation of a s-AL with a v-Ph and a fusion protein p190. It represents additional evidence supporting the idea that changes previously considered specific for *de novo* AL characterize s-AL following treatment with drugs targeting DNA topoisomerase II. The association of v-Ph with  $-7$  and  $dup(3q)$  might be regarded as the consequence of exposure to both alkylators and topoisomerase II inhibitors.

Thus our case combines a number of biological and clinical aspects which were recently shown to be related to distinct mutagenic mechanisms associated with exposure to chemotherapy. Better knowledge of these diseases could be of fundamental importance in understanding the leukemogenic process and might provide targets for specific therapeutic measures.

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048

**AUTOLOGOUS BONE MARROW TRANSPLANTATION IN ACUTE LEUKEMIA: CYTOGENETIC STUDIES IN 32 PATIENTS**

Testoni N, Zaccaria A,\* Miggiano MC, Pelliconi S, Martinelli G, Carboni C, Salini L, Gherlinzoni F, Rizzi S, Motta MR, Visani G, Manfroi S, Rosti G, Cavo M, Tura S  
*Istituto di Ematologia L. e A. Seràgnoli, Università degli Studi di Bologna; \*Servizio di Ematologia, Ospedale S. Maria delle Croci, Ravenna, Italy*

Since April 1987, 93 patients (pts) with acute leukemia (82 acute myeloid leukemia – AML – and 11 acute lymphoblastic leukemia – ALL) have been submitted to autologous bone marrow transplantation (ABMT). Of the 82 AML patients, 71 were studied cytogenetically (40 before ABMT only and 31 serially after ABMT); 11 received no cytogenetic study. Of the 31 studied serially, 12 were females and 19 males; median age was 37 yrs (range 16-54). FAB classification was M1 in 2 patients, M2 in 5 patients, M3 in 16 patients, M4 in 5 patients, M5 in 3 patients; the conditioning regimen was busulphan and cyclophosphamide. One to 11 studies were performed for each patient after BMT. The karyotype was determined in 26 patients at harvest and in 20 at ABMT. Clonal and non-clonal chromosomal abnormalities were found in 14 patients; of these, 9 relapsed hematologically: 4 with the same pattern seen at diagnosis, 1 with a complex karyotype and 4 with additional aberrations with respect to the karyotype found at diagnosis. Four pts in complete remission (CR) showed non clonal abnormalities, only one occasion: in 1 patient the same abnormality as that seen at diagnosis occurred, 2 demonstrated a different karyotype and 1 patient presented 2 different abnormalities in 2 different studies. Finally, the patient with  $t(15;17)$  promyelocytic ANLL showed monosomy 7 in 25% of the observed cells 8 months after ABMT and in 95% from the 10<sup>th</sup> month on, with evolution to a myelodysplastic syndrome.

Regarding ALL, only 1 patient was followed cytogenetically after ABMT. At diagnosis, the karyotype revealed Ph positive hyperdiploid metaphases. The conditioning regimen was total body irradiation (TBI) and CY. In 4 studies performed from the 1<sup>st</sup> to the 11<sup>th</sup> month, the karyotype was normal; at the 11<sup>th</sup> month, 8.8% of the cells were Ph positive. In 3 subsequent studies (19<sup>th</sup>, 22<sup>th</sup> and 24<sup>th</sup> month) we found clonal and non clonal abnormalities with no evidence of the Ph chromosome. The patient relapsed in the 28<sup>th</sup> month, showing 22% abnormal metaphases and a minor percentage of Ph metaphases.

In conclusion, different chromosome patterns may be found after ABMT: 1) non clonal aberrations probably secondary to the conditioning regimen and not associated with hematologic relapse; 2) on the other hand, disease relapse may be associated with the presence of specific clonal abnormalities similar or not similar to those found at diagnosis.

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049

**ASSESSMENT OF MINIMAL RESIDUAL DISEASE IN ACUTE PROMYELOCYTIC LEUKEMIA WITH  $t(15;17)$  BY CHROMOSOME PAINTING**

Giacobbi F, Temperani P, Vaccari P, Gandini G, Longo G, Torelli G, Emilia G  
*Dipartimento di Scienze Mediche, Sezione di Oncologia ed Ematologia, Università di Modena, Italy*

Complete remission (CR) in leukemic patients does not exclude the persistence of a low percentage of leukemic cells in the bone marrow, i.e. *minimal residual disease* (MRD). In this phase it is extremely important to evaluate the extinction or the persistence of the neoplastic clone in order to establish the clinical course of the disease and to undertake additional therapeutical measures. Some authors have demonstrated the feasibility of fluorescence *in situ* hybridization (FISH) for more accurate detection of MRD in leukemia and lymphoma patients. Conventional cytogenetics in acute promyelocytic leukemia (APL) is often limited by the poor morphology of chromosome preparations, making evaluation of the persistence of the t(15;17) clone particularly difficult. In the present study we explored the use of FISH with biotinylated probes specific for the whole chromosome 17 to evaluate of MRD in APL with t(15;17). The patients were examined at diagnosis, I relapse and II relapse after treatment with ATRA.

On the basis of our results, banding procedures and FISH are equivalent at diagnosis; however, conventional cytogenetic analysis of metaphases is often laborious and less rapid than hybridization. Moreover, the use of chromosome painting permitted us to find three distinct signals in three patients in CR indicating rearrangement of chromosome 17 not detected by conventional cytogenetics. Our study demonstrates the feasibility of this method to evaluate MRD in hematologic neoplasias, independently of chromosome morphology and metaphase spread.

050

#### CHROMOSOME REARRANGEMENTS AT THE TELOMERIC LEVEL IN HEMATOLOGIC DISORDERS

Temperani P, Giacobbi F, Gandini G, Vaccari P, Torelli U, Emilia G  
*Dipartimento di Scienze Mediche, Sezione di Oncologia ed Ematologia, Università di Modena, Italy*

Recent research suggests that one of the mechanisms controlling cell aging is the progressive shortening of the ends of chromosomes, the telomeres, constituted by short tandem repeated DNA sequences and synthesized by a ribonucleoprotein enzyme, telomerase. This enzyme is expressed in germline and usually absent in normal somatic differentiated cells. Activation of telomerase either in somatic tissues or in neoplastic cells could lead to enhanced stability of telomeres and, consequently, to unlimited cell proliferation (immortalization). Following retrospective screening of our karyotype data from 414 consecutive non-childhood neoplastic hematologic diseases, we isolated 11 cases with telomeric rearrangements, including: a) non clonal telomeric association (tas), b) clonal terminal rearrangements consisting of additional material of unknown origin fused at the end of the chromosome, and c) clonal telomere-centromere fusion (t telcen) with pseudodentric structure. Most of these abnormalities, 9 of 11, were associated with an evolutive disease

phase: 8 cases of lymphoproliferative diseases (4 NHL, 1 lymphoid b.c. of CML, 1 ALL, 2 CLL), 1 case of erythroid b.c. of CML, 1 case of undifferentiated b.c. of CML, 1 case of PV. The presence of chromosome rearrangements at the telomeric level, particularly in a progression phase of neoplastic process, suggests that these mechanisms may contribute to circumventing the effects of telomeric DNA shortening (senescence) and to stabilizing neoplastic clones.

051

#### FISH ANALYSIS IN THREE CASES OF APL WITH NON-CLASSICAL CYTOGENETIC TRANSLOCATION

Guercini N,\* Di Bona E,<sup>o</sup> Montaldi A,\* Celli P,\* Novella E,\* D'Emilio A,<sup>o</sup> Zambello R,<sup>o</sup> Rodeghiero F,<sup>o</sup> Stella M\*  
 \*Servizio di Genetica Umana e <sup>o</sup>Divisione di Ematologia, Ospedale San Bortolo, Vicenza, Italy

Acute promyelocytic leukemia (APL) is characterized by a specific balanced reciprocal translocation, t(15;17)(q22;q21), that is present in almost all patients. This rearrangement results in the fusion of the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) gene on chromosome 17 with the putative transcription factor gene (PML) on chromosome 15.<sup>1</sup>

Variant translocations have been reported in a small percentage of APL, in most instances complex translocations involving chromosomes 15, 17 and others; molecular data from these cases demonstrated the presence of RAR $\alpha$  and PML gene rearrangements.<sup>2</sup> Non-classical translocations, t(11;17)(q23;q21) and t(5;17)(q32;q12), have recently been observed in a few patients. These rearrangements showed fusion between RAR $\alpha$  and a second gene other than PML.<sup>3,4</sup>

In the present study we report three cases of APL with atypical rearrangements. We performed classical and molecular cytogenetic analysis on bone marrow cells at the time of diagnosis in two patients (AA and CF) and at relapse in the third (GL).

GTG banding results are provided in the following table:

Case	Specimen type	GTG results
AA	Diagnosis	46,XY,-15,-17,+M1,+M2
GL	Relapse	47,XX,t(15;17)(q22;q21),t(4;10),16p+
CF	Diagnosis	46,XY,t(11;17)(q23;q21),-7,+i(7q)

We carried out FISH analysis using: a) chromosome painting probes (#11, #15 and #17); b) cosmid probes for simultaneous detection of RAR $\alpha$  and PML genes; c) cosmid probes mapping at different levels of chromosome 17 (ONCOR).

In case AA the hybridization pattern showed the structure of the two marker chromosomes. M1 appeared as a der(17) in which four regions originating from chromosomes 15 and 17 alternated, giving rise to RAR $\alpha$ /PML fusion at two different levels. Furthermore, FISH data excluded M1 as isochromosome of the long arms of a former der(17)t(15;17). M2 was a der(15)t(15;17), probably with PML/RAR $\alpha$  rearrangement inside.

FISH analysis of chromosome 16p+ (GL) demonstrated

duplication of the 17q region, yet lacking RAR $\alpha$  sequences. The hybridization pattern of t(11;17)(CF) showed that: a) this translocation was not a complex rearrangement; b) the breakpoints did not involve PML sequences.

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052

### COMPLEX PH1 TRANSLOCATION AND UNUSUAL CLINICAL COURSE IN A CML PATIENT

Maffei L, Cox-Froncillo MC, Cantonetti M, Lentini R, Bruno A, Adorno G, Masi M, Giudiceandrea P, Amadori S *Cattedra di Ematologia, Dipartimento di Medicina Interna, Università "Tor Vergata", Ospedale S. Eugenio, Rome, Italy*

Variant Ph1 translocations are described in 4-5% of CML patients; these variant forms are most frequently observed in black people. There is no evidence that patients with variant Ph1 experience an unusual clinical or prognostic course with respect to CML patients bearing the classic t(9;22). Involvement of chromosome 15 in variant Ph1 translocations has only been sporadically described in the literature.

We report the case of a CML patient with a peculiar clinical course whose karyotype at disease onset displayed a complex variant Ph1 translocation: t(9;15;22) (q34;q15;q11). FISH analysis performed with a set of probes complementary to the bcr and abl genes and with a double color detection system revealed a fusion signal in 74% of the cells analyzed.

Complete clinical and cytogenetic remission was achieved after 1 year of IFN- $\alpha$  therapy, though FISH analysis was still positive for the bcr/abl fusion signal in a significant proportion of cells analyzed (16%). Three months after achieving complete remission, a blastic crisis with LANL M2 features and a t(9;15;22) karyotype with no additional cytogenetic alterations was evident. The patient underwent chemotherapy (CT) following the VAC protocol (VP-16, Ara-C, carboplatin) and obtained clinical and cytogenetic remission. FISH analysis showed a fusion signal in 12% of bone marrow cells but was negative in peripheral blood stem cells collected during the aplasia rescue phase under GM-CSF pulse.

We found the clinical course of this Ph1 variant CML patient, who achieved a prompt complete remission with IFN therapy and then suffered an early and unexpected blastic crisis that responded to CT, to be unusual. Notably, although there was a complex Ph1 translocation, FISH analysis detected a bcr/abl signal. Furthermore, this approach was much more informative than classic cytogenetic analysis in detecting residual bcr/abl positive cells. We think that FISH could also be extremely useful in autografting procedures for determining the best source of stem cells (peripheral vs bone marrow) to be used.

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053

### THE FUNCTIONAL CLASSIFICATION OF ANEMIA IN MYELODYSPLASTIC SYNDROMES DOES NOT IDENTIFY SUBGROUPS OF PATIENTS RESPONSIVE TO RECOMBINANT HUMAN ERYTHROPOIETIN THERAPY

Bucalossi A, Marotta G, Galieni P, Bigazzi C, Dispensa E *Divisione di Ematologia, Ospedale "A. Sclavo", Siena, Italy*

The myelodysplastic syndromes (MDS) are clonal stem cell disorders in which anemia, typically refractory to treatment, is the main feature of the disease. For this reason many authors have investigated the utility of recombinant human erythropoietin (rh-Epo) therapy in this patient population.

Previous studies have demonstrated an erythroid response in about 25% of MDS patients. Considering the relatively low response rate, pre-treatment identification of patients who will respond to this therapy is important for avoiding expensive but ineffective treatment. Several parameters have been evaluated in various clinical trials, but none of them have shown certain predictive value.

Since the pathophysiologic characteristics of anemia in MDS can identify homogeneous groups potentially responsive to rh-Epo therapy, we treated 11 patients with myelodysplastic syndromes (MDS) to evaluate the utility of a functional classification of anemia to predict erythroid response. Patients were classified according to the criteria of the French-American-British (FAB) cooperative group; there were five with refractory anemia (RA), three with refractory anemia with ringed sideroblasts (RARS), and three with refractory anemia with excess of blasts (RAEB). Rh-Epo was administered three times weekly by subcutaneous injection at a dose of 150 units/kg, which was escalated to 300 units/kg if a response was not observed after 1 month of treatment. Changes in hemoglobin level, reticulocyte index, and transfusion requirements were compared with pretreatment values.

To construct the reference curves representing the relationships between Hct and serum Epo or serum TfR, we evaluated 40 subjects with different Hct levels, as previously described by Beguin *et al.* The appropriateness of Epo response to anemia in the individual patient was evaluated by the observed/predicted (O/P) ratio; O/P ratio values lower than 0.82 identified patients with inadequate Epo response to anemia.

To establish the adequateness of erythroid proliferation in relation to anemia, we evaluated the TfR O/P ratio in our reference group and found that values lower than 0.96 indicated an inadequate TfR level for anemia. Our patients were subdivided into three groups.

In particular, 8 out of 11 patients showed a condition of *intrinsic marrow hypoproliferation*, as defined by low Hct and decreased O/P TfR in the presence of normal O/P Epo. Two out of 11 patients demonstrated a pattern of *defective Epo production*, characterized by low Hct with decreased O/P TfR and O/P Epo, and 1 out of 11 depicted a pattern of *hyperdestruction* identified by decreased Hct with normal O/P Epo and O/P TfR. Epo response was analyzed in 10 out of 11 enrolled patients.

We did not observe any response either in terms of increased hemoglobin level or reduced transfusion

requirements. In conclusion, the absolute lack of a correlation between the pathophysiology of anemia and response to rh-Epo therapy indicates that other parameters able to predict response must be sought.

054

#### EFFECTS OF IL-12 AND IL-2 ON CYTOTOXIC ACTIVITY MYELODYSPLASTIC SYNDROMES (MDS)

Sarina B, Cortezzi A, Cattaneo C, Silvestris I, Soligo D,\* Hu C,<sup>o</sup> Monza M,<sup>o</sup> Calori R, Di Stefano M, Bertolini F,<sup>#</sup> Maiolo AT

*Servizio di Ematologia, \*CTMO, <sup>o</sup>Ist. Medicina Interna, Università di Milano; <sup>#</sup>Centro Trasfusionale, IRCCS Osp. Maggiore di Milano, Italy*

Produced by B lymphocytes, IL-12 is a cytokine with immunomodulating activity (it stimulates NK and antibody-dependent cytotoxicity, induces NK and T-cell secretion of  $\gamma$ -IFN and  $\alpha$ -TNF and, in association with other cytokines, regulates the survival of committed and non committed stem cells). Since a defect of NK activity (NK-a) has been documented in MDS, the potentiation of such activity could lead to an antileukemic effect.

IL-2 is capable of significantly stimulating NK-a *in vitro* during the course of MDS. We therefore evaluated the effects of IL-12, alone and in association with IL-2, on NK-a during the course of MDS. Eighteen patients (8 RA, 2 RARS, 6 RAEB, 2 CMML) and 8 healthy subjects were enrolled. NK-a (evaluated in terms of Cr51 release by K562 cells) and cytofluorimetric immunophenotypes (IP) (CD3, CD56, CD3/56<sup>+</sup>, CD4, CD8) were determined on bone marrow (BMMNC) and peripheral blood mononucleate (PBMNC) cells separated by density gradient. The BMMNC and PBMNC were resuspended in IMDM + 10% FCS and RPMI + 10% AB serum, respectively, and stimulated with IL-2 500 U/mL, IL-12 0.1 U/mL and IL-2+IL-12. IP and NK-a were evaluated after 7 days.

**Results.** The baseline bone marrow and peripheral NK-a of the MDS patients was significantly less than that of the healthy subjects ( $p < 0.01$ ). IL-12 increased both marrow and peripheral NK-a but this increase, besides not being statistically significant, was less than that obtained with IL-2 alone. The association of the two cytokines led to a significant increase in NK-a ( $p < 0.05$ ), greater than that obtained with IL-12 alone but less than that obtained with IL-2 alone. Following culture with IL-12 and IL-2+IL-12, no significant variations in IP were observed in relation to controls, although we did note the appearance of a CD4/CD8 inversion. However, after the addition of IL-2 a significant increase in CD3<sup>-</sup>/CD56<sup>+</sup> cells was observed ( $p < 0.01$ ) with respect to both baseline and the samples in untreated cultures.

**Conclusions.** Unlike IL-2, IL-12 does not seem to be capable of significantly increasing the defective NK-a of MDS patients. Furthermore, in our *in vitro* system we did not observe any of the synergistic or additive effects of IL-2 and IL-12 reported in the literature. This may be due to the concentration of the cytokine, but it does not exclude *a priori* its possible usefulness during the course of MDS because the antileukemic effects of IL-12 may be due less to direct stimulation of cytotoxic activity and more to indi-

rect activity mediated by cytokine release. Experiments involving the concentrations of  $\gamma$ -IFN and  $\alpha$ -TNF in the supernatant of the liquid cultures are currently ongoing, as are in the evaluation of clonogenic activity and morphological analysis of bone marrow cells after incubation with IL-12.

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055

#### A PROSPECTIVE STUDY ON INTERFERON $\alpha$ -2A VS CHEMOTHERAPY IN CHRONIC MYELOID LEUKEMIA: AN UPDATE OF KARYOTYPE RESPONSE AND SURVIVAL

Zuffa E, for the Italian Cooperative Study Group on Chronic Myeloid Leukemia  
*Institute of Hematology "L. e A. Seràgnoli", S. Orsola University Hospital, Bologna, Italy*

We compared recombinant interferon  $\alpha$ -2A with conventional chemotherapy (hydroxyurea or busulfan) in a prospective trial that enrolled 322 patients with chronic myeloid leukemia between July 1986 and July 1988; 218 patients were randomly assigned to be treated with interferon ( $\alpha$ -IFN) and 104 were randomly assigned to be treated with chemotherapy (CT). The dose and schedule of CT were free; the dose of  $\alpha$ -IFN was 9 MIU daily for the first 14 months and thereafter treatment was either reduced to 3 MIU 3 times a week in the case of no karyotype response, or was continued at the maximum tolerated dose. Serial statistical analysis showed that median survival was significantly longer in the  $\alpha$ -IFN than in the CT group, and the karyotype response rate (defined as  $>33\%$  Ph negative metaphases) was higher in the  $\alpha$ -IFN treated patients than in the CT ones (32% vs 5%, respectively). Updating the study as of March 1995, when the median follow-up of living patients was 80 months (range 71 and 100 months), confirmed that survival was longer with  $\alpha$ -IFN than with chemotherapy (median 76 mo vs 52 mo,  $p = 0.0018$ ) and that the difference was accounted for by a delay in the progression to accelerated or blastic phase (56% in the IFN group vs 74% in the CT group after 84 mo,  $p = 0.0013$ ). Survival length was strongly associated with a karyotype response; in the  $\alpha$ -IFN group, patients with a karyotypic response survived longer than those with minimal or no karyotype conversion (68% vs 32% after 84 months,  $p < 0.0001$ ), while no statistically significant difference in survival was detected between CT patients and  $\alpha$ -IFN patients with no karyotype response.

In our study response to  $\alpha$ -IFN was related with a low Sokal score and, in particular, to a low percentage of blast cells in the peripheral blood at diagnosis; thereafter there was a strong relationship between hematologic and karyotype response.

056

#### BCR-ABL ANTISENSE OLIGONUCLEOTIDES AND $\alpha$ -INTERFERON IN COMBINATION: BIOLOGICAL AND ANTILEUKEMIC EFFECT ON CELLS FROM PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA

Sala R, Venditti A, De Propriis MS, Lisci A, de Fabritiis P  
Hematology, Dept. of Human Biopathology, University La Sapienza and Hematology, University Tor Vergata, Rome, Italy

Experimental studies with BCR-ABL antisense oligonucleotides (ODN-As) in chronic myelogenous leukemia (CML) have clearly shown that these agents can inhibit the *in vitro* growth of leukemic cell lines and of primary cells from patients in blastic crisis.

We previously demonstrated that a proportion of Ph-pos mononuclear and CD34<sup>+</sup> cells from patients in chronic phase are eliminated by 26- and 16-mer ODN-As. To increase the antileukemic effect and to define further the biological aspects of ODN-As treatment, we investigated *in vitro* effects of ODN-As in combination with  $\alpha$ -interferon ( $\alpha$ -IFN). Incubation of the BV173 cell line with increasing concentrations of  $\alpha$ -IFN (100-10,000 U/mL) showed a dose-dependent CFU-GM inhibition, ranging from 16.5 and 38.5%. Combinations of  $\alpha$ -IFN and ODN-As demonstrated a greater antiproliferative effect than the two agents used individually. In particular, a concentration of 80 ug/mL ODN-As and  $\alpha$ -IFN at concentrations up to 1000 U/mL showed greater inhibition than  $\alpha$ -IFN alone. The addition of greater doses of  $\alpha$ -IFN did not produce a further increase in the antileukemic effect. Subsequently, 80 ug/mL ODN-As and 1000 U/mL  $\alpha$ -IFN were tested on primary cells from patients with CML in chronic phase. Combinations of  $\alpha$ -IFN and ODN-As produced an antiproliferative effect of  $69.6 \pm 3.2\%$  in the evaluated patients, which was greater than that observed after treatment with ODN-As alone ( $46 \pm 7.5\%$ ) or  $\alpha$ -IFN alone ( $32.5 \pm 3.9\%$ ).

To evaluate the role of  $\alpha$ -IFN in association with ODN-As in restoring the capacity of Ph-pos cells to adhere to preformed stroma, we studied the clonogenic ability of adherent and non-adherent cells before and after 72h of incubation. Using double-labelled immunofluorescence evaluation we also estimated the expression of a number of adhesion molecules (CD11c, CD54, CD18, CD11b, CD11a) on mononuclear and CD34<sup>+</sup> cells before and after incubation with ODN-As and/or  $\alpha$ -IFN. Preliminary results indicated that incubation of ODN-As and  $\alpha$ -IFN with mononuclear cells results in adhesion molecule expression similar to that found on untreated groups; however, CD11b expression was noted on CD34<sup>+</sup> cells after treatment, while it was undetectable on untreated groups. Demonstration of the *in vitro* antileukemic efficacy of ODN-As in combination with agents currently used for this treatment of the disease suggests that this gene modulation-based therapy might have future clinical applications in patients with CML.

057

**PREFERENTIAL EFFECT OF BCR-ABL ANTISENSE OLIGONUCLEOTIDES ON CD34-POSITIVE CELLS: SELECTION OF PATIENTS WITH CML FOR *IN VITRO* BONE MARROW PURGING**

De Propriis MS, Sala R, Lisci A, Buffolino S, Paggi MG, Calabretta B, de Fabritiis P  
Hematology, University La Sapienza and Regina Elena Institute for Cancer Research, Rome, Italy; Jefferson Cancer Center, Thomas Jefferson University, Philadelphia, USA

We studied the effect of 26- and/or 16-mer phosphorothioate oligodeoxynucleotides ([S]ODNs) complementary to the BCR-ABL junction on the colony-forming ability of mononuclear and/or CD34<sup>+</sup> enriched cells from 33 patients with chronic myelogenous leukemia (CML) in chronic phase. Twenty-seven patients were tested with 26-mer [S]ODNs and mean colony recovery was 41.7% of the untreated samples after 120 hours of incubation. Fifteen cases showed a significant reduction in colony formation after incubation with junction-specific antisense (J-sp AS) [S]ODNs as compared to untreated samples; however, in comparison to treatment with junction-non specific [S]ODNs, the effect was statistically significant in only 6 of them. Eight of 11 cases tested on CD34<sup>+</sup> enriched cells showed a significant inhibition of colony formation, but the effect was junction-specific in only 3 of them. The effect of J-sp AS ODNs on CD34<sup>+</sup> enriched cells appeared to be dependent on the proportion of CD34<sup>+</sup> cells present in the samples. In the 8 cases in which the number of CD34<sup>+</sup> cells was available after double-step concentration, a correlation was found between the proportion of CD34<sup>+</sup> cells and colony inhibition after 120h of incubation ( $r=0.88$ ;  $p=0.004$ ). Cases with the highest numbers of CD34<sup>+</sup> cells were also those in which the specific effect of AS ODNs treatment was most pronounced. Compared to 26-mer antisense [S]ODNs, the antileukemic effect of 16-mer antisense [S]ODNs was slightly less marked on mononuclear cells; it was, however, more specific than that of 26-mer antisense [S]ODNs on both mononuclear and on CD34<sup>+</sup> cells. Comparisons of 24- versus 120-hour incubation time revealed that there was a marked increase in the inhibition of Ph-positive colony formation after 120h, in agreement with a progressive increase in intracellular [S]ODN concentration from day 1 to day 5 of incubation that correlates with downregulation of p210 BCR-ABL levels. Hybridization experiments to evaluate non-specific effects of antisense [S]ODNs showed partial cross-hybridization when the 26-mer B2A2 antisense [S]ODNs was hybridized with B3A2 sense [S]ODN, but cross-reactivity was absent when 16-mer [S]ODNs were used. Downregulation of p210 was observed in 3/6 cases tested for its expression, and a good correlation was found between the antisense [S]ODN effect on leukemic colony formation and protein expression. These studies confirm that under optimal conditions for target cell culture and oligodeoxynucleotide size antisense [S]ODNs complementary to the BCR-ABL junction have specific antileukemic effects.

058

***IN CELL* RT-PCR IN CHRONIC MYELOID LEUKEMIA**

Testoni N, Martinelli G, Farabegoli P, Carboni C, Raspadori D, Pelliconi S, Salini L, Zaccaria A,\* Tura S  
Istituto di Ematologia "L. e A. Seràgnoli", Università degli Studi di Bologna, \*Servizio di Ematologia, Ospedale S. Maria delle Croci, Ravenna, Italy

We employed *in cell* retro transcriptase polymerase chain reaction (RT-PCR) to detect the hybrid BCR-ABL transcript within individual cells of chronic myeloid leukemia (CML) patients. Following cell permeabilization

and fixation, the hybrid m-RNA was reverse transcribed in c-DNA and then amplified by PCR, using fluorescent specific primers. The amplified DNA was detected within the individual cells by fluorescent microscopy and, in a few cases, by flow cytometry.

With this technique we studied 10 Ph<sup>+</sup> CML patients in different disease phases and 3 normal controls. Four patients were studied at diagnosis and their karyotypes were 100% Ph positive, while *in cell* RT-PCR positivity ranged from 96.2 to 98.7%. The other 6 patients were studied in various phases. Of these, 2 were Ph negative by cytogenetic studies because they had been submitted to allogeneic bone marrow transplantation (BMT) 8 months and 9 years earlier, respectively; only the latter showed a weak positivity at *in cell* RT-PCR (0.9%). The last 4 cases showed different amounts of Ph<sup>+</sup> metaphases: two patients were in  $\alpha$ -IFN treatment, one was in relapse after BMT and the last one had been treated with  $\alpha$ -IFN and then submitted to autologous BMT. These 4 patients showed 96, 60, 57.4 and 10.4% Ph-positive metaphases and 97.2, 81.7, 67.3 and 18.6% *in cell* RT-PCR positive cells, respectively. We observed that on the same sample *in cell* RT-PCR positivity was higher than cytogenetic positivity. *Classic* RT-PCR was positive in all patients but the one submitted to BMT 9 years earlier. The last three studies were performed on Ph-negative controls and no positive cells were revealed by *in cell* RT-PCR.

This technique seems to be specific and quantitative for the detection of the BCR-ABL transcript within individual cells, and it can be performed in other hematologic malignancies associated with chromosomal translocations and the formation of hybrid genes through the use of specific primers during PCR.

059

#### GEMCITABINE AND GEMCITABINE PLUS ARA-C HAVE SIGNIFICANT ACTIVITY ON BLAST CELLS FROM PATIENTS WITH BLAST CRISIS CHRONIC MYELOPROLIFERATIVE DISORDERS

Santini V, Bernabei PA, Figuccia M, D'Ippolito G, Zoccolante A, Rossi Ferrini PL

Dept. of Hematology, University of Florence, Italy

The pyrimidine analogue Gemcitabine (dFdC) inhibits the *in vitro* growth of acute myeloid leukemia cells and increases Ara-C cellular uptake, thus potentiating its cytotoxic activity.

We investigated the effects of dFdC on peripheral blood or/and bone marrow blast cells from 13 patients affected by chronic myeloproliferative disorders (9 CML and 4 Ph1 negative) in blastic phase. Mononuclear cells were separated by 1.077 g/mL density gradient centrifugation and cultured in the presence of IL-3 10 ng/mL with the addition of dFdC (1, 10, 100  $\mu$ M), Ara-C 5  $\mu$ M and Ara-C plus dFdC (1, 10  $\mu$ M). After 48 hours of culture we determined the following parameters: tritiated thymidine uptake, cell cycle distribution by propidium iodide incorporation and FACS scan analysis, percentage of apoptotic cells both by FACS analysis and agarose gel electrophoresis of DNA endonucleosomic cleavage products, p210 expression in treated Ph1-positive blast cells by SDS-PAGE of the total cell lysate and immunoblot with specific antibody. Blast cells recov-

ered after culture in the presence of the drugs were also plated in semisolid medium, and colony growth was assessed at day 7. dFdC showed a remarkable cytotoxic effect on blasts from all patients in terms of inhibition of thymidine uptake (up to 75% of control cell uptake), the appearance of apoptotic cells (indicated by the presence of an aploid peak at cytofluorimetric analysis), and the appearance of the characteristic DNA ladder at electrophoresis. Colony growth was equally inhibited when cells had been exposed to dFdC. Ara-C also demonstrated an intrinsic effect on leukemic blasts but the combination of Ara-C and dFdC yielded major cytotoxic activity, as shown by an increase in the inhibition of thymidine uptake and colony formation and by the appearance of apoptotic cells. Immunoblotting indicated a parallel decrease in p210 expression.

These observations suggest the possibility of overcoming the resistance to chemotherapy shown by CML and myeloproliferative disease blast cells. The combination of Ara-C and dFdC seems to be an alternative scheduling able to induce the maximal cytotoxic effect of Ara-C in otherwise poorly responding cases.

060

#### CLINICAL AND BIOLOGICAL FEATURES IN 30 CML PATIENTS (PTS) WITH COMPLETE KARYOTYPE RESPONSE (CCR) AFTER $\alpha$ -INTERFERON (IFN)

Montefusco E, Meloni G, Buffolino S, Cedrone M, Fenu S, Luzi G, Latagliata R, Spadea A, Alimena G

Hematology, Department of Human Biopathology, University "La Sapienza" of Rome, Italy

From September 1985 to December 1993, 200 adults patients with Ph<sup>+</sup> chronic myelogenous leukemia (CML) in chronic phase received IFN 2a or 2b for a median period of 35 months (r 1-102). Thirty patients are at present in CCR; in 8 pts CCR has been confirmed in at least 2 samples with an interval period > 6 mo (median CCR 19 mo; r 9-43). Ten of 30 pts underwent autologous bone marrow transplantation (ABMT), either before (3 pts) or after (7 pts) IFN treatment. All pts were examined for the following parameters: Sokal risk assessment at diagnosis, IFN tolerance (WHO), peripheral blood cell count, NAP score, minimal residual disease detection with PCR amplification. Clonogenic assay with X-chromosome inactivation was performed in 4 women with DXS255 locus heterozygosity; 27/30 patients showed a low-risk Sokal classification. Good tolerance to IFN and a complete hematological response with grade I leuco-thrombocytopenia were observed in all patients. Twelve of 30 patients achieved NAP normalization and the 4 evaluated women showed a polyclonal marrow population during CCR. Moreover, 5 out of 7 patients with ABMT after IFN treatment, are in CCR without any further therapy (median 57 mo; r 20-71). A longer follow-up and deeper biological analysis (HLA, IFN cellular receptors/mediators, *in situ* hybridization) are needed to establish the clinical and biological significance of our data and those of other researchers.

061

**TREATMENT WITH LYMPHOBLASTOID INTERFERON  $\alpha$  (nIFN $\alpha$ ) OF 17 PH<sup>+</sup> CML PATIENTS RESISTANT TO HUMAN RECOMBINANT INTERFERON- $\alpha$ 2A (IFN $\alpha$ 2A): EVALUATION OF HEMATOLOGIC AND KARYOTYPE RESPONSE**

Russo D, Candoni A, Zuffa E,\* Minisini R,<sup>o</sup> Silvestri F, Fanin R, Lorenzon A,<sup>o</sup> Martinelli G,\* Zaja F, Infanti L, Tura S,\* Baccarani M

*Division of Hematology and <sup>o</sup>Chair of Microbiology, Udine University Hospital, \*Institute of Hematology "L. e A. Seràgnoli", University of Bologna, Italy*

Interferon- $\alpha$  (IFN $\alpha$ ) as a single agent not only produces a hematologic response in 60-80% of CML patients, but also delays disease progression towards the blastic phase in the ones with a complete or partial karyotype conversion (Ph neg. metaphases from >33% to 100%). However, more than 30% of CML patients show early resistance to treatment and a significant number of them develop late resistance to IFN $\alpha$ , due in part to the induction of neutralizing IFN $\alpha$  antibodies (nIFN $\alpha$  Abs). For these patients a change in therapy to a non cross-reactive type of IFN $\alpha$  could be considered. For these reasons nIFN $\alpha$  was given at escalating doses of 3,6,9 MU daily in 17 patients with Ph<sup>+</sup> CML in chronic phase who had discontinued IFN $\alpha$ 2a between the 5th and 60th month (mean=24, median=13), because they were hematologically (12/17) and/or karyotypically (17/17) unresponsive, and/or neutralizing IFN $\alpha$ 2a antibody (nIFN $\alpha$ 2a Ab) positive (9/17). The hematologic response was checked every three months, while the karyotype response was assessed only in responders at the 12<sup>th</sup> month. After 12 months of nIFN $\alpha$  treatment a hematologic response, defined as complete (WBC <10 $\times$ 10<sup>9</sup>/L, without precursors in the differential; PLT <500 $\times$ 10<sup>9</sup>/L; spleen not palpable) or partial (lack of one of the above mentioned criteria) was obtained in 8/12 hematologically and karyotypically unresponsive patients and was maintained in 2 out of the remaining 5 pts who were hematologically responsive but karyotypically unresponsive (4 cases) or nIFN $\alpha$  2a Ab positive (1 case). Out of 10 hematologically responsive patients who completed 12 months of treatment with nIFN $\alpha$ , 9 did not achieve any karyotype conversion (Ph<sup>+</sup> 100%) and 1 obtained a minimal karyotype response (Ph neg 21%).

No difference in response was observed between the nIFN $\alpha$  Ab-positive patients and those who were negative. nIFN $\alpha$  was discontinued in 3 pts because of neurologic toxicity (grade III/IV) and fatigue (grade III/IV). These results show that a change in therapy to a non cross-reactive type of IFN $\alpha$  (nIFN $\alpha$ ) can induce a hematologic response in most CML patients unresponsive to IFN $\alpha$ 2a (whether nIFN $\alpha$  Ab positive or negative), but it does not seem to be able to produce a karyotype conversion.

062

**MODULATION OF ALL-TRANS RETINOIC ACID (ATRA) PHARMACOKINETICS IN ACUTE PROMYELOCYTIC LEUKEMIA (APL) BY PROLONGED INTERFERON- $\alpha$  (IFN) THERAPY**

Corso A, Lazzarino M, Regazzi MB,\* Iacona I,\* Bernasconi C

*Institute of Hematology, University of Pavia, and \*Dept. of Pharmacology, IRCCS Policlinico S. Matteo, Pavia, Italy*

Continuous treatment with ATRA induces accelerated drug catabolism, which is considered to be responsible for acquired resistance to ATRA. We studied the effect of IFN on ATRA pharmacokinetics in two patients with APL in molecular remission who were maintained by alternating 15 days of IFN- $\alpha$ 2a (3 M.U./m<sup>2</sup> every other day) and 15 days of ATRA (45 mg/m<sup>2</sup>/day). ATRA pharmacokinetics were also assessed on day 1 and day 15 of therapy in two additional APL patients who received daily ATRA alone. Plasma ATRA levels were measured prior to therapy and hourly for 6 hours after a single oral ATRA dose of 45 mg/m<sup>2</sup>, on days 1 and 15 of each course of ATRA. For the two patients on IFN+ATRA, the pharmacokinetics of ATRA during IFN+ATRA maintenance showed a mean decrease in AUC levels by day 15 of only 26.7% of day 1 values (from a mean of 696.0 ng  $\times$  h/mL to a mean of 510.2 ng  $\times$  h/mL). In the other two patients on ATRA alone, by day 15 of continuous ATRA administration plasma AUCs decreased by 93.2% of day 1 values (from a mean of 1010.4 ng  $\times$  h/mL to a mean of 68.1 ng  $\times$  h/mL). In one of the patients receiving IFN+ATRA, IFN- $\alpha$  was discontinued and ATRA pharmacokinetics were repeated monthly for a 3-month period during which this patient was maintained on ATRA alone for 2 weeks per month. After suspension of IFN, successive courses of ATRA were characterized by a progressive decrease of day 15 plasma AUCs; this reduction after 3 months reached a value of 88.3%, approaching that observed in patients not receiving IFN.

In conclusion, prolonged IFN treatment may lead to downregulation of catabolic mechanisms involved in ATRA clearance through still unknown pathways.

063

**CLINICAL RELEVANCE OF MOLECULAR MONITORING OF MINIMAL RESIDUAL DISEASE IN PATIENTS WITH ACUTE LYMPHOID LEUKEMIA (ALL) WITH THE t(4;11) ABNORMALITY**

Elia L, Rivolta A, Rapanotti MC, Ferrari A, Mancini M, Lo Coco F, Biondi A, Cimino G

*Dip. di Biopatologia Umana, Ematologia, Università La Sapienza, Rome; Clinica Pediatrica, Università di Milano, Ospedale S.Gerardo, Monza, Italy*

Following identification of the AF-4 (FEL) and ALL-1 (MLL, HRX, Hrtx 1) genes fused in leukemic cells with t(4;11) translocation, we designed specific primers to amplify by reverse transcriptase polymerase chain reaction (RT-PCR) the ALL-1/AF-4 fusion gene in all patients with t(4;11)-positive ALL. RT-PCR amplification was a two round *nested* PCR with a sensitivity of 1 $\times$ 10<sup>4</sup> cells. For amplification of cDNA products we used the Ex 5 (Exon 5 - ALL-1 gene) forward primer and the AF 4.1 (Exon 4 - AF-4 gene) reverse primer. The first PCR product was utilized for a second round of amplification using the Ex 6 (Exon 6 - ALL-1 gene) nested primer. Amplification of the normal ALL-1 exons 5-7 transcript was performed with 1 g of the same RNA preparation as the RNA quality control to verify the efficiency of the RT step. Negative control were included in all PCR experiments.

In the present study we used this technique to monitor minimal residual disease (MRD) during the follow-up of 12 ALL patients (10 females and 2 males) with cytogenetic and/or molecular evidence of t(4;11). The ALL-1/AF-4 fusion transcript was detected in all patients at diagnosis. Seven out of the 12 patients were infants between the ages of 1 and 15 months. The remaining 5 were adults. Hyperleukocytosis (WBC > 150 × 10<sup>9</sup>/L) was present in 11 cases. The immunophenotype was pre-pre-B in 10 cases and CD10<sup>+</sup> in 2. A co-expression of myeloid markers was demonstrated in 7 cases. One patient showed a shift towards a monocytic phenotype two months after diagnosis of ALL. All patients received intensive conventional chemotherapy as induction and consolidation (no one received a BMT). Five patients underwent an early relapse after achieving a CR and died within 18 months; five are in continuous complete remission (CCR) at 32, 39, 52, 53 months from diagnosis, respectively, and 2 died of resistant disease.

Sequential analysis of the ALL-1/AF-4 hybrid transcript showed a persistently negative RT-PCR in the 5 CCR long-term survivors. On the other hand, the RT-PCR was consistently positive in the remaining 7 cases, including the 5 who relapsed after achieving a clinical CR. To our knowledge, this is the first demonstration of the clinical relevance of RT-PCR in the monitoring of t(4;11)-positive ALL. In fact, our results indicate: 1) that this neoplastic clone can be eradicated, as demonstrated by the 5 long-term survivors in CCR with negative PCR; and 2) that a positive PCR is predictive of disease relapse, since this always occurred in our cases within 12 months from diagnosis.

064

#### CYTOGENETIC PROFILE AND CLINICOBIOLOGICAL FINDINGS IN MINIMALLY DIFFERENTIATED (FAB M0) ACUTE MYELOID LEUKEMIA

Cuneo A, Michaux JL, Boogaerts M, Dal Cin P, Doyen C, Van Den Berghe H, Rigolin GM, Bardi A, Castoldi GL  
*Istituto di Ematologia, Università di Ferrara, Italy and Institutes of Hematology, UCL de Louvain in Brussels and Mont-Godinne; Institutes of Hematology and Genetic Centre KUL Leuven, Belgium*

Cytogenetic data were studied in 26 patients with *de novo* acute myeloid leukemia (AML) with minimal myeloid differentiation (corresponding to the M0 subtype of the FAB classification), in correlation with cytoimmunologic and clinical findings.

Clonal abnormalities were detected in 21 cases (80.7%), 12 of which showed a complex karyotype. Partial or total monosomy 5q and/or 7q was found, either as the sole aberration, or in all abnormal metaphases, in 11 patients; in 8 cases additional chromosome changes were present, including rearrangements involving 12p12-13 and 2p12-15, seen in 3 cases each. Five patients demonstrated trisomy 13 as the possible primary chromosome change, and non-recurrent chromosome abnormalities were seen in 5 others. Comparison of these findings with chromosome data from 42 patients with AML-M1 shows that abnormal karyotypes, complex karyotypes, unbalanced chromosome

changes, -5/5q- and/or -7/7q-, and +13 are seen more frequently in AML-M0 than in AML-M1. Patients with abnormalities of chromosome 5 and/or 7 frequently demonstrated trilineage myelodysplasia and low WBC count. Despite a median age of 44, complete remission (CR) was achieved in 4/11 patients only. Patients with +13 were elderly males with a history of frequent exposure to myelotoxic agents in the workplace. Unlike patients with clonal abnormalities, most AML-M0 cases with normal karyotypes showed 1-2% peroxidase-positive blast cells at light microscopy and frequently achieved CR.

It is concluded that: a) AML-M0 shows a distinct cytogenetic profile, partially reminiscent of that of therapy-related AML; b) different cytogenetic subgroups of AML-M0 that show characteristic clinicobiological features can be identified, and c) chromosome findings may account in part for the unfavorable outcome frequently observed in these patients.

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#### P170 EXPRESSION IN ADULT ACUTE LEUKEMIA: CORRELATION WITH TREATMENT OUTCOME

Damiani D, Michieli M, Michelutti A, Grimaz S, Masolini P, Candoni A, Fanin R, Russo D, Baccarani M  
*Division of Hematology and Department of Bone Marrow Transplantation, University Hospital, Udine, Italy*

The incidence of the MDR-1 gene product, p170 glycoprotein was investigated in the blast cells of 98 patients with acute leukemia (73 ANLL and 25 ALL) at diagnosis by flow cytometry (FACScan, BD) using the MRK16 antibody (Immunotech, Marseille, France). MRK16 positivity was expressed as the mean fluorescence index (MFI) defined as the ratio of the mean fluorescence of the test sample and to isotypic control. Forty-one of 48 patients (85%) had a MFI ≥ 6 (i.e. a MFI higher than normal leukocytes) and were considered MDR<sup>+</sup>. All patients had received MDR related drugs and 76/98 (77.5%) were evaluable for treatment response; 27/35 (77%) with MFI < 6, but only 15/41 (36.5%) with MFI ≥ 6 achieved a complete remission (p=0.0009). Complete remission was significantly longer in MDR<sup>-</sup> patients (p=0.04 logrank test). In 46 cases the p170 function was assayed by flow cytometry after a 2-hour incubation with 1 µg/mL of daunorubicin (DNR) and expressed as NMFI, according to Luk (*JNCI*, 1989). By multiple regression DNR content correlated negatively with MRK16 MFI (R=0.434, p=0.001). MDR<sup>+</sup> cells showed a significantly lower DNR content than MDR<sup>-</sup> blasts (NMFI 270±79 vs 365±136, p=0.006). Moreover, the reversal agent PSC 833 (Sandoz) significantly increased the DNR content (NMFI 270±79 vs 332±80; p=0.000) in MDR<sup>+</sup> cells, whereas no significant modification in DNR content was found in MDR<sup>-</sup> cells. Our data confirm the negative prognostic role of p170 in acute leukemia and suggest that p170 function could be efficiently counteracted with PSC 833 by increasing drug toxicity in MDR<sup>+</sup> blast cells but not in normal tissues.

066

### **SURFACE MARKER EXPRESSION IN ADULT ACUTE MYELOID LEUKEMIA (AML): CORRELATION WITH INITIAL CHARACTERISTICS AND RESPONSE TO THERAPY**

Pagnucco G, Livraghi A, Santagostino A, Bellio L, Vanelli L, Canevari Sciorati A, Maiocchi M, Zanaboni M, Corso A, Brusamolino E, Lazzarino M, Bernasconi C  
*Istituto di Ematologia, Università di Pavia, Divisione di Ematologia, IRCCS Policlinico San Matteo, Pavia, Italy*

From May 1985 to December 1994, we studied 211 previously untreated pts with adult *de novo* AML (M1=25, M2=56, M3=48, M4=55, M5=27). Bone marrow and/or peripheral blood blasts were tested for reactivity with monoclonal antibodies (MAb) to early (CD34, CD33, CD31, CD13, DR) and late (CD15, CD14, CD11b, CD11c) differentiation-associated antigens of the myeloid lineage. Furthermore, we studied the concomitant expression of lymphoid markers using MAb to B-lineage (CD19) or to T-lineage (CD7, CD2) antigens. Antigen expression was determined by immunofluorescence and flow cytometry using a FACS (EPICS-C). A cell population was considered positive for a given surface marker if more than 20% of the gated cells stained positively. Individual antigen expression was found to correlate with FABc, age (DR), leukocyte (DR, CD11c) and platelet count (DR, CD11b), and serum LDH (CD13, CD11c). Expression of CD14 and DR antigens was closely associated with extramedullary disease. The incidence of DIC and early death from bleeding was higher in the DR<sup>-</sup> cases. Induction remission therapy included standard regimens with DNR or IDR or mitoxantrone and ARA-C or VP16. Analysis of response and survival in the 152 (72%) evaluable patients showed these results: a CR rate higher in the DR<sup>-</sup> and CD15<sup>+</sup> pts (DR<sup>-</sup> 76% vs. DR<sup>+</sup> 59%,  $p=.05$ ; CD15<sup>+</sup> 73% vs. CD15<sup>-</sup> 56%,  $p=.05$ ); the 4-year rates of DFS were higher for the DR<sup>-</sup> and CD13<sup>+</sup> patients and for patients with a CD33/CD13 ratio >1 (DR<sup>-</sup> 35% vs. DR<sup>+</sup> 17%,  $p=.02$ ; CD13<sup>+</sup> 39% vs. CD13<sup>-</sup> 18%,  $p=.04$ ; CD33/CD13 >1 34% vs. CD33/CD13 <1 19%); overall survival was higher in DR<sup>-</sup>, CD15<sup>+</sup>, CD33<sup>+</sup> and for patients with a CD33/CD13 ratio >1 (4-year survival: DR<sup>-</sup> 40% vs. DR<sup>+</sup> 15%,  $p=.0001$ ; CD15<sup>+</sup> 25% vs. CD15<sup>-</sup> 15%,  $p=.04$ ; CD33<sup>+</sup> 26% vs. CD33<sup>-</sup> 12%,  $p=.01$ ; CD33/CD13 >1 28% vs. CD33/CD13 <1 14%,  $p=.09$ ). CD34 expression was not associated to a better response to induction therapy or longer survival. Of interest, we observed expression of the lymphoid markers CD7, CD2 and CD19 in 33/211 cases (17%). The expression of these antigens was correlated with an unfavorable prognosis (4-year DFS: AML/Ly<sup>-</sup> 31% vs. AML/Ly<sup>+</sup> 10%,  $p=.05$ ; 4-year survival: AML/Ly<sup>-</sup> 25% vs. AML/Ly<sup>+</sup> 15%,  $p=.05$ ). We conclude that immunophenotype studies have proven clinically useful in the characterization of adult AML.

067

### **TREATMENT WITH ARABYNOSILCYTOSINE INCREASES P170 EXPRESSION IN ACUTE NON-LYMPHOCTIC LEUKEMIA**

Grimaz S, Michelutti A, Damiani D, Melli C, Michieli M, Masolini P, Infanti L, Barillari G, Fanin R, Russo D, Baccarani M

*Division of Hematology and Department of Bone Marrow Transplantation, Udine University Hospital, Udine, Italy*

P170 (also known as Pgp)-mediated multidrug resistance confers cross resistance to a wide group of cytotoxic drugs. Malignancies arising from tissues normally overexpressing P170 show the highest levels of Pgp. In other types of cancer as well as in hematological malignancies, P170 expression often increases after chemotherapy and during disease progression. This may be due to a selection of cells in the neoplastic population overexpressing P170, but there is also some *in vitro* evidence suggesting that *mdr1* gene expression may be induced by exposure to some drugs. Chaudary and Roninson (*JNCI* 1993; 85:632-9) observed that some non MDR-related drugs, such as cytarabine, are also able to induce *mdr1* in cell lines. In order to investigate whether this may also occur *in vivo*, we evaluated Pgp expression in 32 cases of ANLL before and after 4 days of cytarabine administration, during a course of chemotherapy including 4 days of cytarabine + 3 days of idarubicin ± reversal agents. We used an indirect cytofluorimetric assay with the monoclonal antibody MRK16 (that is directed against an extramembrane domain of P170) and a FITC conjugated anti-mouse antibody; mean fluorescence index (MFI) was calculated by dividing the mean FL1 intensity of the positive sample by the mean FL1 isotypic control. The mean value of the MFI was 6.6 (±3.4) before arabinosyl cytosine treatment and 8.2 (±3.3) after 4 days of treatment with it ( $p=0.002$  paired data t-test,  $p=0.06$  independent data Student's t-test). These data suggest that P170 induction following exposure to cytotoxic drugs may also occur *in vivo* and may account for the development of MDR during treatment; moreover, these observations could stimulate the use of reversal agents in combination with MDR-related drugs during first-line treatment.

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### **FLOW CYTOMETRIC DETECTION OF P-GLYCOPROTEIN (PGP) AT DIAGNOSIS IDENTIFIES HIGH RISK PATIENTS IN ACUTE MYELOID LEUKEMIA (AML)**

Aronica G, Del Poeta G, Stasi R, Venditti A, Bruno A, Buccisano F, Santinelli S, Suppo G, Cox C, Masi M, Papa G, Amadori S  
*Cattedra e Divisione di Ematologia, Università Tor Vergata, Ospedale S. Eugenio, Rome, Italy*

Several studies have indicated that expression of the multidrug resistance phenotype (MDR) in AML specimens at diagnosis is associated with a significantly lower probability of successful treatment outcome (*Sato et al, 1990; Campos et al, 1992*). Nevertheless, few studies (*Ino et al, 1994*) have applied flow cytometry to the detection of PGP in AML. This may reflect problems related to the precise semiquantitative measurement of low level antigen expression, which is frequently found in AML. We studied 180 patients with newly diagnosed AML, 82 females and 98 males, median age 57 yrs (range 17-81), admitted to our unit between June 1990 and December 1994. All patients were treated with intensive chemotherapy based on the association of an anthracycline, etoposide and

intermediate to high doses of cytarabine. P-170 expression was demonstrated by flow cytometry, using the C-219 (Cis Diagnostici, Italy) monoclonal antibody (MoAb), an IgG2a that recognizes an epitope on the inner surface of the cytoplasmic membrane. Bone marrow and/or peripheral blast cells were fixed and permeabilized in two steps: first in 3.5% paraformaldehyde/PBS and, then, after washing in PBS, in 50% cold acetone/PBS. Next, samples were incubated at 4° C for 30 minutes with unconjugated C-219 at optimal concentration. After two further washings in PBS, cells were incubated at 4° C with a FITC-conjugated F(ab)2 fragment of goat anti-mouse Ig (dilution 1:20) (Technogenetics, Milan, Italy). Negative controls were performed by incubating cells with non relevant isotype IgG2a antibody and by incubating C-219 with cells of a sensitive or resistant Lovo cell line. Analysis was carried out by flow cytometry. At onset, P-170 was detected in 48.3% of cases and no relationship was found between sex, age, organomegaly or MDR phenotype. C-219 positivity was significantly associated with a leukocyte count greater than  $50 \times 10^9/L$  ( $p = 0.004$ ). With respect to the FAB subgroups, strict correlations were noted between C-219 negativity and M3 subtype and between C-219 positivity and M0-M4-M5 subtypes ( $p = 0.001$ ). There was a close association between PGP phenotype and CD7, since 117 of 180 samples had similar patterns of staining with C-219 ( $P < 0.001$ ). With regard to clinical outcome, remission induction rates differed significantly between C-219<sup>+</sup> and C-219<sup>-</sup> cases (30% vs 72%,  $p < 0.001$ ). Relapse was more frequent in C-219<sup>+</sup> patients (19/23) than in C-219<sup>-</sup> ones (29/70) ( $p < 0.001$ ). Survival and remission duration rates were significantly shorter in C-219<sup>+</sup> patients ( $P < 0.001$  and  $p = 0.01$ , respectively). In a multivariate analysis, C-219 was confirmed as an independent prognostic factor for CR achievement and duration, together with age, CD7 and CD14. The flow cytometric procedure described and statistical data analysis allowed us to detect low levels of PGP expression and identify patients with poor prognosis who might benefit from P-170 modulators.

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069

#### ACUTE MYELOID LEUKEMIA (AML): COEXPRESSION OF T-CELL ANTIGENS AND STUDY OF RESIDUAL DISEASE (RD) WITH AN IMMUNOLOGIC METHOD

Pezzetti L, Cairoli R, Valletto AF, Brando B,\* Marenco P, Perutelli PF, Mori PGF, Morra E  
Department of Hematology and \*Nephrology, Ospedale Niguarda, Milano; IV Department of Pediatrics, Istituto G. Gaslini, Genova, Italy

We studied the clinical, cytogenetic and cytochemical features of 100 cases of AML in which immunophenotype analysis was performed in our laboratory between 1992 and 1995: T-cell antigen coexpression was evident in 14 patients (pts)(see Table).

Gene rearrangement analysis of T-cell receptor  $\beta$ ,  $\gamma$  and  $\delta$  chains disclosed that the  $\delta$  chain was clonally rearranged in only 1 pt (#8); in all cases either the  $\beta$  or the  $\gamma$  chain

was in germline configuration. No patient presented adenopathy or central nervous system involvement. Nine of 12 patients (mean age 52 y., range 26-70) treated with intensive chemotherapy achieved complete remission (CR), 8 after the first course of induction. The patients #2 and #13 received alternative therapy. Using dual color flow cytometer analysis we studied the residual leukemia-associated phenotype in 8 pts after CR had been obtained; 37 bone marrow samples were analyzed (extended gate, 50,000 events). In 2/37 samples (pts #5,6) the anomalous coexpression recurred after +26, +8 months in CR. In one patient (#3) a phenotypic shift occurred at relapse. Five patients are in continuous CR (follow-up 4-40 months).

N.	WBC $\mu L$	Bt. %	Co- expression*	CD 34	CD 117	Karyotype	FAB
1	70,000	96	CD7+CD33	POS	POS	46XY	M1
2	320,000	95	CD7+CD33	POS	POS	ND	M1
3	80,000	95	CD7+CD13	POS	POS	47XY+22	M1
4	47,000	75	CD7+CD13	POS	POS	46XY	M1
5	7,000	68	CD7+CD33	NEG	POS	46XX del 11q	M1
6	160,000	97	CD7+CD13	POS	POS	47XX +8	M1
7	38,000	54	CD4+CD33	NEG	POS	46XY	M1
8	16,000	87	CD7+CD13	POS	POS	46XY	M1
9	4,000	37	CD7+CD13	POS	POS	46XY <sup>®</sup>	M1
10	78,000	97	CD7+CD33	POS	ND	46XX del 5q	M1
11	360,000	95	CD7+CD33	POS	ND	46XX	ND
12	92,000	70	CD7+CD13	POS	NEG	46XX del 5q	M1
13	13,000	38	CD7+CD33	POS	POS	46XX del 5q <sup>®</sup>	M1
14	17,000	74	CD7+CD33	POS	POS	46XY	M1

Bl.: blasts; \*Blast positivity  $\geq 20\%$ ; sensitivity  $\leq 1\%$  (75 normal controls); <sup>®</sup>complex karyotype; ND: not done.

**Conclusions.** The frequency of positive AML T-cell antigens was 14%; morphologically, all cases were classified FAB M1 with immature immunophenotype (12/14 CD34<sup>+</sup>, 11/12 CD117<sup>+</sup>). Thirteen out of 14 pts showed germline configuration of the TCR genes. The immunologic study of RD provided concordant results with morphological and cytogenetic analysis for the evaluation of CR in 7/8 patients.

070

#### PROGNOSTIC IMPACT OF TERMINAL TRANSFERASE (TdT) DETECTED BY FLOW CYTOMETRY IN ACUTE MYELOID LEUKEMIA (AML)

Aronica G, Del Poeta G, Stasi R, Venditti A, Bruno A, Suppo G, Pisani F, Franchi A, Rainaldi A, Masi M, Papa G, Amadori S  
Cattedra e Divisione di Ematologia, Università Tor Vergata, Ospedale S. Eugenio, Rome, Italy

The advantages of demonstrating nuclear TdT expression in leukemic cells by flow cytometry rather than with the conventional slide staining technique are represented by a rapid and objective analysis of a high number of cells as well as detection of residual leukemic cells through multicolor staining for TdT/surface antigen combinations. Difficulties in developing a reliable method for the flow cytometric determination of TdT have been primarily due

to the need for cell fixation and permeabilization and to the use of indirect immunofluorescence staining with polyclonal anti-TdT antibodies, which results in high background fluorescence. It has been reported (*Paietta et al, 1993; Drexler, 1993*) that the fluorescence intensity of TdT staining in AML or acute lymphoid leukemia (ALL) expressing myeloid antigens is markedly weaker than in ALL. We describe here an optimized methodology for the detection of TdT by flow cytometry based on double-step fixation and permeabilization and the use of a directly FITC-conjugated mixture of TdT monoclonal antibodies (MoAbs).

Our study included 243 consecutive patients with *de novo* AML, median age 55 yrs (range 17-81), 104 females and 139 males, all treated with intensive chemotherapy regimens, enrolled between 1988 and 1994. Mononuclear cells from bone marrow and/or peripheral blood samples were resuspended in immunofluorescence medium (IFA) (10 mM HEPES, pH 7.4, 150 mM NaCl, 4% fetal bovine serum) and fixed in 2 mL of 1% paraformaldehyde/PBS for 15 min at room temperature. Subsequently, the cell membrane was permeabilized in 2 mL of 0.1% triton X-100 in IFA for 3 min at room temperature to allow penetration of the anti-TdT FITC-conjugated MoAb mixture (H-TdT, Technogenetics). After resuspension in IFA with 2% human AB serum, cells were incubated with 10 µL of anti-H-TdT MoAb for 1h at 4°C. Next, the stained cells were washed twice with PBS and measured on an Epics Profile (Coulter) flow cytometer. A non specific FITC-mouse monoclonal IgG1 was used as negative control. A positive reaction was defined as 20% of gated cells being more fluorescent than the control. This criterion was adopted to avoid the fluorescence background.

TdT was detected in 61 patients (25.1%). There were no differences between the TdT negative and TdT positive groups with respect to white blood cell count (higher or lower than  $50 \times 10^9/L$ ), organomegaly, age, or extramedullary disease. A strict relationship was found between TdT positivity and FAB M0 and M1 classes (67% and 38%, respectively;  $p < 0.0005$ ). Lower levels of CD15 and CD14 expression were found in TdT<sup>+</sup> AML ( $p < 0.001$  and  $< 0.05$ , respectively). TdT expression was strongly associated with HLA-DR positivity (56/61,  $p = 0.015$ ) or CD34 phenotype (55/60,  $p < 0.0001$ ). A lower complete response frequency was observed among TdT<sup>+</sup> patients (32% vs 75%), and this difference was statistically significant ( $p = 0.015$ ). Moreover, TdT positivity in our cases was associated with a deterioration of overall survival and continuous complete remission rate ( $p = 0.012$  and  $= 0.059$ , respectively). Although the prognostic implication of TdT positivity in AML has remained controversial (*Paietta et al, 1993*), the above mentioned methodology which allows detection of low level fluorescent TdT<sup>+</sup> myeloblasts, suggests that TdT<sup>+</sup> AML may represent a biologically and perhaps clinically distinct leukemia subtype.

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#### P170 GLYCOPROTEIN EXPRESSION IN ACUTE NON LYMPHO-CYTIC LEUKEMIA (ANLL)

Melli C, Michieli M, Damiani D, Michelutti A, Grimaz S, Masolini P, Candoni A, Fanin R, Russo D, Baccarani M  
*Division of Hematology, University Hospital, Udine, Italy*

The study of P170 glycoprotein expression may be of value when investigating the outcome of ANLL. P170 expression was therefore evaluated in 92 cases of ANLL observed from 1989 to 1994 by means of the APAAP immunocytochemical technique, using the MRK16 monoclonal antibody. Leukemic cells were defined as P170 positive when P170 expression was comparable to that of a positive control P170<sup>+</sup> cell line. P170 positivity was detected in 65 out of 92 cases at disease onset.

All 92 cases were evaluable for the outcome of therapy; a complete remission with first-line chemotherapy was achieved in 29 of the 65 P170-positive ANLL patients and in 20 of the 27 P170-negative cases ( $p = 0.018$ ). A relapse occurred in 18 of the 29 P170-positive ANLL and in 10 of the 20 P170 negative cases. Overall treatment failure was noted in 54 of the 65 P170-positive ANLL and in 17 of the 27 P170-negative cases ( $p = 0.03$ ).

These data indicate that a high proportion of ANLL are P170 positive at their onset, and that such cases are poorly responsive to first-line chemotherapy. Thus, the possible usefulness of reversal agents should be considered in P170-positive ANLL.

072

#### SUBCUTANEOUS LOW-DOSE IL-2 FOR REMISSION MAINTENANCE IN ELDERLY AML PATIENTS

Notaro R, Selleri C, Caparrotti G,<sup>o</sup> Picardi M, di Grazia C, Pagnini D,<sup>o</sup> Cantore N,<sup>#</sup> Andriani A,<sup>@</sup> Carella M,<sup>^</sup> Catalano L, Del Vecchio L\*, Di Girolamo R<sup>o</sup>, Volpe E,<sup>#</sup> Carotenuto M,<sup>^</sup> Rotoli B  
*Cattedra di Ematologia, Univ. Federico II, Naples; Divisioni di Ematologia <sup>o</sup>Osp. S.Felice a Cancellò, <sup>#</sup>Osp. Civile, Avellino, <sup>@</sup>Osp. S. Giacomo, Roma, <sup>^</sup>IRCCS Casa Sollievo della Sofferenza, S. Giovanni Rotondo, \*Servizio di Immunematologia, Osp. Cardarelli, Naples, Italy*

The outcome of therapy in elderly AML patients is disappointing; low-dose treatment leads to complete remission (CR) only occasionally, while intensive chemotherapy (CT) can only be applied to select patients. The results are less than 50% CR and 15% of patients surviving more than two years.

Elderly patients with AML (excluding M3) who were not eligible for the GIMEMA 0491 protocol because of age or poor performance status entered a study aimed at exploring the efficacy and toxicity of intermediate dose CT (induction: idarubicin 8 mg/m<sup>2</sup> + etoposide 100 mg/m<sup>2</sup> days 1 to 3, cytarabine 50 mg/m<sup>2</sup> bid days 1 to 5; consolidation: idarubicin 12 mg/m<sup>2</sup> day 1, etoposide and cytarabine same as in induction), followed by a maintenance therapy with subcutaneous low dose IL-2 (1.5 MU/m<sup>2</sup> day 1, then 3 MU/m<sup>2</sup> days 2 to 5, monthly) in an attempt to prolong CR duration.

From March 1993 to July 1994, 26 patients (median age 69, range 60-82) were enrolled in the study. Performance status (WHO) was 2 or more in 13 patients, 11 presented infection at diagnosis, 4 suffered from cardiopathy and one

from nephropathy. CR was achieved in 13 patients (in 2 after a second induction course), 3 died early and 10 were resistant. The 13 patients in CR received monthly courses with subcutaneous IL-2 (mean 6 courses per patient); 11 relapsed. DFS was 15% after 2 years, with a median of 7 months. OS was 39% after 2 years, with a median of 10 months. CT was well tolerated, systemic and hematological toxicity was low. Consolidation was particularly well tolerated: hospitalization was brief (mean 19.5 days) and few RBC and plt transfusions were needed (mean 3 and 0.5 units, respectively). Home treatment with IL-2 was inoffensive (only one patient was withdrawn due to a vasculitis relapse). IL-2 treatment significantly increased peripheral levels of lymphocyte subsets: CD25<sup>+</sup> from 6.8 to 16%, CD4<sup>+</sup>/DR<sup>+</sup> from 5.8 to 11%, CD8<sup>+</sup>/DR<sup>+</sup> from 5.4 to 12.2%.

Our results in a negatively selected cohort of patients (age, infection, performance status) are similar to those obtained with more intensive courses in patients selected for the absence of risk factors. Additional studies will be conducted to try to reduce the resistance rate and to prolong DFS by: (a) modelling a salvage protocol for resistant patients; (b) intensifying consolidation; (c) reducing the interval between low dose IL-2 courses.

073

#### ADULT PHILADELPHIA-CHROMOSOME POSITIVE (PH1<sup>+</sup>) OR BCR/ABL REARRANGED (BCR/ABL<sup>+</sup>) ACUTE LYMPHOBLASTIC LEUKEMIA (ALL): RESULTS OF A GIMEMA ALL 0288 PILOT STUDY

Mandelli F, Annino L, Ferrari A, Camera A, Ciulli S, Fabiano F, Ferrara F, Invernizzi R, Miraglia E, Recchia A, Resegotti L, Sica S, Specchia G, Stasi R, Zagonel V. and Saglio G for the GIMEMA Group

In May 1990, in the context of the GIMEMA ALL 0288 trial, the GIMEMA Group initiated a pilot study specifically designed for adult Ph1<sup>+</sup> or BCR/ABL<sup>+</sup> ALL patients that included early transplantation (Mandelli *et al*, 1993). At diagnosis, centralized molecular analysis was to be carried out, whereas cytogenetics was to be performed in each individual center. From May 1990 to March 1994, of the 496 adult (<60 yrs.) ALL patients enrolled in this protocol, 200 were studied at the molecular level and 35 (17.5%) were found to carry rearrangement (15 p190<sup>+</sup>, 18 p210<sup>+</sup> and 2 p190/p210<sup>+</sup>). Cytogenetic analysis was performed in 26 out of these 35 rearranged patients: 20 were Ph1<sup>+</sup>, 2 Ph1 negative and 4 did not show metaphases. Another 9 patients, for whom molecular biology was not carried out, were found to be Ph1<sup>+</sup>. Thus a total of 44 patients (30 males and 14 females, median age 35.5 years, range 13-57 yrs), entered the pilot study. At diagnosis, median WBC count was 20×10<sup>9</sup>/L (range 1.4-144×10<sup>9</sup>/L); as regards immunophenotype, 26 (59%) were B-lineage ALL, 1 T-ALL, 15 hybrid leukemia (My<sup>+</sup>), 1 AUL and 1 was not evaluable. Complete response (CR) was achieved in 36/44 (82%), 7 (16%) were resistant and 1 (2%) died during induction. Among the 36 CRs, 11 went off-study early because of relapse (8), death in CR (1) and chemotherapy-related toxicity (2). Of the remaining 25 CRs, 14 underwent transplantation (7 BMT and 7 ABMT) and 11 continued on chemotherapy because of transplant refusal (4) or other causes (7). Regarding the follow-up of

the 14 transplanted, 8 (57%) (4 BMT and 4 ABMT) relapsed in a median time of 6.2 months (range 4-9 mos.) from transplant, while 6 patients (3 BMT and 3 ABMT) are in 1<sup>st</sup> continuous CR (CCR) for a median of 20.4 mos. Overall median CR duration of all transplanted patients is 12.8 mos. (range 6-60 mos.); overall median survival (OS) is 24.6 mos. (range 13.2-60.3). As regards the follow-up of the 11 non-transplanted patients, 5 (45%) relapsed in a median time of 11.4 mos. (range 6.4-38.1 mos.), 1 patient developed chronic phase CML 30 mos. post CR, 1 died in CR, and 4 are in 1<sup>st</sup> CCR for a median of 24.3 mos. The median OS of these non-transplanted patients is 21 mos. (range 9.5-46 mos.); the median CR duration is 18.1 mos. (range 6.4-38.1 mos.). The median OS of all 44 patients entered in this study is 15.9 mos. (range 1-60.3 mos.). To date, 15/44 are alive: 10 in 1<sup>st</sup> CCR (6 transplanted and 4 non transplanted), 1 in 3<sup>rd</sup> CR, 1 in CML chronic phase and 3 with active disease.

074

#### IMMUNOLOGICAL PHENOTYPE IN ACUTE PROMYELOCYTIC LEUKEMIA: A STUDY OF 48 CASES

Buonanno MC, Pagnucco G, Pastorini A, Bellio L, Livraghi A, Vanelli L, Canevari Sciorati A, Nozza A, Passamonti F, Castelli G, Brusamolino E, Canevari A, Lazzarino M, Bernasconi C

*Istituto di Ematologia, Università di Pavia; Divisione di Ematologia, IRCCS Policlinico San Matteo, Pavia, Italy*

In order to investigate the clinical importance of immunophenotype in acute promyelocytic leukemia (APL), we studied 48 patients with this condition from May 1985 to December 1994. A diagnosis of the M3 variant (M3v) was ascertained in 10 cases. Bone marrow and/or peripheral blasts were tested with monoclonal antibodies (mAb) to early (CD34, CD33, CD31, CD13, HLA-DR) and late (CD15, CD14, CD11b, CD11c) differentiation-associated antigens of the myeloid lineage and with monoclonal antibodies of the lymphoid lineage (CD7, CD2, CD19). Antigen expression was determined by immunofluorescence and flow cytometry using a FACS (EPICS-C) after appropriate forward light scatter gating of total bone marrow samples. We considered a mAb positive when at least 20% of blast cells shared the positivity. Positivity to the myeloid (early and late) markers considered was as follow: DR 17%, CD34 12%, CD33 98%, CD15 27%, CD14 11%, CD13 51%, CD11b 35%, CD11c 6%. Twelve cases (25%) were positive for lymphoid antigens. We observed the CD7 expression in 6/42 cases (14%), CD2 in 10/43 (23%), and CD19 in 4/45 (19%). We found a higher expression of CD2 (60%, p<.01), DR (44%, p<.02), and CD11b (67%, p<.05) in M3v cases. The expression of lymphoid markers was associated with significantly higher mean WBC counts but had no impact on disease-free or overall survival. Our data indicate that: 1) APL is generally (but not always) DR- and CD34-negative, CD33- and frequently CD13-positive; 2) monocyte-associated membrane CD14 and CD11c expression is low; 3) certain lymphoid markers (CD7, CD2, CD19) may be expressed; 4) the expression of CD2, DR and CD11b is associated with hypogranular morphology (M3v). Because a diagnosis of

APL is important in order to establish the specific therapeutic approach, the discriminant capacity of immunological typing by flow cytometry to identify M3 and M3v could be decisive for a rapid diagnosis.

075

#### **RHODAMINE-123 EFFLUX AND P-gp 170 MoAb DETECTION IN ACUTE LYMPHOID LEUKEMIA: CLINICAL CORRELATIONS**

Maurillo L, Burba L, Mascolo MG, Guglielmi C, Annino L, Testi AM, Petrucci MT, Tafuri A  
*Hematology, Department of Human Biopathology, University "La Sapienza", Rome, Italy*

Expression of the multidrug resistance (MDR) gene and its product P-gp 170 is reported to have prognostic importance in cancer. We investigated MDR expression in acute lymphoid leukemia (ALL) patients (pts) with two flow cytometric tests: 1) a functional assay based on the fluorescent dye rhodamine-123 (Rhd) efflux (Rhd-E), performed in the presence or absence of cyclosporin A (CsA) used as MDR-reversing agent; 2) immunofluorescence evaluation of external epitopes of P-gp 170 by means of the 4E3.16 and MM4.17 monoclonal antibodies.

Results were correlated with disease status (diagnosis/relapse), age of pts (pediatric/adult) and clinical response. We studied 28 pts with ALL: 17 at diagnosis and 11 in relapse, 16 pediatric and 12 adult. A significant difference ( $p=0.03$ ) in mean (m) Rhd-E was found in overall pts at diagnosis between cases who achieved complete remission (CR) ( $m=4.5\%$ ) and those who were resistant ( $m=15\%$ ). These results were confirmed using 4E3.16 ( $m=0.4\%$  vs  $m=2.6\%$ ,  $p=0.01$ ) and MM4.17 ( $m=0.7\%$  vs  $m=2.6\%$ ). When we analyzed the pediatric pts at diagnosis, we found they presented a lower Rhd-E, 4E3.16 and MM4.17 values ( $m=5.5\%$ ,  $m=0.4\%$ ,  $m=0.6\%$ , respectively) compared to adults ( $m=10.1\%$ ,  $m=1.4\%$ ,  $m=1.3\%$ ). No differences in MDR expression were found in the overall group between pts, at diagnosis and in relapse, whereas there were in the pediatric pts: Rhd-E ( $m=5.5\%$  vs  $10.3\%$ ), 4E3.16 ( $m=0.4\%$  vs  $2.6\%$ ), MM4.17 ( $m=0.6\%$  vs  $1.6\%$ ). Among the 7 adults pts studied at diagnosis, we observed lower Rhd-E ( $m=0\%$ ), 4E3.16 ( $m=0.3\%$ ) and MM4.17 ( $m=0\%$ ) in the 2 cases that achieved CR than in the 5 that did not ( $14\%$ ,  $2.6\%$  and  $1.3\%$ , respectively). All pediatric pts achieved CR (Rhd-E  $m=5.5\%$ ). In conclusion, we found: 1) a correlation between the functional and immunofluorescence tests; 2) lower MDR value at diagnosis in pediatric pts than in adults; 3) lower MDR values in pediatric pts at diagnosis than at relapse; 4) higher MDR values at diagnosis in adult pts unable to achieve CR.

076

#### **SURFACE MARKER EXPRESSION IN ADULT T ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL): CORRELATION WITH INITIAL CHARACTERISTICS AND RESPONSE TO THERAPY**

Livraghi A, Pagnucco G, Santagostino A, Bellio L, Vanelli L, Castelli G, Canevari A, Lazzarino M, Bernasconi C  
*Cattedra di Ematologia, Università di Pavia; Divisione di Ematologia, Policlinico S. Matteo, IRCCS, Pavia, Italy*

From June 1985 to December 1994 we studied the immunophenotype and clinical characteristics of 45 patients with adult T-ALL. Antigen expression was determined by immunofluorescence and flow cytometry using a FACS (EPICS-C) after appropriate forward light scatter gating of total bone marrow samples.

Leukemic cells were tested for reactivity with monoclonal antibodies to T-cell (CD1a, CD2, CD3, CD4, CD5, CD7, CD8), B-cell (CD19, CD20, CD21) and myeloid (My) antigens (CD33, CD13, CD14, CD15, CD11b), common ALL antigen (CD10), class II antigen (HLA-DR) and CD34. For the immunological study we used the Roper classification of T-ALL into groups I, II and III (Roper M. *et al. Blood* 1983; 61:830). Furthermore, we examined the main biological and clinical features into establish a correlation with the degree of antigenic differentiation of the leukemic blasts (pro-T ALL and late-T ALL).

*Immunological data.* Distribution of the 45 cases according to the Roper classification showed the following results: group I, 12 patients (27%); group II, 22 pts (49%) and group III, 11 pts (24%). The overall incidence of HLA-DR was 16% and was significantly higher in group III (group III vs II:  $p=0.004$ ; group III vs II vs I:  $p=0.09$ ); CD10 was positive in 30% of the cases and its expression was never observed in group I (I vs II:  $p=0.08$ ; I vs III:  $p=0.09$ ); CD34 expression (10% of the cases) was more frequent in group I, but the difference with the other groups was not significant. The incidence of myeloid antigen was 13% without any correlation with the degree of antigenic differentiation.

The other classification into pro-T and late-T confirmed that a low degree of differentiation is more frequent in adult T-ALL (76% vs 24%;  $p=0.0001$ ). The differences in the distribution of the other antigens were similar to those mentioned above.

*Clinical data.* Analysis of clinical features showed these results: a lower incidence of mediastinal mass in group III (9% vs 58% and 41% for group I and II;  $p=0.04$ ); lower mean value of LDH in group III (689 U/mL vs 1893 and 1612 for groups I and II;  $p=0.1$ ); a lower mean value of uric acid in group III vs group II (4.7 mg/dL vs 7.9 mg/dL;  $p=0.03$ ). The other clinical features (median age, splenomegaly, adenomegaly, central nervous system involvement and bulky disease) showed no significative differences either among the Roper groups or between pro-T and late-T.

Analysis of response to therapy and survival showed no differences among the groups as regards CR rate (58%, 68%, 81% for groups I, II and III;  $p=0.4$ ), DFS (25%, 18%, 15% at 3 years;  $p=0.3$ ), EFS (18%, 15%, 15% at 3 years;  $p=0.4$ ), and overall survival (20%, 22%, 30% at 3 years;  $p=0.8$ ). Only the expression of CD10 influenced overall survival; the CD10<sup>+</sup> group showed a median survival of 32 months vs 15 months for the CD10<sup>-</sup> group ( $p=0.1$ ).

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#### **ANLL WITH BASOPHILIC DIFFERENTIATION: SIX CASES STUDIED WITH ULTRASTRUCTURAL CYTOCHEMICAL ANALYSIS**

Tomasi P, Mejak C, Scapoli GL, Spanedda R, Castoldi GL  
*Istituto di Ematologia, Università di Ferrara, Italy*

The differentiation pattern in blast cells is revealed by the expression of lineage-specific cellular antigens recognized by Moab and cytoenzymatic activities; however, identification of early basophil blast cells is difficult and controversial because of the overlapping of immunophenotyping with granulocytic blasts and the absence of a peculiar cytochemical pattern, so that additional studies such as electron microscope analysis, which detects specific basophil lineage granulations, are required.

In this report basophilic blast proliferation was demonstrated in 6/52 cases of *de novo* ANLL on the basis of ultrastructural cytochemical analysis: these cases were previously classified by conventional morphological, cytochemical, immunologic analysis as M1 (one case), M2 (three cases), M4 (two cases).

Ultrastructural examination showed that a variable percentage of the blasts, ranging from 20 to 50%, contained organelles which resembled immature basophil/mast cell granules. The blast cells were extremely heteromorphic with regard to cell size, nuclear configuration, peroxidase activity and organelle content. A wide spectrum of cytoplasmic granules were seen: membrane bound granules 0.1-0.4  $\mu$ m in diameter containing arrangements of granulo-filamentous material, granules showing clear vacuolar structures and sometimes myelin figures, membrane bound granules internally bisected by electron dense membranes (theta granules). Mast cell-like granules containing electron dense material arranged in a pattern of scrolls and lamellae or loosely packed amorphous material were also present at the same time in some blasts. In most blast cells the nuclear envelope, RER, Golgi apparatus and granulations were peroxidase positive; some cells contained both positive and negative granules. Rare precursors manifested peroxidase activity in the nuclear envelope and RER, with negative basophil/mast cell granules.

Four patients showed clinical signs of excess histamine release (bronchospasm, dermatographism, diarrhea and peptic ulceration). The cytogenetic findings were heterogeneous and none of the patients had a 6;9 translocation, an abnormality that has been associated with acute basophilic leukemias.

In conclusion, recognition of basophil lineage involvement in *de novo* ANLL requires ultrastructural examination and this procedure helps us to refine the definition of new distinct subtypes of ANLL.

Grant Murst 60% and regional funds.

078

#### GF RECEPTOR EXPRESSION AND CELL PROLIFERATION KINETIC CHANGES IN AML PATIENTS TREATED WITH GM-CSF PRIMING: CLINICAL CORRELATION

Ricciardi MR, Petrucci MT, Mascolo MG, Tafuri A  
Hematology, Department of Human Biopathology, University "La Sapienza", Rome, Italy

Previous studies suggested that growth factors (GF) can recruit quiescent acute myeloid leukemia (AML) cells into the cell cycle, thereby increasing the cytotoxic effects of cycle-specific drugs. In the present study, we investigated the expression of several GF receptors (GM-CSF R, G-CSF

R, IL-3 R, SCF R), the effects of GM-CSF on blasts proliferation and their correlation with clinical response in AML patients (pts) treated with GM-CSF and chemotherapy. We studied 12 newly diagnosed AML pts belonging to larger randomized clinical trials in which participants were treated with (7 pts) and without (5 pts) GM-CSF (5ug/kg $\times$ 7-28 days) initiated 24 hours before chemotherapy (CT) (Ara-C+DNR). Cell proliferation was evaluated just before GM-CSF was started (t=0) and after 24 hours (day +1) by measuring cell cycle changes (acridine-orange) and/or bromodeoxyuridine incorporation. The expression of GF receptors was examined by multiparameter flow cytometry after staining with biologically active, biotin-labeled, human GF secondarily conjugated with avidin-FITC (Fluorokines, R&D Systems, Minneapolis). Several cell lines (CEM, U937 and Mo7e) were used as negative/positive control. Expression of receptors for GM-CSF, G-CSF, IL-3 and SCF was observed in 58%, 75%, 30% and 89%, respectively, of the cases tested. Among the 12 pts studied, 5 of the 7 treated with GM-CSF and 2 of the 5 treated without GM-CSF expressed GM-CSF R. Evaluation of changes induced by 24 hrs of *in vivo* GM-CSF infusion revealed an increase in S-phase (m=7.8% to 9.8% from t=0 to day+1). Moreover, pts achieving CR showed lower S-phase (m=5.4%) at diagnosis in this group than those who were resistant (m=10.9%) and a significant increase in S-phase from t=0 to day +1 (m=5.4% to 9.2%; p=0.03). Analysis of clinical response in the pts treated with GM-CSF and positive for GM-CSF R showed that 2 pts who received GF during CT achieved CR, whereas 3 pts who were also given GF after CT until PMN recovery, were resistant. The preliminary conclusions of this study indicate the importance of evaluating GF R and cell proliferation in AML priming and suggest that AML cases with GM-CSF receptors may not benefit from priming strategies if GF are administered after discontinuation of CT.

079

#### DETECTION OF P-GLYCOPROTEIN ACTIVITY IN CELL LINES USING IDARUBICIN AND DAUNORUBICIN.

Bellio L, Pagnucco G, Buonanno MC, Vanelli L, Zanaboni M, Consensi E, Balduini A, Corso A, Brusamolino E, Lazzarino M, Bernasconi C

Istituto di Ematologia, Università di Pavia, Divisione di Ematologia, IRCCS Policlinico San Matteo, Pavia, Italy

Exploiting the fluorescent properties of idarubicin (IDR) and daunorubicin (DNR), which are transported by the transmembrane efflux pump P-glycoprotein (P-gp), we investigated by flow cytometry with a FACS (EPICS-C) the P-gp activity in a sensitive cell line (CCRS-CEM) and in a cell line with MDR phenotype (CEM 300 VLB). The cell lines were a kind gift from Dr. D. Damiani (Hematology Institute, Udine University). One $\times$ 10<sup>6</sup> cells/mL were incubated with either IDR or DNR at a concentration of 250 ng/mL for 15 min, washed twice in PBS and resuspended in free medium. Then IDR or DNR retention was determined at different time intervals (15, 30, 45, 60, 90, 120 min). The threshold for IDR and DNR uptake was set for each sample according to the upper

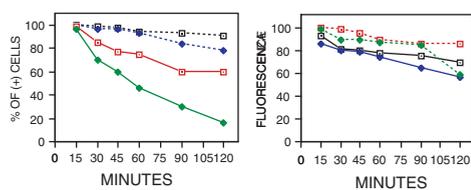


Fig. 1. IDR and DNR retention in CEM and CEM-VBL cells. (---□--- CEM+IDR, ---●--- CEM+DNR, ---□--- CEM-VBL+IDR, ---●--- CEM-VBL+DNR)

limit of cell autofluorescence; IDR or DNR uptake was the percentage of cells above this threshold. Fluorescence was obtained by dividing the mean channel at several time intervals by the mean channel measured after removing the anthracyclines (time=0). Retention of IDR and DNR in the two cell lines is reported in Figure 1. We confirm that IDR is retained to a greater degree than DNR.

#### 080

### RISK OF CENTRAL NERVOUS SYSTEM (CNS) INVOLVEMENT IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA (ALL). ANALYSIS OF 168 CASES

Livraghi A, Pagnucco G, Santagostino A, Bellio L, Vanelli L, Brusamolino E, Canevari A, Lazzarino M, Bernasconi C *Cattedra di Ematologia, Università di Pavia. Divisione di Ematologia, Policlinico S. Matteo, IRCCS, Pavia, Italy*

The aim of this study was to evaluate the main factors that can increase the risk of CNS involvement in adult ALL at onset or during clinical course. From June 1985 to December 1994 we studied the immunophenotype and clinical characteristics of 168 patients with adult T-ALL. Antigen expression was determined by immunofluorescence and flow cytometry using a FACS (EPICS-C) after appropriate forward light scatter gating of total bone marrow samples. A cell population was considered positive for a given surface marker if more than 20% of the gated cell stained positively. CNS leukemia was defined as the presence of unequivocal blasts in a cytocentrifuged preparation of uncontaminated cerebrospinal fluid. CNS disease was present at onset in 12 patients (7%) and developed during the course of ALL in 27 others (16%), while 129 patients (77%) did not experience meningeal leukemia. We compared clinical data at onset with the immunological phenotype of these groups: A) patients with CNS+ at onset; B) patients CNS+ during the course of the disease; C) patients without CNS involvement. As regards *CNS involvement at onset*, analysis of the immunophenotype and the presenting clinical features showed these results: 1) a higher incidence in T-ALL ( $p=0.0001$ ); 2) a lower incidence in patients HLA-DR<sup>+</sup> ( $p=0.0001$ ), CD10<sup>+</sup> ( $p=0.01$ ) and CD34<sup>+</sup> ( $p=0.05$ ); 3) a higher frequency of mediastinal mass ( $p=0.01$ ) and bulky disease ( $p=0.03$ ); 4) higher mean values of uric acid ( $p=0.004$ ), alkaline phosphatase ( $p=0.0002$ ) and LDH ( $p=0.1$ ). In order to evaluate the risk of *CNS involvement during the course of the disease*, we compared the immunological and clinical data for groups B and C. The results demonstrated that there was a significantly higher risk of meningeal leukemia in the following

cases: T-ALL ( $p=0.05$ ), HLA-DR<sup>+</sup> ( $p=0.04$ ), the presence of bulky disease ( $p=0.05$ ); furthermore, other factors were closely associated, but not a significantly correlated with CNS disease: B-ALL-SmIg<sup>+</sup> phenotype ( $p=0.09$ ), CD34<sup>+</sup> phenotype ( $p=0.09$ ), and the presence of mediastinal mass ( $p=0.1$ ). Moreover, analysis of the actuarial risk of meningeal leukemia showed a cut-off value for WBC ( $12 \times 10^9/L$ ;  $p=0.03$ ) and LDH (450 U/mL;  $p=0.05$ ).

Our study indicates that there are certain subgroups of ALL at with high risk of developing CNS leukemia at onset or during the course of the disease.

#### 081

### MOLECULAR ANALYSIS OF THE BCL6 AND BCL2 GENE CONFIGURATION IN DIFFUSE NON-HODGKIN'S LYMPHOMA

Lo Coco F, Pistilli A, Pescarmona E, Pacchiarotti A, Cimino G *Hematology, Dept. of Human Biopathology; Istopathology, Dept of Experimental Medicine, University La Sapienza of Rome, Italy*

Unlike other NHL subsets (i.e. follicular NHL, Burkitt's NHL), diffuse large cell lymphoma still represents a subtype whose molecular pathogenesis is poorly understood. Recently, an important genetic lesion associated with diffuse NHL was characterized at the molecular level. This allowed description of a new gene, BCL6, normally located on chromosome band 3q27, that codes for a putative transcription factor. Following cytogenetic translocations involving band 3q27 and various partner chromosomes, the BCL6 gene is rearranged and presumably deregulated since it is placed under the control of heterologous promoters. Preliminary clinical studies have indicated an interesting association between BCL6 alterations and the diffuse large cell NHL histotype. However, it is also known that a variable fraction of diffuse NHL may show a rearranged BCL2 gene, probably reflecting their origin from a previous follicular form (so-called *transformed* NHL). In this study, we analyzed by Southern blot the BCL2 and BCL6 gene configuration in 80 NHL and in 17 HD cases. Our aim was to investigate the incidence of rearrangements and to correlate the configuration of these two genes in the different histologic subsets. To this purpose, we used probes PFL1 and PFL2 to explore the major and minor BCL2 bcr sequences and probe Sac4.0, which specifically detects breakpoints in the BCL6 5' region. In all NHL patients, lymph node DNAs were previously probed with an Ig heavy chain gene in order to confirm B-cell origin and the presence of a significant tumor cell infiltration in the examined sample. BCL6 rearrangements were detected in 24/80 (30%) diffuse NHLs (19/55 centroblastic, 2/14 centrocytic-centroblastic, 3/11 immunoblastic) and in no HD cases. Two centroblastic NHL were rearranged at the BCL2 locus only. These data indicate that the BCL6 rearrangement represents the most frequent genetic alteration presently detectable in diffuse NHL, and that this abnormality is preferentially observed in *de novo* diffuse NHL carrying normal BCL2 genes.

082

**MULTIPARAMETRIC LYMPH NODE STUDY OF 62 CASES OF NON-HODGKIN'S B-CELL LYMPHOMAS (NHLs)**

Baldini L, Cro L, Ferrari A,\* Bianchi F,\* Fracchiolla N, Colombi M, Nobili L, Boletini A, Zucal N, Neri A, Maiolo AT  
*Serv. Ematologia, Osp. Maggiore IRCCS, Ist. Scienze Mediche, Università degli Studi, Milano, \*Serv. Anatomia Patologica, Osp. Fatebenefratelli e Oftalmico, Milan, Italy*

The recent REAL classification proposal (*Revised European-American Classification of Lymphoid Neoplasms*) of the *International Lymphoma Study Group* emphasizes the need for a multiparametric approach (morphologic, immunophenotypic and molecular biology) for a better diagnostic definition of NHLs. We analyzed 62 lymph nodes from B-cell NHLs at diagnosis by means of: 1) conventional histology and immunohistochemistry (CD20, CD21, CD45RA, CD45RO); 2) flow cytometry on cell suspensions for the evaluation of immunophenotype (CD1a, CD1c, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11a-c, CD19, CD23, CD25, CD49c-d, FMC7, DR, SIg, CyIg) and the expression of bcl-2 and p53 proteins, and 3) molecular biology (bcl-1, bcl-2 and bcl-6 gene rearrangement). Histologically, our panel was represented by 11 cases of *small lymphocytic lymphoma/CLL* (SLL); 9 cases of *lymphoplasmacytoid-immunocytoma* (LP-IC); 17 cases of *follicular center lymphoma* (FCL); 6 cases of *follicular center lymphoma, diffuse* (FCL-d); 7 cases of *mantle cell lymphoma* (MCL); 1 case of *splenic marginal zone lymphoma* (SMZL); 8 cases of *large cell lymphoma* (LCL); 2 cases of *lymphoblastic lymphoma* (LL); 1 case of *Burkitt's lymphoma* (BL). Flow cytometry (FCM) allowed fast and accurate characterization of the cases examined in terms of cell lineage definition, clonality and presumed cell origin (cell subsets of the mantle or germinal center, lymphoblasts). We observed an overall good correspondence between histologic-immunohistochemical and flow cytometric diagnoses; in three cases FCM and molecular biology analyses differ from the histologic diagnosis: one case (histologically FCL) in which flow cytometry and molecular biology failed to detect clonality; one (histologically MCL) in which flow cytometry failed to detect the CD5 expression characteristic of MCL, while immunohistochemistry revealed bcl-2 protein expression and molecular biology, the bcl-2 gene rearrangement characteristic of FCL; a third identical to the second one. Our flow cytometry analysis proved the central role of CD10, CD19/CD5, CD23 and SIg (class and intensity) in the classification of B-NHLs. The expression of CD1c and FMC7, not frequent in SLL, is variable in the other subsets of NHL. CD25 is rarely expressed in NHLs originating from the follicular-center or in LCLs, but is more frequently expressed in tumors from the mantle zone. As far as expression of the  $\beta 2$  integrins is concerned, CD11a is constantly expressed except in cases of simil-Burkitt tumor, CD11b is frequently positive in SLL/LP-IC and LCL, while CD11c is most frequently expressed in SLL. Regarding  $\beta 1$  expression,  $\alpha 3$ -VLA is almost exclusively expressed in SLL, confirming in the lymph node the expression pattern found in leukemic forms of NHLs (Baldini et al, *Blood* 1992; 10:26-88);  $\alpha 4$ -VLA is expressed almost constantly in all histotypes, with the exception of

LCLs. Molecular analysis detected bcl-2 gene rearrangement (MBR and mcr) in 10/17 FCL cases and in 2/8 LCL cases; bcl-1 locus rearrangement (MTC) in 2/7 MCL and bcl-6 rearrangement in 1/17 FCL and in 2/8 LCL cases. In conclusion, our study confirms the importance of a multiparametric approach in the diagnosis of NHLs and the central role of complete FCM immunophenotypic characterization.

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083

**MYELODYSPLASIA AND LEUKEMIA IN HODGKIN'S DISEASE ARE RELATED TO AGE, SPLENECTOMY, AND COMBINED RADIO-CHEMOTHERAPY WITH MOPP AND NITROSOUREAS**

Brusamolino E, Orlandi E, Astori C, Passamonti F, Nozza A, Pagnucco G, Lazzarino M, Bernasconi C

*Istituto di Ematologia, Università di Pavia, Divisione di Ematologia, Policlinico S. Matteo IRCCS, Pavia, Italy*

*Objectives.* Survivors of Hodgkin's disease are at risk for the development of acute leukemia. This study analyzes the leukemia and myelodysplasia (MDS) risk in different therapy groups and provides data on duration of risk, the role of pretreatment variables and splenectomy, and the influence of length of therapy.

*Methods.* This survey includes 607 consecutive patients followed for a median time of 12 years. Actuarial risk in the different groups was calculated according to Kaplan-Meier; analysis of predictive factors was carried out with multiple regression and case-control study.

*Results.* The overall 10-yr actuarial risk of leukemia and MDS was 5.4% and 7.1%, respectively. No cases of leukemia or MDS occurred among patients given radiotherapy (RT) alone. The ANLL/MDS risk among patients given CT only was 2%; 1.9% for MOPP alone and zero for ABVD or MOPP/ABVD as initial treatment. In the group of adjuvant MOPP after RT, the actuarial risk was 7.5%; in splenectomized patients given MOPP as adjuvant to RT, the risk was 10.5%. In patients receiving combined RT+MOPP as salvage, the overall risk was 11.1%; patients treated with lomustine and melphalan as salvage after MOPP and ABVD showed a risk of 18%. Multiple regression and case-control analyses indicated age over 40 and splenectomy as significant pretreatment risk factors. Combined modality therapy including MOPP and lomustine and CT duration > 6 months were the most significant covariates for both ANLL and MDS risk. The hazard function curve showed peaks at the 4<sup>th</sup> and 9<sup>th</sup> year; no cases of ANLL/MDS occurred beyond the 10<sup>th</sup> year.

*Conclusions.* RT+adjuvant MOPP and RT+salvage nitrosoureas after MOPP and ABVD carry a higher risk of leukemia and MDS than RT or CT alone. This risk is related to the duration of therapy and ceases after the 10<sup>th</sup> year of follow-up.

084

**NODULAR LYMPHOCYTE PREDOMINANCE HODGKIN'S DISEASE (NLP-HD): CLINICAL BEHAVIOR AND PATTERN OF PROGRESSION IN 67 PATIENTS**

Orlandi E, Lazzarino M, Brusamolino E, Paulli M,\* Astori C, Magrini U,\* Bernasconi C  
*Institute of Hematology, University of Pavia; Division of Hematology, IRCCS Policlinico San Matteo, Pavia; \*Dept. of Pathology and Research Unit, University of Pavia, IRCCS Policlinico San Matteo, Pavia, Italy*

NLP-HD displays distinctive pathologic and immunophenotypic features that suggest a relationship with low-grade B-cell non-Hodgkin's lymphoma (NHL). In order to evaluate presenting characteristics, response to treatment and pattern of evolution, the records of 67 consecutive pts observed between 1975 and 1994 were reviewed (8% of all cases of HD). Eight pts showed benign hyperplasia on lymph node biopsies performed 7 to 36 mos before the diagnosis of NLP-HD. M/F ratio was 2:1 and median age 35 yrs (range 14-86); B symptoms were present in 15% of pts, and 34 pts (51%) were in stage I, 15 (22%) in stage II, 8 (12%) in stage III, 10 (15%) in stage IV. The sites primarily involved were peripheral lymph nodes (72%). One pt revealed synchronous NLP-HD and NHL (WF:H) at diagnosis. At a median follow-up of 70 mos (range:12-240), 61 pts were evaluable for response to treatment (RXT in 25, CHT in 21, combined therapy in 15) and 57 of them (93%) had achieved complete remission (CR). There were 18 relapses (31%), 4 to 172 mos after CR (median 37 mos), with HD mixed cellularity histology in 2 pts and NLP-HD in 16. Second CR was obtained in 11 pts (61%), 2 of whom experienced a second relapse. During follow-up 5 pts (8%) developed a NHL (WF: A:1; G:3; H:1) 36-185 mos from disease onset (3 while in CR and 2 in PR of HD). Only 1 case of NHL was observed in the whole series of HD pts with other histologic types. Three pts had a total of 4 episodes of lymph node enlargement during remission, all with a histologic diagnosis of benign follicular hyperplasia. Thirteen pts died: 7 of HD, 1 of NHL, 1 of therapy-related toxicity, 1 of lung cancer and 3 of non-related causes in CR. Overall survival and disease-free survival at 5, 10, and 15 yrs were: 78% and 71%, 70% and 55%, 63% and 41%, respectively. At prognostic analysis, disease-free survival was influenced by disease stage (I-II vs III-IV,  $p=0.004$ ).

**Conclusions.** NLP-HD, as compared to other histologic subtypes of HD, shows peculiar clinical behavior characterized by: 1) frequent occurrence of lymph node enlargement with histologic features of benign follicular hyperplasia, both before diagnosis and during remission; 2) a constant pattern of relapse over time; 3) a histologic evolution to NHL at progression or relapse.

085

#### A NOVEL SCHEME WITH INTENSIFIED CHEMOTHERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) AS FIRST-LINE THERAPY IN HIGH-RISK DIFFUSE LARGE CELL LYMPHOMA (DLCL)

Vitolo U,\* Cortellazzo S,<sup>o</sup> Liberati AM,<sup>#</sup> Freilone R,\* Falda M,\* Bertini M,\* Botto B,\* Cinieri S,<sup>#</sup> Locatelli F,\* Lovisone E,\* Marmont F,\* Pizzuti M,<sup>o</sup> Rossi A,<sup>o</sup> Viero P,<sup>o</sup> Grignani F,<sup>#</sup> Barbui T,<sup>o</sup> Resegotti L\*

*Divisioni di Ematologia degli ospedali: \*Molinette, Torino; <sup>o</sup>Riuniti Bergamo; <sup>o</sup>S. Carlo, Potenza; <sup>#</sup>Cattedra di Clinica Medica, Perugia; Italy*

From January 1992 through December 1994, 57 pts with high-risk DLCL were enrolled in a pilot cooperative study. High-risk pts were defined as those presenting advanced stage with high tumor burden and elevated LDH level or bone marrow (BM) involvement at diagnosis, or those who achieved only PR after conventional chemotherapy. The therapeutic scheme included three phases: 1) induction with MACOPB for 8 weeks; 2) intensification with a 3-day course of mitoxantrone 8 mg/sqm + HD-ARAC 2 g/sqm/q12h + dexamethasone 4 mg/sqm/q12h (MAD protocol) with G-CSF (kindly provided by AMGEN) 5 µg/kg on days 4-17 to harvest peripheral blood progenitor cells (PBPC); 3) consolidation with BEAM + ASCT with PBPC or marrow or both. Median age was 45 yrs (19-59). Fifty pts were previously untreated high-risk DLCL and 7 were PRs after conventional chemotherapy; 48 showed high tumor burden, 46 elevated LDH level, 39 stage IV, 28 presented with two or more extranodal sites and 19 with BM involvement. MAD was well tolerated; median number of days with  $N < 0.5 \times 10^9/L$  was 6. Severe infections (WHO grade  $> 2$ ) occurred in 5 pts, one of whom died of mucormycosis. One patient died of brain hemorrhage due to severe thrombocytopenia. Leukapheresis yielded a median of  $29 \times 10^6/kg$  CD34<sup>+</sup> cells and  $76 \times 10^4/kg$  CFU-GM. Forty-six pts were autografted: 29 with PBPC alone, 7 with marrow only and 10 with both; 11 pts were not transplanted: 8 because of progressive disease and 3 due to toxicity. The median times to achieve engraftment were 11 days (5-19) for neutrophils  $> 0.5 \times 10^9/L$  and 12 days (8-90) for platelets  $> 50 \times 10^9/L$ . One HBsAg<sup>+</sup> patient died after BEAM because of acute hepatitis reactivation. At the end of the complete therapy 43 patients were in CR, 11 showed NR and 3 had died from toxicity-related complications. Among the 35 PRs or NRs after the induction phase with MACOPB, 68% achieved a CR with the subsequent intensified therapy. Factors significantly affecting CR were: B symptoms (68% vs 90%), PS  $> 1$  (68% vs 92%) and BM involvement (61% vs 89%). DFS was 77% at a median follow-up of 16 months. These results compare favorably with those achieved in a historical, age-matched control group having the same clinical characteristics that was treated with MACOPB alone (CR 76% vs 43%, DFS 77% vs 41%). This sequential scheme is feasible with moderate toxicity and may improve the outcome in high-risk DLCL.

086

#### MOLECULAR ANALYSIS OF CUTANEOUS B AND T CELL LYMPHOMAS

Neri A,\* Fracchiolla NS,\* Roscetti E,<sup>o</sup> Garatti S,<sup>o</sup> Trecca D,\* Boletini A,\* Perletti L,\* Baldini L,\* Berti E,<sup>o</sup> Maiolo AT\*

*\*Serv. Ematologia, Ist. Scienze Mediche; <sup>o</sup>Ist. Scienze Dermatologiche, Università di Milano, Osp. Maggiore IRCCS, Milan, Italy*

Among extranodal non-Hodgkin's lymphomas (NHL), primary cutaneous lymphomas (CL) represent an important group of B and T cell malignancies. We investigated the arrangement of immunoglobulin (Ig) and T-cell receptor (TCR) genes, together with the involvement of several oncogenes and tumor suppressor gene p53, in a

panel of primary cutaneous B and T-cell lymphomas (CBCL and CTCL). Southern blot analysis was performed to detect rearrangements of the Ig, *c-myc*, *bcl-1*, *bcl-2*, *bcl-3*, *bcl-6* and *NFKB2/lyt-10* genes in 52 cases of CBCL, and rearrangements of the TCR, *bcl-3* and *NFKB2/lyt-10* genes in 38 cases of CTCL. *tal-1* gene deletions were analyzed in CTCL by means of polymerase chain reaction (PCR). p53 gene mutations were assayed using PCR, single strand conformation polymorphism analysis (SSCP) and direct DNA sequencing. Clonal rearrangements of Ig genes or oncogenes were found in 25 of the 52 CBCLs. In particular, we detected rearrangements of the *bcl-1* locus (two cases), the *bcl-2* gene (two cases), the *NFKB2/lyt-10* gene (two cases) and the *bcl-6* gene (one case); interestingly, four of these cases showed a germline arrangement of the Ig genes. Clonal rearrangements of TCR genes were detected in 37 of the 38 CTCL. Rearrangements of the *NFKB2/lyt-10* gene were present in two, and *tal-1* gene deletions in three CTCL cases; p53 gene mutations were detected in one CTCL case. Overall, our data indicate that: 1) clonal rearrangement of Ig genes is frequently undetectable by means of Southern blot in CBCL ( $\approx 60\%$ ); 2) genetic lesions are involved in a limited but significant fraction of primary CL showing a molecular marker of clonality (13/62;  $\approx 20\%$ ); 3) rearrangements of the *bcl-1*, *bcl-2* or *bcl-6* loci associated with specific subsets of nodal lymphoid neoplasias, are rarely observed in CBCL.

Moreover, our results suggest that *tal-1* gene deletions may play a pathogenetic role in non-acute T-cell malignancies and that, in the context of lymphoid malignancies, CL may represent a favorable target for the possible oncogenic potential of the *NFKB2/lyt-10* gene.

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087

#### AGGRESSIVE TRANSFORMATION IN SPLENIC MARGINAL ZONE LYMPHOMA

Baldini L, Fracchiolla NS, Guffanti A,\* Cro L, Colombi M, Sita G, Neri A, Maiolo AT  
*Serv. Ematologia, Osp. Maggiore IRCCS, Ist. Scienze Mediche, Univ. Studi Milano, \*Div. di Medicina, Osp. Fatebenefratelli ed Oftalmico, Milan, Italy*

The emergence of clinically and histologically aggressive intermediate/high-grade NHL has been described in many cases of low-grade non-Hodgkin lymphoma (NHL). This behavior may be due either to the selection of a different clone or to the clonal progression of the previous malignancy, which may be caused by additional genetic alterations. While this behavior is well known for B-cell chronic lymphocytic leukemia (Richter's syndrome) and follicular lymphoma, little is known regarding splenic marginal zone lymphoma (SMZL). SMZL was recently recognized as a provisional entity in the *Revised European-American Classification of Lymphoid Neoplasms* by the *International Lymphoma Study Group*. The course is reported to be indolent, with splenomegaly, bone mar-

row infiltration and variable leukemic involvement by villous or non villous lymphocytes. We recently observed and reported 15 cases of non villous SMZL in leukemic phase (Baldini *et al*, *Blood* 1994; 84:270), in which the frequent p53 mutations (6/15 cases) suggested a possible pathogenetic role for this tumor suppressor gene. While our first impression about clinical course was that of low-grade forms regardless of p53 loss/mutation, after a longer follow-up (median 56 mo.) we observed a very different situation. Three patients evolved to aggressive, non responsive NHL. A 61-year-old female (with t(8;14) and *c-myc* rearrangement progressed to a Burkitt tumor, with typical clinical features (abdominal lymphadenopathy, meningeal involvement and hypercalcemia). The other two patients (male, 66 yr; female, 54 yr) evolved to large cell NHL, with one of them showing at progression an impressive osteolytic bone involvement caused by large CD30-positive malignant B-cells. It is interesting that in two of these cases molecular analysis demonstrated tumor progression of the original clone. In the third case molecular evaluation could not be performed. These data tend to support the hypothesis that a Richter-like syndrome could depend on tumor progression of a single clone rather than the emergence of a different neoplastic clone. Furthermore, the finding of p53 alterations in 2 of the 3 cases that progressed may suggest that in SMZL this molecular event could have a role not only in the initial neoplastic transformation but also in possible subsequent malignant progression.

In conclusion, SMZL, like other low-grade NHL, can evolve to more aggressive tumors, often via a mechanism of clonal progression. Moreover the relatively frequent presence of p53 alterations should advise a more cautious approach to a tumor until now considered as an indolent NHL.

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#### FLUDARABINE IS HIGHLY EFFECTIVE IN NON PRETREATED OR RELAPSED B-CHRONIC LYMPHATIC LEUKEMIA AND LOW GRADE NON HODGKIN'S LYMPHOMAS

Spriano M, Clavio M, Bruni R, Pierri I, Rossi E, Carrara P, Balleari E, Gatti AM, Vimercati R, Canepa L, Migliano M, Celesti L, Patrone F, Ghio R, Damasio E, Gobbi M  
*Division of Hematology 1, S. Martino Hospital, Chair of Hematology and Patologia Medica B, DIMI, University of Genoa, Italy*

In the last 5 years reports have been published on the brilliant results of fludarabine treatment of B-chronic lymphatic leukemia (B-CLL) and low-grade non Hodgkin's lymphomas (LG-NHL), both in pretreated patients and particularly in non pretreated ones. Our group recently confirmed the efficacy of this drug in a study performed in pretreated and/or alkylating agent refractory patients.

From 1992 we decided to test the efficacy and the toxicity of FLU as first line treatment of B-CLL and LG-NHL; patients with late relapses were also enrolled in the study. Twenty-eight cases, 17 B-CLL and 11 LG-NHL, were included in the study. Among CLL patients 11 were males, 6 females, median age was 58 years (range 43-73); all had

active disease. Rai stage was I in 2 patients, II in 8, III in 1, IV in 6. Thirteen received FLU as first therapy (76%) and 4 were treated in first or second relapse (24%). The LG-NHL group was composed of 8 males and 3 females, with a median age of 54 years (range 38-72); the histotypes were lymphocytic in 3 patients, follicular centroblastic-centrocytic in 1, immunocytoma in 6. All 11 had fourth stage disease with marrow infiltration; 8 received FLU as first line treatment, 3 were treated in relapse. All 28 participants were administered FLU as single drug at a dosage of 25 mg/sqm/day i.v. for 5 days, every 28 days for 6 cycles. Only those who received at least 3 courses of therapy were evaluated. Four patients experienced hematological toxicity (WHO 3 in only 1 case), while no cases of extrahematological toxicities were recorded. Myelotoxicity, unrelated to the number or type of chemotherapy lines, was observed only in the LG-NHL group. All 17 CLL patients were evaluable: 3 achieved complete remission (CR) (18%), 5 nodular partial response (nPR) (30%), 9 partial response (PR) (52%). No patient showed refractory disease. All the CRs were obtained in non pretreated patients. Of the 9 evaluable LG-NHL patients, 1 reached CR (11%), 7 PR (78%), 1 did not respond (11%). The follow-up was too short for DFS evaluation; nevertheless, 5 relapses occurred in the CLL group (29%) and 2 (25%) in the LG-NHL group. Five out of the 28 enrolled patients died (3 CLL, 2 LG-NHL).

This study confirms that early administration of FLU has a very low toxicity and can produce a response in almost all B-CLL patients; LG-NHL is also responsive but slightly less so than CLL. In conclusion, we recommend FLU as first-line treatment of CLL and LG-NHL, above all for young patients who might then receive ABMT with peripheral stem cell rescue. A multicenter randomized trial, now in progress, will compare FLU and conventional therapy regarding the percentage of therapeutic responses and the impact on survival.

089

#### **IFOSFAMIDE, EPIRUBICIN, ETOPOSIDE (IEV) AND AUTOLOGOUS BONE MARROW TRANSPLANTATION (ABMT) IN RELAPSED/REFRACTORY HODGKIN (HL) AND NON HODGKIN'S LYMPHOMA (NHL)**

De Sanctis V, Tosti S, Capria S, Giovannini M, Proia A, Anselmo AP, Martelli M, Mandelli F

*Dip. di Biopatologia Umana, Ematologia, Università "La Sapienza", Rome, Italy*

*Background.* Unfortunately, no significant improvement has been made in the salvage treatment of refractory/relapsed NHL/HL.

*Purpose.* To evaluate the efficacy of salvage chemotherapy (IEV) followed by high dose chemotherapy (BEAC/BEAM) and ABMT in this category of patients (pts).

*Patients and Methods.* Between August 1991 and August 1994, 62 pts were treated in our Institute. Thirty-nine NHL (14 refractory, 25 relapsed) and 23 HL pts (13 refractory, 10 relapsed) received ifosfamide (2500 mg/sqm/day on days 1-3), epirubicin (100 mg/sqm/day on day 1) and etoposide (150 mg/sqm/day on days 1-3). The courses were repeated every 3-4 weeks for a total of 2-

3 cycles. Twenty-seven of 39 NHL cases were responsive to IEV; 21/27 of these were submitted to high-dose chemotherapy and ABMT; 5 pts were not eligible because of poor performance status and 1 pt refused. Sixteen of 23 HL pts achieved a response after IEV treatment; 4/16 received ABMT, while no further treatment was given to the remaining twelve responders.

*Results.* Twenty-seven of 39 (69%) NHL and 16/23 (69%) HL pts responded to IEV treatment. Among the NHL group, 21/27 responding pts were submitted to ABMT; 20 pts were evaluated as responders because one died from heart failure as a result of ABMT. Thirteen pts relapsed at a mean time of 6.3 months (range 1-16) and 6 pts are still in continuous complete remission (CCR) at a mean time of 14 months (range 3-24). One pt died in CR from pulmonary embolism. All 6 pts who did not receive ABMT consolidation relapsed within a mean time of 4.8 months (range 3-12) and 3 died of lymphoma. In the HL group, 4/16 responding pts received ABMT and are in CCR at a mean time of 15 months (range 3-39). The remaining 12 pts did not receive any intensive treatment; 8 relapsed after a mean time of 6.7 months (range 3-18) and 4 are in CCR at a mean time of 21 months (range 11-26).

*Conclusions.* These results confirm the efficacy of the IEV regimen in inducing a good response rate in refractory-relapsed NHL/HL. As reported by other group, high-dose chemotherapy plus ABMT as consolidation therapy may be warranted, especially in chemosensitive NHL/HL.

*Supported in part by AIRC.*

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#### **PRIMARY GASTRIC LYMPHOMAS: A COMPARISON BETWEEN MALT AND NON-MALT LYMPHOMAS**

Gherlinzoni F, Miggiano MC, Bendandi M, Zinzani PL, Salini L, Salvucci M, Tura S

*Institute of Hematology "L. & A. Seràgnoli", S. Orsola Hospital, Bologna University, Italy*

The best treatment for patients (pts) with primary gastric lymphoma remains uncertain, especially because of the extreme heterogeneity among different series which makes comparisons very difficult. In particular, it has not yet been ascertained whether there is a clinical and prognostic difference between MALT (*mucosa-associated lymphoid tissue*) and non-MALT lymphomas, or whether it is clinically useful to distinguish between low-grade and high-grade patterns within MALT lymphomas. We analyzed 91 consecutive pts with primary gastric lymphoma diagnosed between 1986 and 1994. They all underwent gastrectomy (total or subtotal) followed by radiotherapy and/or chemotherapy according to the presence or the absence of the following poor prognostic factors: positive surgical margins, positive regional lymph nodes, disease extended to the serosa. Forty-seven pts presented MALT lymphomas (31 low grade, 16 with a mixture of high- and low-grade histology), and 44 non-MALT lymphomas (25 diffuse large B-cell lymphomas, 17 follicle center lymphomas, 2 other histology).

We found no differences between the two groups with regard to sex, age, stage (IE, IIE1, IIE2), symptoms, LDH

value, macroscopic tumor features, involvement of surgical margins or regional lymph nodes; the only statistically significant difference concerned the involvement of the serosa, which occurred more frequently in non-MALT lymphomas (59% vs 31%,  $p < 0.05$ ). Median follow-up was 50 months for MALT and 61 months for non-MALT lymphomas. No MALT lymphoma patient relapsed, compared to 8 pts (18%) in the non-MALT group ( $p = 0.002$ ). Overall survival is projected at 98% for MALT lymphomas (1 pt died of lymphoma-unrelated causes), and at 83% for non-MALT lymphomas; relapse-free survival is projected at 100% and 80%, respectively ( $p = 0.01$ ).

These data suggest that MALT lymphomas have peculiar clinical and biological characteristics, that probably account for a different clinical course with respect to non-MALT lymphomas. Among MALT lymphomas, the distinction between low-grade histology and mixed (low and high grade mixed together) does not seem to play a significant prognostic role.

091

#### LOCALIZED INTERMEDIATE-HIGH GRADE NON HODGKIN'S LYMPHOMA TREATED WITH BRIEF CHEMOTHERAPY (ACOP-B) PLUS LOCOREGIONAL RADIOTHERAPY (LRRT)

Freilone R, Botto B, Vitolo U, Bertini M, Depaoli L, Dongiovanni A, Griso L, Infelise V, Musella R, Orsucci L, Ricardi U, Rota Scalabrini D, Salomone A, Secondo V, Volta C, Resegotti L

For Italian Multiregional NHL Study Group (IMRNHLG); Division of Hematology, Molinette Hospital, Torino, Italy

**Background.** Approximately 25-30% of NHL cases show localized disease (I and II) without B symptoms at diagnosis. RT alone produces CR in the majority of patients, but 50% of them usually relapse. Chemotherapy (CT) plus RT was proved to be superior to RT by some randomized studies at the beginning of the 80's.

**Patients and Methods.** From January 1988 through December 1994 the IMRNHLG treated 95 patients with IA and IIA intermediate-high grade NHL with nodal or extranodal involvement. Exclusion criteria included HIV infection and gastrointestinal involvement at diagnosis. All patients were treated with the ACOP-B scheme (doxorubicin 50 mg/sqm and cyclophosphamide 350 mg/sqm during weeks 1, 3, 5; vincristine 1.4 mg/sqm and bleomycin 10 mg/sqm during weeks 2, 4, 6; prednisone 50 mg po daily for the first two weeks and thereafter every other day, followed by LRRT (36 Gy) one month after the completion of CT.

**Results.** Median patient age was 58 y (25-79), with 34% > 65 y; 49 were males and 46 females. Histology according to the WF was D 4, E 7, F 18, G 42, H 24; 63 pts were in stage I and 32 in stage II. Thirty-eight pts presented extranodal involvement alone, the most common sites being Waldeyer's ring (14), bone (11) and testis (4). Five pts showed LDH levels above normal and 3 pts had bulky disease. Treatment was well tolerated; there were no toxic deaths and 28% WHO grade 1 and 2 mucositis. Ninety pts achieved a CR after CT and 94 at the end of the complete therapy. At a median follow-up of 3 years DFS was 80% with 16 relapses, 87% of which were disseminated. Ten pts

achieved a second CR with second line CT and/or RT. Two pts with testis involvement at diagnosis relapsed in the CNS. Only histologic subtype H significantly affected the DFS rate (58% vs 87% for the other subtypes,  $p < 0.01$ ). OS was 86% at 3 years.

**Conclusions.** This brief scheme (ACOP-B plus LRRT) produced a high CR rate (99%) with few relapses. Patients with NHL of the testis as primary site require CNS prophylaxis because of the high risk of CNS relapse (2/4). This scheme is also feasible, with a very low toxicity, in elderly people and on an outpatient basis.

092

#### LONG-TERM RECOMBINANT INTERFERON $\alpha 2b$ THERAPY FOR CRYOGLOBULINEMIAS. FINAL DATA FROM A MULTICENTRIC STUDY

Bibas M, Andriani A, Di Giacomo C, Montuoro A, Deriu L, Guarino S, Montanaro M, Di Vona S, Picardi R  
Latium Cryoglobulinemia Cooperative Study Group, c/o Department of Medicine, Section of Hematology, Ospedale San Giacomo, Rome, Italy

To evaluate the role of recombinant interferon  $\alpha 2b$  in the management of cryoglobulinemia, we started a multicenter controlled therapeutic trial in 1990. Forty-nine pts. (13 men and 36 women; median age 58 years, range 33 to 73) were classified according to the system of Brouet: 5 pts. had type I cryoglobulinemia, 26 type II and 18 type III. Nine were essential cryoglobulinemia and the other 40 were secondary cryoglobulinemia associated with chronic liver disease (35), Waldenström's macroglobulinemia (2), non-Hodgkin's lymphoma (2), and multiple myeloma (1). With regard to HCV and HBV antibodies, 33 pts were negative for HBV and positive for HCV, 4 were positive for HBV and negative for HCV, 3 were positive for HBV and HCV, and 9 were negative for HBV and HCV. We administered four doses of r-IFN- $\alpha$  (1.5 MU) for induction and then 3 MU three times a week for six months. At six months pts. with a complete or partial response received 3 million units per week; those with a minor response or documented disease progression were withdrawn from the study. Two of the pts. with type I cryoglobulinemia showed early progression of their primary disease (multiple myeloma and Waldenström's macroglobulinemia). At present 43 pts. have been evaluated for response and toxicity; the length of follow-up ranges from 20 to 44+ months. Only one patient discontinued treatment because of severe thrombocytopenia. Mild side effects, such as a flu-like syndrome, were frequent, but there was no severe toxicity. Evaluation of the results after 20 months of therapy showed that 22 patients (51.2%) achieved a complete response (complete disappearance of any clinical and serological evidence of disease, with a cyocrit less than 10% of the initial value), 14 (32.6%) achieved a partial response (complete disappearance of any clinical manifestations but with a cryocrit more than 10% of the initial value) and 7 (16.3%) had a minor response (persistence of some clinical signs of vasculitis). The response rate for interferon  $\alpha 2b$  as a single agent was 83% after 20 months of therapy. Our results support the effectiveness of r-IFN- $\alpha 2b$  in the management of cryo-

globulinemia, with a significant reduction of the cryocrit ( $p = 0.005$  at 20 months), and confirm the high prevalence of HCV infection in type II and III cryoglobulinemia. The authors will present the final data and a new protocol.

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#### **DETECTION OF HUMAN HERPES VIRUSES (EBV, HHV-6 AND HHV-8) BY PCR IN LYMPH NODES FROM PATIENTS WITH CASTLEMAN'S DISEASE**

Barozzi P, Luppi M, Marasca R, Bonaccorsi G, Ferrari MG, Morselli M, Torelli G

*Department of Medical Sciences, Section of Hematology, University of Modena, Italy*

Castleman's disease (CD) is a heterogeneous group of lymphoproliferative disorders of uncertain etiology. Two pathologic types, hyaline vascular (HV) and plasma-cell disease (PC), have been recognized. More recently, a disease has been described that is morphologically similar to the plasma-cell type but with a multicentric presentation, systemic manifestations, and an aggressive clinical course, with a high risk of developing malignant disease, most frequently Kaposi's sarcoma or lymphoma. Previous studies of CD suggested a viral etiology for this disorder. In particular, an involvement of Epstein-Barr virus (EBV) or cytomegalovirus in the pathogenesis of this disease was indicated. To investigate a possible role for herpes viruses in the pathogenesis of CD, we analyzed the paraffin wax sections of lymph node biopsies from a total of 15 patients with CD (7 HV; 8 PC) for the presence of EBV, human herpesvirus-6 (HHV-6) and human herpes virus-8 (HHV-8) by polymerase chain reaction (PCR). Nine out of the 15 cases (60%) were positive for the presence of EBV sequences, while HHV-6 sequences were identified in only 3 (20%). Preliminary data showed the negativity of all cases for the specific sequences of HHV-8 examined by PCR and analyzed on ethidium bromide-stained agarose gel. We also used PCR to investigate the distribution of EBV subtypes A and B, generally associated with lymphomas in immunocompetent and immunocompromized patients, respectively. Characterization of EBV was possible in 6 out of 9 cases; 1 case contained the EBV genome of subtype B and 5 contained the subtype A virus. This study provides evidence of the presence of EBV specific sequences in a high proportion of our series of CD cases, suggesting a relationship between CD and this herpesvirus infection. Moreover, the frequent occurrence of EBV type A may indicate the absence of an underlying immunodisturbance in the majority of patients with CD.

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#### **GEOGRAPHIC SEQUENCE VARIATION OF LATENT MEMBRANE PROTEIN 1 (LMP-1) ONCOGENE OF EPSTEIN-BARR VIRUS (EBV) IN HODGKIN'S DISEASE (HD)**

Ferrari MG, Luppi M, Cocconcelli P, Morselli M, Barozzi P, Lin J-C,\* Torelli G

*Department of Medical Sciences, Section of Hematology, University of Modena, Italy; \*Centers for Disease Control (C.D.C), Atlanta, USA*

EBV LMP-1 is a transforming oncogene and partial deletions of this gene are associated with aggressive behavior in nasopharyngeal carcinoma and apparently confer a proliferative phenotype to lymphoid cells in HD. To assess the role of the EBV LMP-1 oncogene in the development of HD, the polymorphism of this gene in EBV isolates from different geographic locations was analyzed. A 497 bp fragment spanning LMP-1 gene exons 1 and 2 was amplified by PCR, using a primer pair bracketing an XhoI restriction site. PCR products were subjected to XhoI digestion and DNA sequencing analysis. Twenty-five HD biopsy specimens from the USA and 5 HD and 4 non-Hodgkin's lymphoma (NHL) biopsy specimens from Italy were examined. Eighty percent of LMP-1 positive samples (12 of 15) from the USA maintained the XhoI restriction site, while the remaining 20% partially lost it. One of 4 EBV-positive HD and 1 of the 3 EBV-positive NHL specimens from Italy lost the restriction site. The other three EBV-positive HD DNAs were partially cut by XhoI. Direct DNA sequencing analysis revealed that those Italian samples not digested by XhoI were due to a G to C transversion at the first base of codon 18, resulting in a glycine to arginine change. Those DNA samples partially cut by XhoI were due to a mixture of G/T at the second base of codon 17. The sequence variation found in the Italian samples differed from that of Asian EBV strains, in which G to T transversion was detected at codon 17, resulting in the substitution of arginine by leucine. Among the 72% (18 of 25) EBV-positive American HD samples, 67% (12 of 18) were associated with type A virus, 17% (3 of 18) with type B, and 17% (3 of 18) with dual viral sequences. EBV DNA was detected in 80% (4 of 5) of the Italian HD biopsy specimens, of which 50% were associated with type A and 50% with type B. Despite these sequence variations at the XhoI site among EBV isolates from different geographic locations, no direct correlation with a specific genotype was observed. These results, to the best of our knowledge, represent the first observation of a specific point mutation at codon 18 of the LMP-1 gene associated with a particular geographic location. It appears that the XhoI polymorphism may be a useful marker for epidemiologic study, and the alteration in the LMP-1 oncogene may have functional significance in the development of HD in certain geographical areas.

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#### **HEPATITIS C VIRUS (HCV) INFECTION IN SUBSETS OF NEOPLASTIC LYMPHOPROLIFERATIONS NOT ASSOCIATED WITH CRYOGLOBULINEMIA**

Luppi M, Ferrari MG, Bonaccorsi G, Longo G, Narni F, Barozzi P, Marasca R, Torelli G

*Department of Medical Sciences, Section of Hematology, Modena, Italy*

HCV is both hepatotropic and lymphotropic and a clearcut association has been proposed between HCV infection and mixed cryoglobulinemia (MC), a benign lymphoproliferative disorder that sometimes evolves into frankly malignant B-cell non-Hodgkin's lymphoma (B-NHL). Moreover, the presence of antibodies to HCV, as well as of HCV-specific genome, has been reported in the

sera of over 37% patients with B-NHL not associated with MC. Thus, we decided to perform both a serologic and a molecular study to gain insights into a possible relationship between HCV infection and neoplastic lymphoproliferations. We used ELISA and RIBA tests to show that anti-HCV antibodies were present in the serum of 29 out of 69 unselected B-NHL patients (42%), while seropositivity in a healthy population was about 1%. The prevalence of anti-HCV antibodies was low in certain subsets of B-lymphoid disorders, including multiple myeloma, Waldenström's macroglobulinemia and monoclonal gammopathies of undetermined significance. Then, using RT-PCR, we detected HCV sequences directly in pathologic lymph node biopsies in 13 out of 34 B-NHL cases, in particular in 5 out of 7 low-grade MALT type lymphomas and in 5 of 8 centroblastic-centrocytic lymphomas. On the other hand, the peripheral neoplastic cellular population from 10 patients with B-cell chronic lymphocytic leukemia proved to be negative for the presence of HCV genomes. Similarly, viral sequences were absent in 10 T-cell NHL, while only 1 out of the 14 Hodgkin's disease cases tested was positive. Finally, we used a PCR-based assay to characterize the genotypes (I-IV) present in positive lymphomatous tissues. The presence of both serologic and molecular markers of HCV infection in a high percentage of certain B-NHL not associated with cryoglobulinemia, and its absence from other lymphoproliferative diseases extends the spectrum of HCV-associated lymphoproliferations, while at the same time arguing in favor of some role for this viral infection in the pathogenesis of malignant proliferations of certain B lymphoid populations.

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#### TREATMENT OF AGGRESSIVE NON-HODGKIN'S LYMPHOMAS WITH THE F-MACHOP REGIMEN

Infanti L, Silvestri F, Salmaso F, Geromin A, Cerno M, Zaja F, Barillari G, Russo D, Fanin R, Bacarani M  
*Division of Hematology and Department of Bone Marrow Transplantation, Udine University Hospital, Udine, Italy*

Several different chemotherapy regimens are currently available for the treatment of aggressive non-Hodgkin's lymphomas (NHL). F-MACHOP, a third generation combination, has been reported to be highly effective in an adequate number of patients with long-term follow-up, yielding a CR rate of 65-80% and DFS of 75-80%. Based on these observations, we applied the F-MACHOP regimen to the patients with aggressive NHL seen at our Institution over the last 3 years with the aim of evaluating its efficacy in the treatment of this disorder. Fifty-three unselected, previously untreated patients entered the study. Male/female ratio was 30/23, median age 42 y (20-60). According to the REAL classification the histologic diagnosis was T- or null-cell anaplastic large cell lymphoma (13 pts), peripheral T-cell, unspecified (6 pts), angioimmunoblastic T-cell (1 pt), diffuse large B-cell (20 pts), follicular center (9 pts), marginal zone B-cell (2 pts) and mantle cell (2 pts). Most of these patients presented with advanced stages disease (33 pts = 62% in stage III and IV); B symptoms were present in 21 cases (39%), bulky disease in 27 (51%) and extranodal involvement in

32 (60%). According to the age-adjusted International Index, prognostic groups were as follow: low-risk 10 pts, low intermediate 23 pts, high-intermediate 14 pts, high 6 pts. All 40 evaluable patients (13 were still on therapy and not evaluable for response) were treated with 6 courses of the F-MACHOP regimen, except for 5 (12.5%) whose treatment was stopped after 1 to 5 courses because of disease progression. In 9 pts radiotherapy on existing bulky disease was employed after the end of CT. Of the 35 subjects who completed the therapeutic program, 18 (51%) entered CR, 9 (26%) entered SPR (significant partial remission, i.e. small residual disease in a previously bulky site) and 8 (23%) PR. Overall, 87.5% of the pts obtained a response. All 5 whose disease progressed on therapy died of lymphoma. Of the 35 pts who responded to CT, 19 (10 in CR, 7 in SPR and 2 in PR) underwent autologous bone marrow transplantation at a median time of 6 months (0.5-19) after the end of therapy. At present, with a median follow-up of 25.5 months (8-43), all those who entered CR after CT ( $\pm$ RT) (n. 18), whether autotransplanted (n. 10) or not (n. 8), are still in CR. All the ones in SPR and PR who underwent ABMT (n. 9) obtained CR; of the other 8 patients, 4 are alive with stable residual disease, 3 have progressed and 1 died of disease.

Our results confirm the efficacy of the F-MACHOP regimen in obtaining a response in many cases of high risk NHL. Whether this response can be maintained over time even without performing ABMT is still a matter of debate. A longer follow-up is required to verify possible relapses. On the other hand, our data indicate that when a PR is obtained, even if significant, it should be consolidated whenever possible with ABMT.

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#### IMMUNOPHENOTYPE OF 21 NHLs IN LEUKEMIC PHASE: COMPARED FLOW CYTOMETRY ANALYSIS OF LYMPH NODE AND PERIPHERAL BLOOD COUNTERPARTS

Cro L, Baldini L, Fracchiolla NS, Colombi M, Nobili L, Zucal N, Guffanti A,\* Neri A, Maiolo AT  
*Serv. di Ematologia, Osp. Maggiore, IRCCS, Ist. Scienze Mediche, Università degli Studi, Milan; \*Div. Medicina Osp. Fatebenefratelli ed Oftalmico, Milan, Italy*

We studied 21 cases of B-NHL, using flow cytometry to compare the immunophenotype of lymph nodes (LN) and peripheral blood (PBL) from the same patient in leukemic phase, with the aim of identifying possible differences in surface molecules with a potential pathogenetic role in the leukemic process. The histologic diagnoses (WF) of the lymph nodes analyzed were as follows: NHL A [11 cases, 5 of which were lymphoplasmocytic (lpc)], NHL C (1 case), NHL E (4 cases), NHL F (2 cases), NHL H (1 case), NHL I (1 case) marginal zone NHL (1 case). The immunophenotype of the LN and of the corresponding PBL samples was evaluated using the following diagnostic panel: CD1c, CD3, CD5, CD10, CD19, CD23, CD25, CD11a-c, CD49c-d, FMC7, SIg/CyIg. Reactivity at the LN level was consistent with what was recently reported by the REAL proposal for the different histotypes. Comparing the LN and PBL immunophenotypes revealed the following differences. FMC7, CD1c, CD11c and CD25

(with decreasing frequency), represented the markers showing the highest degree of variation in expression overall. Sporadic differences were also observed for CD10, CD23, CD11a, CD49c, CD49d. NHL A lpc was the subset with the greatest degree of difference in molecule expression; FMC7 expression was discordant in 6 cases, CD25 and CD11c in 3 cases and CD1c in 2 cases, and these generally presented a lower lymph node reactivity. In 2 of the 6 discordant FMC7 cases we observed a contemporary increase of FMC7, CD11c and CD25 (LN<sup>-</sup>/PBL<sup>+</sup>). CD11a revealed only modest, equivocal reductions in 3 cases. The other adhesion molecules did not show significant differences. In the NHL E group, the most frequent differences were relative to CD49c-d expression (3/4 cases); in two cases LN and PBL presented different CD49c expression and 1 case showed a reduction of CD49d expression in PBL. Interestingly, in one case CD10 was expressed in PBL but not in LN. One case of NHL F expressed CD1c and CD11a in LN but not in PBL. The NHL H case presented a higher expression of CD1c in LN than in PBL. The NHL I case expressed CD23 only in PBL. NHL C and NHL marginal did not reveal significant differences. In nine cases it was also possible to investigate the molecular arrangement of the c-myc, bcl-1 (MTC, p94), bcl-2 (Pfl-1, Pfl-2), bcl-3, bcl-6, Ras (N-H-K-) and p53 (Ex 5-8) genes. We detected c-myc rearrangement in the NHL I case, and bcl-1 (MTC) locus rearrangement in one case of NHL E, without differences between lymph node and PBL.

In conclusion, in NHL A we noted a higher frequency of differences between LN and corresponding PBL immunophenotypes, prevalently in the same direction. These observations suggest further cytogenetic and molecular biology studies. As far as the other histotypes are concerned, although the number of cases is limited, the observed differences appear to be casual. Nevertheless, genotypic characterization indicates a close relationship between the LN and PBL samples.

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#### CLINICAL RELEVANCE OF IMMUNOPHENOTYPIC SUBCLASSIFICATION OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

Bellio L, Pagnucco G, Vanelli L, Livraghi A, Zanaboni M, Buonanno M.C, Castelli G, Lazzarino M, Bernasconi C  
*Istituto di Ematologia, Università di Pavia, Divisione di Ematologia, IRCCS Policlinico San Matteo, Pavia, Italy*

From January 1988 to December 1992, we prospectively studied 84 B-CLL patients with a panel of monoclonal antibodies that detect B-cell (CD19, CD20, CD21, FMC-7, PCA1), T-cell (CD2, CD3, CD4, CD5, CD8) and HLA-DR, CD10 and CD11c antigens. Antigen expression was determined by immunofluorescence and flow cytometry using a FACS (EPICS-C). Surface membrane immunoglobulin analysis and mouse rosette assays were also performed. Patients were classified according to Binet and Rai. Morphological evaluation was performed according to the FAB criteria. This cohort of patients was grouped

according to two distinct immunophenotypic classifications. The first one was based on the expression of CD5 and T antigens: group I (CD5<sup>+</sup>, T Ag<sup>-</sup>) (n=51; 61%), group II (CD5<sup>+</sup>, T Ag<sup>+</sup>) (n=20; 24%), group III (CD5<sup>-</sup>, T Ag<sup>-</sup>) (n=8; 10%). The second one was based on the expression of CD5 and the B-cell associated antigens (B Ag) CD10, CD11c, PCA1: group A (CD5<sup>+</sup>, B Ag<sup>-</sup>) (n=52; 62%), group B (CD5<sup>+</sup>, B Ag<sup>+</sup>) (n=22; 26%), group C (CD5<sup>-</sup>, B Ag<sup>+</sup>) (n=4; 5%). Groups were compared according to clinical and laboratory features, FAB subtypes, Binet and Rai staging, disease progression and survival. Group I had a lower leukocyte count (p<.02) and group B a higher incidence of splenomegaly (p<.05). Typical CLL morphology (>90% small lymphocytes) was strongly associated with group I and group A immunophenotypes (p<.001), whereas mixed morphology was more frequent in groups B and C (p=.05). Interestingly, mixed CLL morphology showed a higher incidence of splenomegaly (p<.02) and a higher leukocyte count (p<.05) than typical CLL morphology. Disease progression to more advanced stages was more frequent in group III (67%) than in groups I-II (22%) (p<.02). The 3-year actuarial risk for progression was higher in group III (50%) than in group I-II (20%) (p=.09) and higher in group A (25%) than in group B (10%). After a median follow-up of 35 mos. (range 1-78 mos.) group III demonstrated poorer survival than groups I and II (median survival of 28 vs. 54 mos. and median not reached, respectively). We conclude that immunophenotype studies may be clinically useful in defining subgroups of B-CLL with prognostic relevance.

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#### IN VITRO CITOTOXICITY OF FLUDARABINE + MITOXANTRONE OR VINOURELBINE ON B-CLL CELLS

Di Raimondo F, Romeo MA, Palumbo GA, Cacciola RR  
*Institute of Hematology, University of Catania, Italy*

The efficacy and safety of fludarabine (Flu) as monotherapy for patients with B-cell chronic lymphocytic leukemia (B-CLL) suggests the possibility of using it in combination with other antineoplastic drugs. For this reason we employed a colorimetric assay (XTT) to test the *in vitro* cytotoxicity of Flu alone and in combination with mitoxantrone (Mx) or vinorelbine (Vnr) from B-CLL cells. We assayed 29 peripheral blood mononuclear cell samples of B-CLL patients (20 stage A, 6 stage B, 3 stage C according to the Binet classification). Flu was tested at doses ranging between 0.03 and 30 µM, Mx between 0.05 and 1 µg/mL and Vnr between 0.01 and 1 µg/mL.

The IC<sub>50</sub> (i.e. the concentration that inhibits 50% of cells) of each individual drug was below concentration levels that are achievable *in vivo*: Flu 1.4 µM, Mx 0.9 µg/mL, Vnr 0.7 µg/mL. Flu + Vnr combination, tested on 14 samples (10 stage A, 2 stage B, 2 stage C), failed to add to the efficacy of the individual drugs. In fact, neither the IC<sub>50</sub> of Flu nor that of Vnr was significantly modified when this combination was tested. By contrast, Flu + Mx showed a greater cytolytic effect. The IC<sub>50</sub> of Mx was markedly reduced by the addition of Flu at a dosage of 0.3 µM (0.2 µg/mL for the combination vs. 0.9 µg/mL for Mx alone), and the efficacy was even more evident on the IC<sub>50</sub>

of Flu (0.14  $\mu\text{M}$  when tested in combination with Mx at a concentration of 0.5  $\mu\text{g}/\text{mL}$  vs. 1.4  $\mu\text{M}$  for Flu alone).

These data demonstrate that the three drugs investigated, and Flu in particular, show cytotoxic activity towards B-CLL cells and that Flu + Mx has an additive effect. These *in vitro* results, obtained with lower dosages than those achievable *in vivo*, may be useful for the development of future clinical trials.

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#### **ANAPLASTIC LARGE CELL LYMPHOMA (CD30<sup>+</sup>ALCL): A RETROSPECTIVE CLINICO-PATHOLOGIC STUDY OF 53 PATIENTS**

Clavio M, Rossi E, Truini M, Ravetti JL, Carrara P, Spriano M, Vimercati R, Canepa L, Pierri I, Celesti L, Miglino M, Santini G, Damasio E, Gobbi M  
*Division of Hematology 1, S. Martino Hospital, Chair of Hematology, DIMI University of Genoa, Italy*

Out of 53 anaplastic large cell lymphomas (ALCL) diagnosed and treated in Genoa between 1989 and 1994, 36 (67%) were classified as Hodgkin related variety (HR) and 17 (33%) as *common type* (CT).

All the cases strongly expressed the CD30 Ki-1 antigen; CD15, CD45 and EMA were expressed in 60%, 44% and 33% of the cases respectively; 18 cases were T (37%), 8 B (17%) 22 Null (46%). The HR and CT subtypes had similar clinical features at presentation. The M/F ratio was 28/25, the median age 43 years (19-80), the stage was I in 12 patients (23%), II in 18 (34%), III in 8 (15%) and IV in 15 (28%). Nineteen patients (36%) had a mediastinal bulky, 13 extranodal disease (25%); the marrow infiltration was documented in 5 patients (9%). B symptoms and elevated serum LDH were recorded in 26 (49%) and 12 (25%) cases, respectively. Three patients had HIV infection.

The patients were treated with the following schemes: VACOP-B/MACOP-B (23), F-MACHOP (5), CHOP (8), MOPP/ABVD (4) and other (10); 2 patients received only radiotherapy, 1 only surgical treatment. Out of 50 evaluable patients 39 (78%) achieved a complete response (33 following the first line therapy, 2 after radiotherapy, 4 salvaged with autologous bone marrow transplantation); 3 patients (6%) obtained only a partial response, 8 (16%) did not respond. Eight patients had disease recurrence (20%).

At the moment 37 patients are alive, 16 died: the death was caused by lymphoma progression in 11 cases (64%), sepsis in 3 (21%), cardiovascular complications in 2 (14%). The median follow up is 18 months (range 4-57); the overall survival of the 53 patients is 70% at 48 months; the DFS at 24 months is 72%. The degree of lymphoma dissemination emerged as the most important prognostic factor.

The patients with I and II stage had better survival ( $p = 0.007$ ) and DFS ( $p = 0.015$ ) compared to those with III and IV stage. Extranodal disease ( $p = 0.007$ ) and B symptoms ( $p = 0.002$ ) had also a negative impact on survival. Bulky disease, LDH, and other biologic factor, such as histotype (HR or CT) and phenotype (T, B, Null), did not influence neither survival nor DFS.

Worth of note was the good responsivity of ALCL to

both III generation schemes (MACOP-B, F-MACHOP) and the more classic CHOP, as far as to Hodgkin oriented protocols, such as MOPP and ABVD. The autologous bone marrow transplantation proved to be an effective salvage therapy, both for refractory and relapsed patients. Our results, although in substantial agreement with the few complete anatomoclinic published reports differ for some aspects (fewer percentage of advanced stages, extranodal disease, above all skin infiltration and different HR/CT ratio).

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#### **DIFFUSE LARGE B CELL LYMPHOMA (REAL CLASSIFICATION) IS CLINICALLY HOMOGENEOUS: RETROSPECTIVE COMPARATIVE CLINICO-PATHOLOGIC STUDY OF 116 PATIENTS**

Clavio M, Vallebella E, Rossi E, Truini M, Ravetti JL, Spriano M, Poggi A, Casciaro S, Mencoboni M, Lerza R, Vimercati R, Congiu M, Nati S, Gatti AM, Ottonello L, Boccaccio P, Ghio R, Pannacciulli I, Santini G, Damasio E, Gobbi M

*Division of Hematology 1, II Division of Internal Medicine, S. Martino Hospital, Chair of Hematology, Clinica Medica R and Patologia Medica B, DIMI, University of Genoa, Italy*

The Revised European-American Classification of Lymphoid Neoplasms, recently proposed by the International Lymphoma Study Group (REAL), introduces the term diffuse diffuse large B-cell lymphoma to group together some histotypes which were separated entities in the preceding classification systems (Kiel and WF). We therefore evaluated if the high grade non Hodgkin's lymphomas classified in the last 4 years in Genoa as centroblastic (64), immunoblastic-B (29), large cell (17) and B-mediastinic with sclerosis (6) and now redefined as diffus B large cell lymphomas had actually homogeneous clinical presentation and therapeutic outcome. The first three histotypes had similar presentation features; the overall median age was 56 years (range 16-85), the M/F ratio 65/51; the stage was I-II in 44 patients (38%), III-IV in 72 (62%); 28 patients (24%) had bulky disease, 48 (41%) B-symptoms, 30 (26%) increased serum LDH values, 68 (59%) extranodal disease, and frequent infiltration of marrow (27%), liver, lung, stomach, bowel; the marrow infiltration was frequently associated to the involvement of other visceral sites. In our series the few B-mediastinic lymphomas with sclerosis had a lower median age and stage I prevalence. The patients were treated according to the policy of the various hemato-oncologic centres (prevalently II-III generation schemes were employed); 109 patients were evaluable for response. Seventy-seven (70%) achieved CR (67 following the first line therapy, 5 following salvage autologous bone marrow transplantation, 1 after RT, 3 salvaged by chemotherapy), 19 (17%) obtained PR; in 14 cases (13%) the lymphoma failed to respond or progressed. The complete response rate of the different histotypes was similar. Sixteen patients (21%) relapsed, 69 are alive, 45 died, 2 were lost to follow up. The overall survival and DFS at 48 months are respectively 40% e 70%; the three mentioned histotypes did not significantly differ but patients with B-mediastinic lymphoma had higher CR and better survival and DFS. Low clinic stage (I-II)

( $p=0.000135$ ), absence of extranodal disease ( $p=0.0078$ ) absence of B symptoms ( $p=0.00000003$ ) had a positive impact on survival, while I and II stage patients had a significant longer DFS. The patients were stratified according to the prognostic factors recognized by the International Index. Low, intermediate-low, high-intermediate, high risk patients had a CR rate of 90, 85, 67 e 38% and a survival of 73, 59. 37 and 10% at 48 months, respectively. In conclusion our analysis confirms the prognostic and clinical homogeneity of diffuse B-large cell lymphomas and the clinical utility to differentiate the primary mediastinal (tymic) large B-cell lymphoma subtype.

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#### THE MANAGEMENT OF PRIMARY GASTRIC LYMPHOMA: A RETROSPECTIVE ANALYSIS OF TREATMENT AT 2 CENTERS OVER AN 18-YEAR PERIOD

Rossi A, Viero P, Motta T, Love S, Norton A, Cortelazzo S, Rohatiner AZS, Lister TA, Barbu T

*Divisione di Ematologia e Istituto di Anatomia Patologica degli Ospedali Riuniti di Bergamo, Italy; ICRF Department of Medical Oncology e Department of Histopathology, St. Bartholomew's Hospital, London, UK*

The stomach is the most frequent primary extra-nodal site for non-Hodgkin's lymphoma (NHL), yet the management of patients (pts) with primary gastric lymphoma (PGL) remains controversial. A retrospective analysis of outcome for a series of newly diagnosed pts with PGL was therefore conducted with a view to identifying patient categories at different prognostic risk and to contributing to a really rational approach to therapy. Between 1974 and 1992, 117 pts with PGL, 62 males and 55 females, median age 60 years (range 16-82), were diagnosed. Seventy seven pts (66%) had localized disease (I, II1); bulky disease (a mass  $\geq 10$  cm in diameter) was present in 21 (18%). Patients were classified histologically according to the concept of MALT derived lymphoma (1): 38 low grade (lg), 21 high grade (hg) with a residual Ig component and 58 hg only. Thirty two pts were treated by surgical resection alone, surgery was followed by chemotherapy (CT)  $\pm$  radiotherapy (XRT) in 52, and 29 received CT  $\pm$  XRT without surgical intervention. Four pts were not treated. Complete remission was achieved in 93/113 (82%) pts overall (93 and 60% respectively for pts with localized and disseminated disease); with a median follow-up of 4 years, 84% of pts (89 and 70% for pts with localised and disseminated disease respectively) are predicted to remain in remission at 5 years. The overall survival was 59% at 5 years (73 and 34% for pts with localized and disseminated disease respectively). There were 13 treatment-related deaths; five pts died of progressive disease; in 2 pts the cause of death is not known. On univariate analysis, localized disease, treatment involving surgical resection ( $p < 0.001$ ), low grade histology and lack of B symptoms ( $p < 0.05$ ) correlated favourably with survival. On multivariate analysis, only histology and surgical resection were found to be independent prognostic factors ( $p < 0.02$ ). Our results support the widely held view that surgery is an essential component of curative therapy for PGL and that both histological subtype and distribution of disease have important prognostic implications.

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#### CD30/Ki-1 POSITIVE ANAPLASTIC LARGE CELL LYMPHOMA (ALCL): CLINICAL AND IMMUNOPHENOTIPIC CHARACTERISTICS IN IMMUNOCOMPETENT AND HIV POSITIVE PATIENTS

Cantoni S, Nosari A, Gargantini L, Landonio G,<sup>#</sup> Oreste P,<sup>o</sup> Caroli Costantini M.<sup>o</sup>, Schiantarelli C,<sup>\*</sup> Morra E

*Department of Hematology, <sup>o</sup>Department of Pathology, <sup>#</sup>Department of Oncology, <sup>\*</sup>Department of Infectious Disease, Niguarda Cà Granda Hospital, Milan, Italy*

Twenty patients (pts) with CD30/Ki-1 positive ALCL were studied. 9 pts (7 M, 2 F, mean age 30 years) were HIV-positive and 11 (7 M, 4 F, mean age 49 years) were immunocompetent.

Among the 9 HIV-positive pts, histological subtype was common type in 5, giant cell rich in 2 and Hodgkin-related in 2 pts. Immunophenotype was *null* in 4, *B* in 4, *T* in 1 pt. 3 cases were EBV positive. At diagnosis, all pts presented with extranodal involvement and 8/9 pts were in stage IV. Bone marrow biopsy was positive in 5/7 pts. No mediastinal involvement was observed in the two pts with the Hodgkin-related subtype; in one pt meningeal involvement was observed.

Among the 11 HIV-negative pts, histological subtype was common type in 8 and Hodgkin-related in 3 pts. Immunophenotype was *null* in 6, *B* in 3, *T* in 2 pts. Only 1 pt was EBV positive. Onset was nodal in 8/11; 4 pts were in stage II, 3 in stage III and 4 in stage IV at presentation. No pt had cutaneous, CNS or bone marrow involvement. Of the 3 pts with Hodgkin-related subtype, 2 had bulky mediastinal involvement.

Our study indicates that: 1) histopathological findings of ALCL are similar in both HIV-positive and negative pts; 2) *T* is not the predominant phenotype; 3) clinical features of ALCL in HIV-positive pts do not differ from HIV related NHL of different histotype; 4) bone marrow involvement, not observed in immunocompetent pts, seems to be characteristic of HIV related ALCL; 5) lung involvement is often seen in both groups (9 of 20 pts, 45%): primary lung involvement, without mediastinal adenopathy, was observed in the 4 HIV positive pts; among the 5 immunocompetent pts, concomitant lung and mediastinal involvement was seen in 4/5pts, while primary lung disease was seen in one pt only with extranodal disease; 6) gastric involvement seems to be rather common: it was seen in 3 of 9 (35%) HIV-positive and in 2 of 11 (18%) HIV-negative pts.

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#### TYPE II MIXED CRYOGLOBULINEMIA (MCII): A LYMPHOPROLIFERATION B-CLL TYPE PATTERN

Bertoncelli MC, Ballaré M, Catania E, Zaccala G, Paccagnino L, Monteverde A

*Ospedale Maggiore di Novara, Italy*

In recent years MCII has acquired a new identity as a lymphoproliferative disorder, related to the expansion of B-cells secreting monoclonal rheumatoid factors (RF) IgM anti-IgG.

HCV, which is very frequently associated with MCII, might drive a polyclonal expansion of a lymphocyte subset leading to the development of a B-cell clone secreting the IgM RF. For some years we have been describing in MCII patients lymphoid portal and bone marrow infiltrates formed by B-cells bearing monotypic surface immunoglobulins; this has suggested that MCII could be considered a lymphoproliferative process similar to a low grade malignant non-Hodgkin lymphoma. Recently, we studied 35 HCV infected MCII with bone marrow and liver infiltrations. Formalin-fixed, paraffin-embedded bone marrow and frozen liver sections were tested with conventional and immunohistological staining (MoAb anti CD20/L26, CD45RA/4KB5, CD43R6/UCHL1, K, L, bcl-2, Ki67). Bone marrow infiltrations were formed by B cells (CD20-CD45Ra) expressing IgMk, bcl-2 and low Ki67. In the liver, lymphoid portal infiltrations with a pseudofollicular appearance presented predominant B-cell component (CD19, CD22, CD5, bcl-2 positive, low Ki67) bearing monotypic surface IgMk. Our data confirm the lymphomatous *indolent* character of the lymphoid population in CMI and permit comparison with B-CLL infiltrates, showing a similar phenotype.

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#### ANALYSIS OF CD44 SPLICE VARIANTS IN NON-HODGKIN'S LYMPHOMAS

Marasca R, Ferrari MG, Luppi M, Barozzi P, Morselli M, Torelli G

*Department of Medical Sciences, Section of Hematology, University of Modena, Italy*

CD44 is a transmembrane group of glycoproteins involved in the interactions between cells and extracellular matrix and supposed to play an important role in lymphocytes recirculation. CD44 gene may generate a large number of different CD44 isoforms by alternative splicing of at least 10 variant exons. The smallest splice CD44 gene product is a 85 KDa protein, called standard form, that is the major isoform expressed on lymphocytes. Activations of T-lymphocytes increase CD44 expression and may generate some CD44 variant forms. Moreover, CD44 variant forms have been found to be over-expressed in a variety of human cancers and tumor cell-lines. Some of these variant forms have been shown to be causally involved in tumor metastasis formation in experimental models. The similarity between tumor spread and lymphocytes recirculation and the supposed role of CD44 in both processes raise the question whether CD44 splice variants might have a role in migration of malignant lymphocytes. Therefore we decided to investigate, by RT-PCR, CD44 splice variants in the RNAs extracted from normal human peripheral mononuclear cells (PBMC), from non-Hodgkin's lymphoma (NHL) tissues of different histological subtypes and from lymph node biopsies derived from patients with inflammatory disease. After 35 cycles of PCR the amplified products, representative of the different forms of CD44 mRNA present, were analyzed by Southern blot and hybridized with an oligonucleotide capable of recognize all CD44 splice products and with the oligoprobe D3 specific for some CD44 splice variants. A

fragment of 482 nt, indicative of the standard form, was present, at almost at the same intensity, in all the samples. In PBMC and in the lymphadenopathy samples were present 3 further forms, containing D3, at a very low level. Four out of 6 aggressive and 1 out of 4 low grade NHLs expressed variant forms with differences in number, size, and intensity of the bands obtained from the control samples. These results indicate the presence of some CD44 splice variants in NHLs, especially in high-grade subtypes, suggesting a possible role in promoting dissemination of lymphoma cells.

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#### INCIDENCE OF HEPATITS C VIRUS INFECTION IN PATIENTS WITH B-CELL NON-HODGKIN'S LYMPHOMAS

Silvestri F, Barillari G, Pipan C,\* Zaja F, Infanti L, Salmaso F, Candoni A, Russo D, Fanin R, Botta GA,\* Baccarani M  
*Division of Hematology and \*Institute of Microbiology, University Hospital, Udine, Italy*

The etiology of most lymphoproliferative disorders is unknown. It has been recently hypothesized that the hepatitis C virus (HCV) might be involved in the pathogenesis of B-cell non-Hodgkin's lymphomas (B-NHL): Ferri and coworkers found antibodies against HCV or HCV viremia in a high percentage of cases (30 and 32% respectively) of B-NHL and postulated a causative role for this virus in such disorders. On the basis of this observation we sought to determine the incidence of HCV infection in the patients affected by B-NHL seen at our Institution in the last 24 months. One hundred and ninety-eight unselected, consecutive patients were included in the study population. Diagnosis of B-NHL was made by morphology and immunophenotypic analysis. They were classified according to the REAL classification. Commercial assays were used to test for anti-HCV, HIV and HBV antibodies and for HBsAg. Detection of HCV-RNA was made by RT-PCR. HCV genotyping was performed on HCV-RNA positive specimens. Overall, anti-HCV antibodies were detected in the serum of 23 of 198 (11.6%) patients. Analysis by histologic pattern revealed that some entity is never or very rarely associated with HCV infection: among them CLL, HCL, PLL, marginal zone lymphomas and aggressive histotypes (lymphoblastic, diffuse large cell, primary mediastinal and Burkitt's lymphomas). Some other type, as follicular center and mantle cell lymphomas, is occasionally associated with HCV, while a strong association was found with the immunocytomas (lymphoplasmacytoid lymphomas): 12 of 35 patients (34.2%) is this group. All these cases showed the presence of a cryoprecipitable IgMK monoclonal component with rheumatoid factor activity (type II). HCV genomic sequences detected by RT-PCR were found in 18 of the 20 tested patients (90%). The viral genotypes identified (No. 10 type 1b and No. 8 type 2) were those most frequent in our region. None of the patients was found positive for anti-HIV antibodies; those against HBV were present in 18% of the cases. HBsAg was present in only 3 (13%) of the HCV-positive subjects: all these 3 had been previously transfused with blood products.

In conclusion, our study shows that the overall inci-

dence of HCV infection among patients with B-NHL is superior to that of the general population (3.2% in our area), but this is mainly due to its very high incidence among the immunocytomas, while other histotypes are only occasionally associated with HCV. If this virus plays a pathogenetic role as a primer for the proliferation or only as a trigger for the production of a type II IgMK monoclonal component in an otherwise born lymphoma has not been so far defined. No specific association with a particular virus genotype was found.

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#### RESULTS OF A ProMACE-CytaBOM PILOT STUDY AS FIRST-LINE THERAPY IN FOLLICULAR NON-HODGKIN'S LYMPHOMAS

Colombi M, Baldini L, Gobbi PG, Federico M, Avanzini P, Cavanna L, Abbadessa V, Brugiattelli M, Di Renzo N, Pitini V, Silingardi V, Maiolo AT for GISL  
*Serv. Ematologia, Osp. Maggiore, IRCCS, Ist. Scienze Mediche, Univ. Studi, Milan, Italy*

In 1988 the GISL carried out a study to verify the efficacy and toxicity of ProMACE-Cyta-BOM as first line therapy in a selected group of patients affected by Fo-NHLs in advanced stage and aged less than 55 yrs. Our therapeutic approach was prompted by a previous favorable GISL experience with ProMACE-CytaBOM in intermediate/high grade NHLs and also by the promising results of NCI group's trial based on the use of ProMACE-MOPP for the treatment of indolent NHLs (*Young et al, Semin Hematol 1988; 2:11*).

Thirty-two patients were included in the study (M/F:9/23; mean age: 42.7±9 yrs); nine had a follicular small cleaved cell NHL and 23 had a follicular mixed NHL. Fifteen pts were in stage III and 17 in stage IV; nine pts (28.1%) showed B symptoms. Twenty-one pts received 6 cycles of ProMACE-CytaBOM, 9 pts 5 cycles and 2 pts stopped the treatment after the third cycle for withdrawal of consent. Four pts received IF-RT after chemotherapy. Sixteen out of 30 pts (53.3%), receiving almost 5 cycles of chemotherapy, achieved a complete remission (CR) and the remaining pts achieved a partial remission (PR). Of the two pts who received only 3 cycles, 1 achieved CR and 1 PR.

The therapeutic response was not related to the main clinico-hematological features of possible prognostic significance (age, P.S, B symptoms, stage, bulky disease, LDH level). The frequency of relapse was evaluable in 25 pts, since 7 pts in PR were immediately treated with an alternative regimen. At a median follow-up of 39 months (12-72) 12/25 pts (7 in CR and 5 in PR) showed a relapse of the disease (TTF: 39 months). Among 32 pts enrolled in the study (median follow-up: 35 months), two pts died for disease progression. Nine pts treated only with ProMACE-CytaBOM remain still disease free after a median follow-up of 43 months (24-51). Toxicity from the ProMACE-CytaBOM chemotherapy was limited: hematological (grade 3, at least once) in 10% of the cases; mucositis and vomiting (grade 1-2) in 21%; neurotoxicity (grade 1-2) in 16%; alopecia (grade 2-3) in 85% and grade 3 cardiotoxicity

was observed in 1 patient. The DI was maintained and G-CSF was sporadically used. Although an objective therapeutic response was obtained in all of the cases, the ProMACE-CytaBOM regimen as first-line therapy in a selected group of advanced Fo-NHLs does not allow to obtain a higher rate of CR than that reached by using less aggressive regimens. The frequency of relapse is quite high even though we have obtained a satisfactory rate of durable CR (28.1% with CR lasting > 24 months). Our therapeutic approach has not been changed by a significant frequency of side effects.

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#### GALLIUM 67 IN ADVANCED LYMPHOMAS

Cavallero GB,\* Bonferroni M\*, Biggi A,<sup>#</sup> Farinelli C,<sup>#</sup> Sturlese U,\* Gallo E<sup>o</sup>  
<sup>\*</sup>Divisione di Medicina II, <sup>#</sup>Servizio di Medicina Nucleare e <sup>o</sup>Divisione di Ematologia, Azienda Ospedaliera S. Croce e Carle, Cuneo, Italy

We evaluated if Gallium 67 (Ga67) may be useful in identifying patients in stable CR after induction treatment for advanced lymphomas. Thirty-eight patients were studied: 19 had intermediate-high grade non-Hodgkin lymphoma (NHL) (W.F.:D-I) and 19 had Hodgkin disease (HD), mean age 55 years (range 20-73) for NHL and 34 years (range 18-47) for HD, mean follow up after treatment 37 months (range 7-129) for NHL and 56 months (range 4-121) for HD. Stages were the following: III (6), IV (13), 11 with bulky disease in NHL; III (10), IV (9), B symptoms (11), 6 with bulky disease in HD. Ga 67 was performed at diagnosis and after induction treatment (polichemotherapy ± radiotherapy). All patients had a positive Ga67 before therapy. CR was judged by disappearance of disease after therapy without considering Ga67 results.

After induction treatment we found: 1) Ga67 was negative in 12/19 (63%) NHL: 5/12 (42%) are alive in first CR and the mean follow up after therapy is 60 months (range 11-129), 1 was in PR and died after 7 months, 6 relapsed after 6, 7, 7, 8, 21, 92 months (1 died, 1 is alive in 2° CR after ABMT, 4 are in PR); 2) Ga67 was positive in 7/19 (37%) NHL: 4 were in PR (only one is alive after ABMT), 2 were in CR and relapsed after 11 and 24 months (one is alive in PR and the latter died), 1 has been in CR for 9 months, 3) Ga67 was negative in 17/19 (90%) HD: 13/17 (76%) are alive in first CR and the mean follow-up after therapy is of 68 months (range 20- 121), 3 relapsed after 9, 17 and 58 months and are alive in 2° CR, 1 died for progressive disease; 4) Ga67 was positive in 2/19 (10%) HD and both died for progressive disease. We calculated the DFS and the OS in Ga67 positive and Ga67 negative patients and the results were analyzed using the Kaplan-Meier method.

We concluded: 1) in HD a statistically significant difference is observed between Ga positive and Ga negative patients: DFS (p=0.0008), OS (p=0.0005). In these patients a Ga negative is associated with a stable CR;

2) in NHL the difference in DFS is not significant (p > 0.05) and in OS is low (p=0.03). In this subgroup of NHL

a Ga67 negative after induction treatment is not useful in identifying patients in stable CR, so other factors must be considered to assess the risk for relapse; 3) in both groups of patients a positive Ga67 after therapy is associated with a bad outcome.

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**RICHTER'S SYNDROME IN A CASE OF ATYPICAL CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) WITH THE t(11;14) (q13;q32): ROLE FOR A p53 EXON 7 GENE MUTATION**

Piva N, Cuneo A, De Angeli C, Roberti G, Bigoni R, Gandini D, Moretti S, Del Senno L, Castoldi GL  
*Istituto di Ematologia e Dipartimento di Biologia Molecolare, Università di Ferrara, Italy*

Clinicobiologic, histologic, cytogenetic and molecular genetic studies were performed in a case of atypical B-cell chronic lymphocytic leukemia (B-CLL) with the t(11;14) (q13;q32) evolving into Richter's syndrome (RS) in order a) to determine the clonal relationship between the cell of origin for B-CLL and for RS and, b) to analyze genetic events underlying disease progression in this patient.

After 4 years of diagnosis, a rapid deterioration of the clinical picture occurred, concomitant with the appearance of large lymphoid blasts in PB, BM and ascites samples. A diagnosis of RS was made and cytogenetic analysis revealed karyotype evolution with trisomy 7 and del(17p) in addition to the t(11;14) in metaphase from PB and effusion samples. Fluorescent *in situ* hybridization showed 78% lymphoid blast cells obtained from ascites sample to be trisomic using a chromosome-7-specific pericentromeric probe. Whereas no rearrangement of the *c-myc* proto-oncogene was detected at disease progression, direct sequencing of p53 gene exon 5 through 9 revealed an exon 7 missense point mutation. This abnormality was not present in the CLL phase. Immunological staining with the monoclonal antibody PAb-1801, detecting the p53 protein product, revealed a negative pattern in the CLL phase, whereas 24% positivity was documented in representative samples obtained at RS, suggesting that an abnormally stable p53 protein was synthesized by lymphoid blast cells carrying the p53 gene mutation and the 17p deletion.

It is concluded that a) an atypical form of B-CLL with the t(11;14), evolving into RS, can be distinguished on clinicobiologic and histologic grounds from the spectrum of neoplasia of follicle mantle lineage; b) RS was cytogenetically related with B-CLL in this patient, suggesting the occurrence of a bona fide transformation and, c) the mutation of p53 exon 7, in association with the development of 17p deletion, played a role in the development of RS.

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**FLUDARABINE AND PREDNISONE VERSUS FLUDARABINE, PREDNISONE AND INTERFERON FOR THE TREATMENT OF B-CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL): PRELIMINARY RESULTS OF A MULTICENTRIC PROSPECTIVE RANDOMIZED TRIAL**

Mauro FR, Mandelli F, Zinzani P.L,\* Baccarani M,<sup>o</sup> Zaja F,<sup>o</sup> Bendandi M,\* Fazi P, D'Elia GM, Di Ienno S, Tura S\*  
*Cattedra di Ematologia, Dip. di Biopatologia Umana, Università "La Sapienza", Roma; \*Istituto di Ematologia "L. e A. Seràgnoli", Università degli Studi di Bologna; <sup>o</sup>Cattedra di Ematologia, Università di Udine, Italy*

Between March 1993 and April 1995, 76 previously untreated B-CLL pts observed at Hematology Institutes of Rome (36 pts), Bologna (22 pts) and Udine (18 pts) were randomized to receive 6 monthly courses of therapy, with either fludarabine (Fluda, Schering SpA: 25 mg/m<sup>2</sup>/iv/day, days 1-5) and prednisone (PDN: 20 mg/m<sup>2</sup>/day, days 1; 3; 5; 7; 14 and 40 mg/m<sup>2</sup>; days 9-13) or the same therapy schedule associated to eight doses of human lymphoblastoid IFN (Wellferon, Wellcome Foundation Ltd, 2 MU/sc/day; every second day from 1<sup>o</sup> to 15<sup>o</sup> day).

Therapy was administered on an outpatient basis. 51 of the 76 enrolled pts have been now assessed for response: 26 pts treated with FLUDA + PDN (arm A) and 25 pts treated with FLUDA + PDN + IFN (arm B). At this time, using NCI criteria for response, the overall response (PR + CR) rate is 86% with no difference of response rate between arm A pts (85%) and arm B pts (88%). The major toxicity consisted primarily of myelotoxicity and infections: pneumonia (3 pts), herpes zoster (2 pts), herpes simplex (2 pts) and HBV hepatitis (2 pts). No neurological toxicity and early deaths were observed.

Responder pts (CR-PR) were then randomized to receive maintenance therapy with IFN (3MU three times a week) or no therapy. The incidence of progressive disease (31%) seems to be lower in CR pts (CR: 17%) vs PR (39%) and in those maintained with IFN (IFN: 22% vs. no therapy 41%). Four pts have died: refractory CLL (1 pts), larynx carcinoma (1 pts), large cell lymphoma (2 pts).

Our preliminary results suggest that FLUDA + PDN ± IFN is an effective regimen with acceptable toxicity for young patients with B-CLL. Further follow-up is needed to assess whether the addition of IFN to FLUDA + PDN may improve the rate and duration of response in B-CLL.

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**ATYPICAL CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) WITH THE t(11;14) (q13;q32): CLINICOBIOLOGIC FEATURES AND EVOLUTION PATTERNS**

Cuneo A, Piva N, Balboni M, Grazia Roberti, Bardi A, Bigoni R, Rigolin GM, Basamo R, Ferrari L, Castoldi GL  
*Istituto di Ematologia, Università di Ferrara, Italy*

In order to better define the features of atypical CLL with the t(11;14)(q13;q32), clinicobiologic data were reviewed in 10 patients, in comparison with similar data from 82 cases without the t(11;14) classified according to the FAB criteria. Cytologic diagnosis was typical CLL in 2 cases and atypical CLL in 8 cases, a diagnosis confirmed histologically in BM biopsies and splenectomy samples in 7 cases. A progressive increase prolymphocytes leading to prolymphocytic transformation occurred in 5 cases over a 6-60 month-period. In one case transformation into Richter's syndrome was documented after 4 years of diagnosis, concurrent with the acquisition of a 17p- chromosome

and a p53 exon 7 gene mutation.

Immunophenotyping showed a mature B-cell phenotype with CD19, CD22, CD24 positivity and CD10 negativity in all patients. A bright staining pattern for SIg was detected in 9/10 cases, CD5 and FMC7 positivity in 9/10 cases, CD23 positivity in 1/10 cases only. Chromosome changes in addition to the t(11;14) were seen in 8 cases; karyotype evolution that was associated with disease progression was recorded in 4/7 assessable patients.

Median age at presentation was 60 years (range 51-78), the lymphocyte count was  $25 \times 10^9/L$ . Only 1 patient showed lymph node involvement at presentation, whereas mild to moderate splenomegaly was recorded in 7/10 cases. Progressive lymphocytosis and splenomegaly developed in 9/10 cases, and all patients showed partial, short lasting responses to single agent (chlorambucil) and multi-agent chemotherapy (C(H)OP). Median survival was 60 months in 6 cases (range 6-72); 4 patients are alive at 24, 36, 72 and 84 months. Comparison of these findings with similar data from 82 unselected B-CLL without the t(11;14) shows that a) atypical morphology and karyotype evolution are more frequently seen in cases with the t(11;14) ( $p=0.015$  and  $0.04$ , respectively); b) the frequency of positivity for CD23 and bright SIg staining differed significantly in the two groups. It is concluded that the t(11;14) identifies a subset of atypical CLL by FAB criteria showing distinct features with respect to other forms of lymphoproliferative disorders of follicle mantle lineage. These patients are characterized by atypical morphology and by frequent cytogenetic evolution; they may undergo polymphocytic transformation or develop Richter's syndrome. Because response to conventional treatment is unsatisfactory, they are good candidates for the assessment of efficacy of alternative cytotoxic agents.

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**LONG-TERM FOLLOW-UP IN ADVANCED STAGE DIFFUSE LARGE CELL LYMPHOMA (DLCL) TREATED WITH MACOPB: ANALYSIS OF LATE RELAPSES AND TOXICITIES AND VALIDATION OF THE INTERNATIONAL PROGNOSTIC INDEX (IPI)**

Vitolo U, Brusamolino E, Cortellazzo S, Bertini B, Botto B, Comotti B, Freilone R, Gallamini A, Gallo E, Gatti AM, Levis A, Luxi G, Meneghini V, Orlandi E, Orsucci L, Rota-Scalabrini D, Tarella C, Todeschini G, Viero P, Barbui T, Bernasconi C, Perona G, Pileri A, Resegotti L

For the Italian Multiregional Non-Hodgkin Lymphoma Study Group (IMRNHLSG): Divisione di Ematologia, Ospedale Molinette, Torino, Italy

Two hundred patients with advanced stage DLCL, age < 61 years, treated with MACOPB in a cooperative study undertaken by the IMRNHLSG (*J Cancer Oncol* 1992; 10:219) between June 1986 and December 1990, have been analyzed for late relapses or late toxicities. Median follow-up was 81 months. 71% achieved a CR, 12% a PR, 12% showed a NR and 5% died of acute toxicity. Among the 48 PRs or NRs only 9 were salvaged and are alive, 39 died (37 of lymphoma). Forty pts relapsed: 11 are alive in

further CR and 29 died (25 of lymphoma). The DFS at 7 years is 69%. The relapse rate was 18% in the 1st year, 6% in the 2nd, 2% in the 3rd and 1% thereafter till the 8th year off therapy. Only 4 relapses were observed after 3 years, the last one was at 90 months. Overall survival (OS) at 3, 5 and 7 years is 65%, 61% and 59% respectively. A second cancer occurred in 5 patients: 4 solid tumors (head and neck, gastric, breast, thyroid) and 1 ANLL. Four died of this cancer (3 while in CR of the lymphoma). Five pts suffered from late cardiac disorders: 3 cardiomyopathies and 2 arrhythmias (2 were given mediastinal RT after MACOPB). Femoral head osteonecrosis was diagnosed in 7 patients with a median time off MACOPB of 15 months, probably due to the prolonged steroid therapy used in this regimen. Moreover the age-adjusted IPI has been tested in this series with LDH, PS and Stage used as prognostic factors as suggested to define the risk groups.

	CR%	DFS%	OS%
SCORE 0	90	81	72
SCORE 1	71	76	67
SCORE 2	67	62	48
SCORE 3	50	44	28

The IPI proved to be an excellent prognostic scheme for CR, DFS and OS ( $P<0.01$ ). However a better discrimination between patients with good or poor outcome is possible subdividing patients in only two prognostic groups, Score 0+1 (low risk) vs Score 2+3 (high risk): CR 79% vs 62%, DFS 72% vs 59%, OS 69% vs 43% ( $P<0.01$ ). These data obtained in a large series of pts with a long follow-up show that more than 50% of advanced stage DLCL are alive and free of disease after MACOPB with few late relapses and toxicities. The new indispensable therapeutic approaches need to be compared with these results.

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**NEONATAL ALLOIMMUNE THROMBOCYTOPENIA (NATP) CAUSED BY A NEW LOW-FREQUENCY PLATELET-SPECIFIC ANTIGEN, MAX<sup>a</sup>, LOCATED ON GLYCOPROTEIN (GP) IIB**

Noris P,\* Simsek S, de Bruijne-Admiraal LG, Porcelijn L, Huiskes E, van der Vlist G, van Leeuwen EF, van der Schoot CE, von dem Borne AEGKr

\*Institute of Internal Medicine and Medical Oncology, University of Pavia-IRCCS San Matteo, Pavia, Italy. Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands

A new human platelet-specific antigen (HPA), Max<sup>a</sup>, was responsible for a case of NATP in a Dutch family without history of bleeding. Soon after the birth a newborn developed typical signs of NATP. In the platelet immunofluorescence test (PIFT) the maternal serum strongly reacted with the paternal platelets but no incompatibility between the platelets of the parents and the newborn for the known HPA was found. In the monoclonal antibody (MoAb)-specific immobilization of platelet antigens (MAIPA) assay the new antigen was located on GPIIb-IIIa complex. Because in the MAIPA assay the binding of anti-Max<sup>a</sup> antibodies from the maternal serum was inhibited by one anti-GPIIb MoAb, the Max<sup>a</sup> antigen

appeared to be located on GPIIb. Family studies showed that the Max<sup>a</sup> antigen segregates as an autosomal dominant character. In further serological investigations, 3 out of 500 donors were typed Max(a+), indicating a phenotype frequency of 0.6% in the Dutch population. Platelet aggregation investigated in Max(a+) individuals was normal. The anti-Max<sup>a</sup> maternal serum showed a weak, probably aspecific, inhibitory effect on Max(a+) platelet function. In order to unravel the molecular basis of the Max<sup>a</sup> antigen, platelet RNA was isolated from the newborn's Maxa-positive father, reverse transcribed and the entire GPIIb coding region was amplified by polymerase chain reaction. Subsequent nucleotide sequence analysis revealed a single G→A substitution predicting a Val→Met amino-acid substitution at position 837 of the mature GPIIb. The association between the new antigen and this point mutation was confirmed by allele-specific restriction enzyme analysis on cDNA (with the endonuclease BsiYI) and on genomic DNA (with BstNI) as well as by allele-specific primer amplification on genomic DNA. Since the new mutation is only 19 bp upstream of the mutation underlying the HPA-3 system, we studied the association between Max and the HPA-3 polymorphism. Until now, all Max(a+) subjects were found to be also HPA-3b, whereas fifty HPA-3a donors were all typed Max(a-). Therefore, the Max<sup>a</sup> antigen might be considered a molecular variant of the HPA-3b allele.

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#### STIMULATORY EFFECT OF flt3/flk2 LIGAND ON HUMAN MEGAKARYOCYTOPOIESIS

Garetto L, Sanavio F, Severino A, Fubini L, Stacchini A, Aglietta M, Piacibello W  
*Dept of Biomedical Sciences and Human Oncology, University of Torino, Faculty of Novara, Italy*

The human homologue of the murine flt3/flk2 gene product is a tyrosine kinase receptor that plays a role in regulating the proliferation of the hematopoietic cells. Using different clonal assay systems (agar, methylcellulose, plasma-clot) and a long-term assay, we studied the effect of the recently cloned human flt3/flk2 ligand (FL), alone or in combination with other hematopoietic growth factors (HGF: IL-3, GM-CSF, G-CSF, SCF and EPO) on the growth of CFU-GM, BFU-E, CFU-MK and BFU-MK. Target cells were obtained from normal bone marrow (BM) donors, after Ficoll separation, immunomagnetic beads negative selection (Lin<sup>-</sup>) and positive selection for CD34 expressing cells. 2×10<sup>3</sup>Lin<sup>-</sup>CD34<sup>+</sup> BM cells were seeded per dish. FL revealed a potent stimulator of CFU-GM growth in the presence of GM-CSF, IL-3±SCF. By contrast it had no effect on BFU-E growth. The effect of FL on the primitive megakaryocyte (MK) progenitor cells, the BFU-MK and the more differentiated CFU-MK were determined: FL alone had no effect of both CFU-MK and BFU-MK growth; however it strongly enhanced the number and the size of MK progenitors in the presence of IL-3 (up to 200%) or GM-CSF (up to 158%). FL synergized with GM-CSF and IL-3 both at the CFU-MK and BFU-MK level. Moreover FL was capable of enhancing the growth of MK progenitors which was already maximally

stimulated by IL-3±SCF. FL alone exhibited limited potential in sustaining long-term megakaryocytopoiesis *in vitro*. It strongly augmented the ability of IL-3 and SCF alone, to promote long term megakaryocytopoiesis: the association of IL-3+FL was significantly more potent than that of IL-3+SCF in maintaining long term MK. These data indicate that multiple cytokines are necessary to optimally stimulate the proliferation of both classes of MK progenitor cells and that FL plays a significant role in this process by amplifying the MK-CSA of both GM-CSF, IL-3 and SCF.

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#### PLATELET-SPECIFIC ANTIBODIES IN ITP: COMPARATIVE EVALUATION BETWEEN CYTOFLUORIMETRIC (FCM) AND STANDARD FLUORESCENCE MICROSCOPE (LM)

Latorraca A, Lanza F, Ferrari L, Moretti S, Castagnari B, Galluccio L,\* Reverberi R,\* Castoldi GL  
*Institute of Hematology, and \*Blood Bank Unit, University of Ferrara, Italy*

Platelet antibodies (Plt Abs) research provides useful information on the causes of thrombocytopenic diseases. In a previous study, we employed a flow cytometry analysis to detect the presence of Plt Abs either in the serum or on the platelet surface from patients with ITP or other thrombocytopenic disorders. The aim of this investigation is to evaluate the presence of anti-plt-specific Abs in ITP patients. During the last 12 months we analyzed 22 ITP patients who were positive for plt Abs, with the aim of differentiating between anti-platelet-specific antibodies and class I HLA antigens by using citric acid and/or chloroquine agents. In the direct technique we used platelet suspension from EDTA-anticoagulated blood incubated with anti-human immunoglobulin reagent (B-cell SIg marker; Ortho), and plt from RBC group 0+ healthy donors as negative controls. Detection of serum anti-platelet antibodies (indirect technique) accomplished as follow: after fixation with PFD 1% in PBS, platelets from 0+ donors were exposed to patients and control sera, and stained with the same reagent. Serum from pts with transfused Cooley disease were utilized as positive control; as negative control we used serum from AB healthy volunteers. Samples were analyzed with a FACScan flow cytometer (B.D.) and with a standard light microscope (LM). In accordance with findings derived from LM methods, FCM analysis (for indirect technique) found the serum of 15/7 pts positive for Plt Abs. Surface Plt Abs (direct technique) showed a marked positivity for Plt Abs in 14/22 cases. Platelets of 4/22 cases were treated both with a pH 3.0 solution of citric acid - Na<sub>2</sub>HPO<sub>4</sub> buffer, or with 0.4 M solution of chloroquine diphosphate; then platelet were washed and fixed with PFD 1% as described above. Platelets from those 4 cases resulted all positive for surface platelet Abs while, after chloroquine and citric acid treatment platelets from 2/4 pts proved to be negative and the other 2 showed presence of surface platelet-specific Abs. Our data seem to support the reproducibility of the two techniques proposed, which were capable of differentiating between HLA and platelet-specific antibodies with FCM.

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### AN AMINO-ACID SUBSTITUTION (PHE55→SER) IN THE UNIQUE LEUCINE RICH MOTIF (LRM) OF GLYCOPROTEIN (GP) IX IS ASSOCIATED WITH BERNARD-SOULIER SYNDROME

Noris P,\* Simsek S, Stibbe J, von dem Borne AEGKr  
 \*Institute of Internal Medicine and Medical Oncology, University of Pavia-IRCCS San Matteo, Pavia, Italy. Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands

The Bernard-Soulier syndrome (BSS) is a rare congenital bleeding disorder caused by the absence and/or the dysfunction of the platelet GP Ib-IX-V complex, the major von Willebrand receptor on the platelet membrane. The complex consists of four distinct GPs: Ib $\alpha$  and Ib $\beta$  subunits are disulphide linked in GPIb heterodimer that is non-covalently bound either to GPIX and GPV. All components of the GPIb-IX-V complex belong to the family of GPs rich in LRM. Their function is not yet completely understood, but they are believed to be involved in molecule-molecule and cell-cell interactions. While several LRM are present in GPIb $\alpha$  and GPV molecules, a single motif is present in GPIb $\beta$  and GPIX. The GPIb-IX-V complex subunits are encoded by four different genes, all of them characterized completely and located on at least three different chromosomes.

Molecular genetic defects have been recently described in several BSS patients due to specific mutations in either the GPIb $\alpha$  or GPIX genes. We have investigated the molecular basis of the defect in a BSS patient suffering from hemorrhagic diathesis from his childhood; in a few occasions hospitalization was necessary but neither red cell nor platelet transfusions were required. Inter-marriage occurred between his parents who were first cousins and had a negative bleeding history. Flow cytometric analysis of the platelet membrane GPs showed that the surface expression of GPIb $\alpha$  and GPV was severely reduced. Three anti-GPIX monoclonal antibodies directed against different epitopes did not show any residual GPIX on the surface of the platelets from the patient. Normal expression of GPIIb-IIIa, Ia-IIa and IV was also shown. Genomic DNA was isolated from peripheral lymphocytes and GPIX gene was amplified by polymerase chain reaction. The entire coding sequence of the patient's GPIX gene was determined and the only difference with the known sequence was a homozygous T→C substitution predicting a Phe55→Ser substitution within the unique LRM presents on GPIX. By allele-specific oligonucleotide hybridization we confirmed the homozygosity of the patient and detected the heterozygosity of both unaffected children. In this patient, the association between BSS phenotype and homozygosity for the Phe55→Ser mutation suggests that the Ser55 might be responsible for an abnormal GPIX biosynthesis resulting in an altered expression of the entire GPIb-IX-V complex.

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### ACQUIRED RESISTANCE TO ACTIVATED PROTEIN C IN WOMEN TAKING ESTROGEN THERAPY

Guella A, Friso S, Olivieri O, Manzato F,\* Bernardi F,<sup>o</sup> Girelli D, Russo C, Azzini M, Corrocher R  
 Institute of Medical Pathology and \*Clinical Chemistry, University of Verona; <sup>o</sup>Department of Biological Chemistry, University of Ferrara, Italy

Recently a poor anticoagulant response to activated protein C has been recognized to be the most frequent marker of thrombophilia in patients with deep-vein thrombosis. Bertina R *et al.* (*Nature* 1994; 369:64-7) found that a significant number of subjects presenting a resistance to activated protein C (APC-R) carried a single point mutation in the factor V gene. Nevertheless, a percentage of individuals (~20%) with APC-R does not present the genetic defect suggesting that other factors capable to induce a poor anti-coagulant response to activated protein C may be involved.

*Aims of the study:* i) to evaluate the prevalence of APC-R in a population of young healthy women taking oral contraceptives (OC) compared with a control group without OC treatment; ii) to investigate a possible relationship between OC use and this new coagulation abnormality.

*Materials and methods:* 100 volunteers, healthy women (range of age: 18 to 40 yrs) were referred during a period between April and June 1994. Fifty women took OC with a content of estrogen less than 50  $\mu$ g during a period ranging to 3 months to 8 years and fifty controls matched for age, smoke-habit and the main clinical and biochemical parameters did not use OC. None of them took any other pharmacological therapy neither presented usual predisposing risk factors for thrombosis. The anticoagulant response to APC was measured with a modified version by the APC-dependent prolongation of the activated partial thromboplastin time-test (Coatest APC R -Chromogenix, Mölndal, Svezia) in an ACL-300 coagulometer (Instrumentation Laboratory, Milan, Italy). The results were expressed as a APC-sensitivity ratio defined as APTT (+APC) divided by the APTT (-APC). Resistance to APC was defined when ratio  $\leq$  2. Women presenting APC-R phenotype were studied for the genetic defect. Results: the mean values of APC-s-ratio in OC group were significant lower ( $P < 0.01$ ) than those observed in the other group; 8 women had a ratio  $\leq$  2 and the estimated prevalence of APC-R was 14% in OC group vs. 2% in the other. Checking again the APC sensitivity ratio we confirmed the APC-R in 6 of the 8 women; the only two subjects who resulted normally APC sensitive had discontinued the OC use for personal reasons. The genetic defect was found in the two subjects with the lowest APC ratio ( $< 1.85$ ): 1 of them was OC user while the other was non-user.

*Conclusions:* the OC use can induce a resistance to activated protein C when determined with a APTT dependent test.

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### ANTIPHOSPHOLIPID ANTIBODIES IN RETINAL VASCULAR OCCLUSION

Arcieri P, Coniglio M, Modrowska R, Di Nucci GD, Mariani G  
 Centro Trombosi, Dipartimento di Biopatologia Umana, Università "La Sapienza" di Roma, Italy

The primary antiphospholipid syndrome (PAPS) is characterized by venous and/or arterial thromboses and recurrent fetal losses, in the presence of the lupus anticoagulant (LA), elevated antibodies to cardiolipin (ACA) or both. We have performed an investigation to evaluate the relation between the APS and retinal vascular occlusion disorders. 52 consecutive patients (25 F, 27 M, mean age 45.5 y.), were screened for ACA and LA. ACA were detected by an enzyme immunoassay, LA was detected by Kaolin Clotting Time. 42 patients had central retinal vein occlusion (CVRO), 6 branch venous occlusion (BVO) and 4 arterial occlusion (RAO).

Our results showed that APS was present in the 48% of the patients with retinal vascular occlusion, 80% of them with central vein occlusion (see Table). Our results suggest that antiphospholipid antibody tests are useful in patients with retinal vascular occlusion, particularly in the case of venous occlusion, since this may have an impact on therapy and prevention of relapses, which are frequent.

	CVRO	BVO	RAO	TOTAL
PAPS	20	5	0	25 (48%)

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#### HEMOSTATIC MARKERS IN JUVENILE ISCHEMIC STROKE

Ruggeri M, Tosetto A, Castaman G, Rodeghiero F  
 Department of Hematology, San Bortolo Hospital, Vicenza, Italy

In the recent years conflicting results have been reported on the possible link between ischemic cerebrovascular disease and coagulation abnormalities in patients with age below 45 years. In this study, 23 young patients (mean age 31 years, range 18-45, males 8, females 15), with a previous diagnosis of ischemic stroke (confirmed by CT and/or nuclear magnetic resonance) have been evaluated. Patients with heart disease, dyslipidemia, hypertension, diabetes, chronic myeloproliferative disorders were not included. One patient was pregnant and one in puerperium; 5 patients were smokers (at least 10 cigarettes/die), 5 patients were on oral contraceptive at least 3 months, 1 patient was smoker and taking oral contraceptive). Venous blood samples were taken at least 3 months from the onset of symptoms for the following tests: PTT, PT, TT, KCT (for lupus anticoagulant, LA, diagnosis) and fibrinogen determination on ACL 300 R automated coagulometer (IL, Paderborn Dugnano, Italia); AT-III, protein C (PC), plasminogen (PLG), heparin cofactor II (HCII) (chromogenic assays), protein S (PS) level and antiphospholipid (APA) screening (ELISA), activated protein C resistance (aPCR). 1/23 was LA+, 3/23 were APA+, 1/23 was LA+ and APA+; AT-III (101%, 82%-121%), PC (100%, 67%-155%), PS (104%, 83%-125%), PLG (105%, 84%-143%), HCII (100%, 77%-126%), aPCR (mean ratio 3.46, 1.7-5.0) were in the normal range (reference range obtained from an ongoing epidemiological study, VITA Project, 4.703 determinations) in all the patients.

We concluded that juvenile cerebral thrombosis is not associated with inherited defects of natural coagulation inhibitors. The significance of the presence of LA and/or APA need to be clarified by prospective studies.

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#### HIGH-DOSE CHEMOTHERAPY IN MULTIPLE MYELOMA: RESIDUAL TUMOR CELLS ARE DETECTABLE IN PB AND BM CELL HARVESTS AND AFTER AUTOGRAFTING

Corradini P, Ladetto M, Astolfi M, Campana S, Comoglio F, Tarella C, Boccadoro M, Pileri A  
 Dipartimento di Medicina ed Oncologia Sperimentale, Divisione Universitaria di Ematologia, Az. Ospedaliera S. Giovanni, Torino, Italy

We have developed a novel PCR-based strategy to detect residual myeloma cells using clone-specific sequences derived from the rearranged variable regions (VDJ) of immunoglobulin heavy-chain (IgH) genes. VDJ's have been amplified from bone marrow (BM) RNA samples using sense primers derived from the leader, or first framework regions, and an antisense primer derived from the six joining regions. Tumor-specific oligonucleotides have been generated from the second and third complementarity determining regions (CDR2 and CDR3). Residual disease has been evaluated by amplifying RNA samples with primers derived from the CDR2 and IgH constant region. CDR3 has been used to probe amplified DNAs. The presence of residual myeloma cells has been evaluated in 17 of 39 patients enrolled in pilot HD programs. Thirteen patients were enrolled in a program involving the administration of etoposide 2 g/sm, methotrexate 8g/sm or mitoxantrone (escalating doses from 30 to 60 mg/sm), and cyclophosphamide 7 g/sm (followed by 2-4 leukaphereses) followed by a submyeloablative phase with melphalan 120 mg/sm, and fractionated total body irradiation (10 Gy). Four patients have been enrolled in a novel double-transplant study in which mitoxantrone has been replaced by cyclophosphamide 5g/sm, and the conditioning regimen consists of melphalan 200 mg/sm (Tx1) followed 60-90 days later by mitoxantrone 60 mg/sm and melphalan 180 mg/sm (Tx2). Molecular monitoring of the disease was performed in 17 patients: 16 autologous, and one allogeneic BMT (7 were in CR with disappearance of monoclonal protein in immunofixation). All PBPC and BM harvests contained residual myeloma cells. In addition, myeloma cells were detectable after single or double transplantation in all patients. Our findings indicate that HD chemotherapy and even double transplantation are unable to achieve molecular remission.

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#### AUTOLOGOUS PERIPHERAL BLOOD STEM CELLS (PBSC) TRANSPLANTATION AS FIRST LINE TREATMENT FOR MULTIPLE MYELOMA: MULTICENTRIC STUDY

Tribalto M, Bugliosi R, Caravita T, Cudillo L, Del Poeta G, Avvisati G, La Verde G, Meloni G, Leone G, Menichella G, Martelli M, Tabilio A, Fioritoni G, Iacone P, Majolino I, Marcenò R, Amadori S, Mandelli F, Papa G  
 A GITMO (Italian Group Bone Marrow Transplantation) Study

Starting from May, 1991, 51 untreated myeloma patients entered a multicentric pilot study in 6 Italian centres: 2-3 monthly cycles of VAD followed by CY, 7 g/sm + G-CSF (Granulokine, Roche) 5 mcg/kg b.w./day c.i. for 14 consecutive days, to mobilize and collect PBSC. The subsequent conditioning regimen was melphalan (60 mg/sm) + busulfan (16 mg/kg/ t.d.) followed by G-CSF. As maintenance R- $\alpha$ 2 IFN was given, at the dose of 3 M.U. t.d. 3 times a week, until relapse. The median age was 49 (33-61), 66% of patients had bone lesions, the median value of s $\beta$ 2 microglobulin was 7.1 mcg/mL (1.0-41.3). After a median follow-up of 29 months, 49 patients received at least 2 cycles of VAD, 40 were submitted to PBSC collection, 38 received the conditioning regimen plus PBSC and 26 of them are in the maintenance phase with IFN. Eight patients progressed during the VAD (6)-HDCY (2) phase. All the 38 patients evaluable for PBSC showed at least an objective response, with 14 (37%) CR, (disappearance of serum MC by immunofixation and <5% plasmacells in the bone marrow). Considering all the 51 enrolled patients for an intention to treat evaluation, responding patients are 74.5% with 27.4% achieving a CR. White cells and platelets rose to >1000/mm<sup>3</sup> and >50.000/mm<sup>3</sup> after a median period of 8 and 9 days, from CY, and 8 and 10 days from transplant, respectively. 12 patients have relapsed, 2 other died while in PR (+70, +155) because of CMV hepatitis and candida pneumonia. The median number of CD34<sup>+</sup> cells and CFU-GM was 24.75 $\times$ 10<sup>6</sup>/kg b.w. and 28.1 $\times$ 10<sup>7</sup>/kg b.w., respectively. In conclusion this treatment appears to be feasible with low toxicity, but a longer follow-up in a prospective randomized study is needed to evaluate the possible role on survival duration of the more aggressive regimens for treatment of myeloma patients.

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#### AL AMYLOIDOSIS: UPDATE OF A MULTICENTER STUDY

Merlini G, Anesi E, Banfi G, Baraldi A, Bergesio F, Bertani T, Bucalossi A, Cavallero G, Conte N, De Luca M, Di Girolamo M, Fabris A, Faedda R, Fregoni V, Frosi A, Furci L, Girelli D, Grassi C, Iannaccone S, Imbasciati E, Loni C, Manganaro M, Manno C, Marcenò R, Marenzi V, Marinone MG, Marzani F, Melis P, Miliani A, Montanaro M, Musto P, Pannacciulli I, Pecchini F, Perfetti V, Pintus F, Pozzi C, Resegotti L, Schena FP, Uziel L, Visconti G, Ascari E

*Italian AL Amyloidosis Study Group; Internal Medicine and Medical Oncology, IRCCS, Policlinico S. Matteo, University of Pavia, Italy*

AL amyloidosis is a rare disease characterized by extracellular deposition of fibrils with typical structural features composed predominantly of monoclonal light chains. A study protocol was started in 1988 with the aim of gathering a scattered patient population and unifying the diagnostic and therapeutic approach to AL amyloidosis.

In this work we report the results of 125 evaluable patients with AL amyloidosis referred to the coordinating Center in Pavia in the period June 1988-December 1994 by

38 Centers.

In all patients the diagnosis was established by biopsy: fine-needle abdominal fat aspiration in 19 patients, tissue biopsy in 34, both techniques in 72. Median follow-up time was 24 months (range 6-78); male/female ratio was 1.2 and median age 59 years (range 34-85). Estimated median survival (Kaplan and Meier) was 40 months. The monoclonal component was IgG in 49%, IgA in 8%, IgM in 3%, light chain only in 40%, with preponderance of lambda chains (lambda/kappa ratio: 2.2). Clinically overt organ involvement in descending order of frequency included: kidney (66%), GI (24%), heart (23%), peripheral nerves (16%), liver (14%), skin (6%), spleen (5%), pulmonary tract (4%). A single organ was involved in 30%, two organs in 46%, three or more organs were affected in 24% of cases.

Ninety-seven patients were treated with melphalan and prednisone for at least six months: 38 of these (39%) benefited with an important ( $p < 0.001$ ) improvement in survival (median value >120 months vs 14 months). The impact of prognostic factors has been evaluated using Cox univariate and multivariate hazard model. Congestive heart failure was the most powerful prognostic variable followed by performance status, hepatomegaly and number of organs involvement (disease extension).

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#### MULTIPLE MYELOMA: TOXICITY AND EFFICACY EVALUATION OF AN INTENSIFIED INDUCTION TREATMENT

Frieri R, Boccardo M, Gallone G, Argentino C, Avvisati G, Baldini L, Bidoli P, Capaldi A, Callea V, Cimino R, Comotti B, De Renzo A, Dominiotto A, Falini B, Gallamini A, Iannitto E, Lauta VM, Liberati M, Marcenò R, Marmont F, Molica S, Musto P, Palumbo A, Tribalto M, Pileri A, Mandelli F

*Italian Multiple Myeloma Study Group (IMMSG), Italy*

In 1990 the IMMSG started the M90 protocol to evaluate toxicity and efficacy of a real intensification of induction treatment. Patients were stratified by stage and randomized to receive alternating cycles of DAV (*dexamethasone, doxorubicine and vincristine*), IDCTX (*intermediate dose cyclophosphamide*) and CED (*cisplatin, etoposide and dexamethasone*) or standard MP chemotherapy. Alternating induction treatment was administered at 28-day interval. After 3 cycles (DAV, IDCTX, CED), patients who did not progressed received further 3 alternating cycles. MP was administered at 28-day interval for a total of 6 cycles. Patients who obtained an objective response or had a stable disease at the end of induction treatment were stratified by induction treatment and randomized to  $\alpha$ -interferon alone (IFN) or  $\alpha$ -interferon plus dexamethasone (IFN+DEX) until relapse. At diagnosis, the major prognostic factors were evaluated, including labelling index and  $\beta$ 2-microglobulin.

Four hundred twenty five consecutive patients from twenty Italian hematological centers entered in this protocol from October 1990 to December 1994. The analysis was performed on 361 patients, 182 patients were treated with alternating regimen (EXP), and 179 with MP. 199 patients entered maintenance regimens. Objective

response was observed in 74/129 (57.3%) patients treated with alternating chemotherapy and in 84/144 (61.1%) with MP. Surprisingly, response rate to MP was higher than that observed in all randomized studies carried out of the last fifteen years. Alternating chemotherapy was highly effective and the response rate was in the range of the best recent regimens. A further reduction of M-component (25%), was achieved by IFN+DEX therapy, in 20% patients ( $p < 0.02$ ).

Statistical analysis was performed in the four groups of treatment induction/maintenance: EXP/IFN, EXP/IFN+DEX, MP/IFN and MP/IFN+DEX. Remission duration was 15, 19, 23, 27 months respectively. Longer remission duration was obtained with MP/IFN+DEX, shorted with EXP/IFN (27 vs 15 months- $p < 0.01$ ). Remission duration was not statistically different in the other treatment groups: this feature can be attributed to the short follow-up. For the time being, the analysis of survival patients randomized in the four groups of treatment is not available. Finally, an intensified induction treatment does not appear to be superior to MP. This interim analysis showed that the addition of DEX to IFN maintenance therapy prolongs response duration.

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#### SEQUENTIAL CYCLOPHOSPHAMIDE-MELPHALAN (CM) WITH NON-CRYOPRESERVED CIRCULATING PROGENITOR CELL SUPPORT FOR REFRACTORY MYELOMA

Boccardo M, Palumbo A, Bruno B, Omedè P, Argentino C, Dominietto A, Triolo S, Pileri A  
Dipartimento di Medicina ed Oncologia Sperimentale, Divisione Universitaria di Ematologia, Azienda Ospedaliera S.Giovanni, Torino, Italy

In the attempt to intensify salvage treatment in refractory multiple myeloma we conducted a pilot study with sequential cyclophosphamide-melphalan (CM) with circulating progenitor cell (CPC) support. The protocol included: cyclophosphamide (2-3 g/m<sup>2</sup>, day 0), G-CSF (10 µg/kg, days 3-9) and melphalan (60 mg/m<sup>2</sup>, day 11) with CPC collected at day 10, kept at 4 °C for 48 hours and reinfused unprocessed at day 12. Twenty-three consecutive refractory MM patients (10 primary resistant, 7 resistant relapse, 6 relapsed) enter the study. This regimen was delivered on an out-patient basis. An adequate CPC harvest with a single leukapheresis (reinfused CD34<sup>+</sup> cells: 2.92×10<sup>6</sup>/kg; CFU-GM: 15×10<sup>4</sup>/kg) allowed a median duration of severe neutropenia (neut <500/µL) and thrombocytopenia (plts <25000/µL) of 5 and 2 days, respectively. 4/23 patients were admitted for broad-spectrum antibiotic therapy for major infections (2 pneumonia, 1 necrotizing fasciitis, 1 enteritis).

All patients were eligible for a second course, that has been delivered to 11 patients so far, without increasing toxicity. After the first course, monoclonal immunoglobulin reduction > 50% was seen in 65% (15/23) of patients, > 75% in 22% (5/23), complete disappearance in 17% (4/23); the second course further increased

response: > 50% in 82% (9/11) of patients, > 75% in 55% (6/11), complete disappearance in 36% (4/23). All patients except one are alive after a median follow-up of 10.5 months. In conclusion, this out-patient procedure with non-cryopreserved CPC was shown safe, cost-efficient and clinically effective. Adequate CPC can be harvested with a single leukapheresis reducing toxicity to that observed in conventional chemotherapy regimens.

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#### HIGH PREVALENCE OF HEPATITIS C VIRUS INFECTION IN IGM MONOCLONAL GAMMOPATHIES

Sampietro M, Baldini L,\* Corbetta N, Colombi M,\* Brunetti E, Salvadori S, Brianzoli V,<sup>o</sup> Fiorelli G, Maiolo AT\*

Ist. Medicina Interna e Fisiopatologia Medica, \*Serv. Ematologia, Ist Scienze Mediche, Osp. Maggiore IRCCS, Università Studi di Milano, <sup>o</sup>Lab Analisi, C.d.C. S.Rita, Milan, Italy

Hepatitis C virus (HCV), the major etiologic agent of post-transfusion and sporadic non-A, non-B hepatitis, has been found in close association with cryoglobulinemia, usually type II, characterized by the presence of a monoclonal IgM with rheumatoid activity. This observation prompted us to investigate the possible role of HCV in other IgM lymphoproliferative disorders in the absence of cryoglobulinemia. We examined 43 subjects (age range 44-87y) with IgM monoclonal disorders negative for cryoglobulins, 22 with monoclonal gammopathy of undetermined significance (MGUS), 11 with Waldenström's macroglobulinemia (WM) and 10 with a previously undiagnosed IgM monoclonal component occasionally found at electrophoresis. HCV antibodies (HCV-Ab) were detected with a second-generation enzyme-linked immunoassay and HCV-RNA by single-tube heminested PCR in the 5' untranslated region (5'UTR) of the viral genome. HCV typing was performed by enzyme restriction of the amplified 5'UTR and subtypes denominated according to the classification proposed by Simmonds *et al.* HCV-Ab were detected in 6 patients (13.9%) and HCV-RNA in 14 (32.5%). Nine patients have evidence of viremia in the absence of detectable antibodies directed against HCV. HCV infection was more frequently found in MGUS and in individuals with previously undiagnosed occasionally found IgM monoclonal components than in WM. Only 3 patients have clinical or biochemical evidence of chronic liver disease and, among risk factors, no blood transfusions of i.v. immunoglobulin therapy are reported for patients with positive HCV markers. Only HCV type 1b was observed among 9 patients so far characterized. In conclusion, a high prevalence of HCV infection is described in patients with IgM monoclonal disorders, confirming a possible role of this virus in inducing B-cell proliferation. A high proportion of viremic patients test negative at standard diagnostic assays; whether this represents a peculiarity of their immune response associated, for instance, to the reduced levels of polyclonal immunoglobulins, or an artifactual interference in antibody detection remains to be assessed.

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**COLLAGEN CROSS LINKS: SKELETON HOMEOSTASIS MARKERS IN MULTIPLE MYELOMA**

Fremiotti A, Persiani M, Mehraban M, Bartolini M, Iacovino P, Ronci B

*Divisione di Ematologia, Laboratorio di Patologia Clinica, Ospedale San Giovanni, Rome, Italy*

Increased osteoclastic activity in multiple myeloma (MM) can induce serious anomalies of skeleton homeostasis. On diagnosis 72% of the patients have a profound unbalanced ratio in skeleton reabsorption/remodelling with significant mass reduction (unbalanced MM), 20% have normal balance (balanced MM) even if with increased turnover, while only 8% (probably patients of the indolent clinic variety) have a MM without skeletal anomalies (Bataille *et al*). Skeleton radiology only shows the most evident clinical lesions while among the osteoclast activity biochemical markers, urinary hydroxyprolin is the most used but it has obvious limits of usefulness. In order to investigate more reliable skeletal reabsorption markers, we are studying the piridinium cross links (hydroxypyridoline, HP, and lysilpyridoline, LP). Piridinium is an amino-acid which makes up covalence bonds between collagen adjacent chains. For this purpose we are adopting a liquid chromatographic method which is not difficult to carry out. HP, although present in many connective tissues is prevalent in the skeleton and in the cartilage, while LP as well as being in the skeleton is only in negligible amounts in the dentine. Moreover, these compounds result only from mature collagens and not neofomed ones. In Table 1 the basal values observed by us in healthy subjects are as shown:

Table 1. Piridinium cross-links. Values in healthy subjects (expressed in  $\mu\text{mol}/\mu\text{mol}$  of creatinine)

	Age	HP	LP
Females	30-50	45-95	9-20
	50-60	50-110	10-22
Males	30-60	38-90	5-19
	60-70	40-130	6-21

We observed these values in 33 MM patients under test: HP range 116-273, LP range 19.1-49, median 31; in three indolent myeloma patients the values were: HP 65, 66, 68. In three MGUS patients: HP 90, 97, 110; LP 16, 17, 22 respectively. These series are still too limited to draw a conclusion but we think the data of this in progress study point out the clinical utility of these osteoclastic markers in the differential diagnosis of MM, indolent myeloma and MGUS.

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**ATYPICAL CASE OF PLASMA CELL NEOPLASIA: COEXISTENCE OF BONE MARROW AND ADRENAL GLAND INVOLVEMENT AT DIAGNOSIS**

Bandieri E, Luppi M, Torelli G, Maiorana A,\* Sabbatini R, Federico M

*Department of Medical Sciences. Section of Oncology and Hematology; \*Department of Pathology. University of Modena, Italy*

The occurrence of a plasma cell infiltration in the adrenals is an extraordinary event, observed only with widespread, long lived bone marrow disease and very rarely recognized in life. We report here the atypical case of a non-secreting plasma cell tumor with a unique clinical feature, represented by the coexistence of bone marrow involvement and of a single osteolytic lesion of the rotula in association with the left adrenal gland localization at diagnosis. The patient achieved a partial clinical remission following chemotherapy, still persisting after 16 months from diagnosis. We describe here the unusual biologic properties of the neoplastic plasma cell population, which might account for the exceptional clinical presentation of the disease. Plasma cell population in the bone marrow showed, at diagnosis, an extraordinary high labeling index (LI%: 6.15), evaluated by the bromodeoxyuridine immunofluorescence technique. A strong nuclear p53 immunoreactivity was demonstrated in about the 60% and the 20% of the neoplastic cells in the left adrenal gland and the bone marrow respectively, suggesting that p53 aberration might have conferred an atypical growth behavior to plasma cell population. The plasma cell population infiltrating both the bone marrow and the left adrenal gland showed a diffuse cytoplasmic staining for lambda light chains. Of interest, an identical  $\lambda$  light chain gene rearrangement was simultaneously detectable both in the bone marrow and in the peripheral blood mononuclear cells, suggesting that circulating B lymphocytes are part of the malignant clone and represent the putative neoplastic cells responsible for dissemination of the disease to atypical sites like adrenal gland. Interleukin-6 (IL-6) serum levels measured twice daily for three days, by a sensitive ELISA (Quantikine) technique, were invariably low. The high percentage of myeloma cells infiltrating the bone marrow, the almost complete absence of synthetic rates of monoclonal protein, the high LI% and the high tumor burden, as determined by a high serum  $\beta$ 2-microglobulin, are consistent with the undifferentiated state of tumor plasma cell population, which may account for the ability to disseminate to an unusual site, like the left adrenal gland. The low levels of IL-6 secretion may be associated with the presence of an undifferentiated tumor plasma cell compartment unable to progress into terminal differentiation, in absence of the appropriate cytokine stimulus.

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**INTERFERON  $\alpha$ -2B MAINTENANCE THERAPY IN PATIENTS AFFECTED BY LOW RISK MULTIPLE MYELOMA: A RANDOMIZED COMPARISON OF TWO SCHEDULES**

Offidani M, Olivieri A, Tedeschi A, Rupoli S, Centurioni R, Alesiani F, Marchegiani G, Pieroni S, Catarini M, Pelliccia G, Altiglia F, Leoni P for the Gruppo Marchigiano per lo Studio del Mieloma Multiplo

Maintenance therapy with interferon (IFN) significantly prolonge response duration but only occasionally overall survival in patients with multiple myeloma (MM)

responding to induction chemotherapy and with disease in plateau phase. Hence the role of IFN in the remission phase of MM is still an open question particularly about the schedule and the subset of patients who could benefit by this approach. This multicenter, prospective, randomized study attempted to assess the better schedule of IFN administration in the maintenance treatment of MM in plateau phase in terms of toxicity, progression free survival (PFS) and overall survival (OS); the second aim was to assess factors affecting the length of plateau phase and survival during IFN therapy.

We enrolled 52 patients affected by low-risk MM according to Bataille *et al.* staging system i.e. patients with serum  $\beta 2$ -microglobulin ( $s\beta 2m$ ) < 6.0 mg/L and serum albumin (sA) > 3.0 g/dL; patients were randomly assigned to receive IFN $\alpha$ -2b (Intron-A Schering-Plough Corp.) subcutaneously 3 megaunits (MU) three times a week (27 patients: group A) or 3 MU/day (25 patients: group B) until disease progression. The patients with stage I MM and stable disease at least for 3 months, were just treated with IFN $\alpha$ -2b whereas patients with evolutive disease were treated with IFN $\alpha$ -2b after obtaining a plateau phase by chemotherapy (almost 6 courses). As of march 1995, with a median follow-up period of 36 months (range 12-71.7), 31 patients (59.6%) had disease progression, 21 (77.8%) in the group A and 10 (40.0%) in the group B ( $p = 0.01273$ ). Total median PFS was 24.8 months; median PFS was 11.9 months and 38.3 months in group A and B respectively ( $p = 0.0038$ ). Eighteen patients (34.6%) died at the time of this report, 11 (40.7%) in the group A and 7 (28%) in the group B ( $p = 0.50084$ ). All patients in the group A died because of disease progression; on the contrary, in the group B, 3 patients died on stable disease. Total median duration of survival was 63.2 months; survival was 63.2 months in group A and 61.9 months in group B ( $p = 0.489$ ). Univariate analysis showed that performance status, hemoglobin,  $s\alpha 2m$ , sA, disease status at random, were significantly predictive of the PFS duration; on the contrary age, sex, stage, bone marrow plasma cells, platelets and LDH were not predictive.

Stepwise Cox analysis showed that only  $s\alpha 2m$  and sA were predictive of PFS. Introducing IFN $\alpha$ -2b schedule in the stepwise analysis, it resulted predictive ( $p = 0.0056$ ) but  $s\beta 2m$  and sA held highly predictive (respectively  $p < 0.001$  and  $p = 0.0044$ ). Stratifying randomized groups using the cut-off of  $s\beta 2m$  (3.0 mg/L), the patients treated with IFN $\alpha$ -2b 3 MU/day had longer PFS than expected in both entire groups, whereas it did not happen in the patients treated with IFN $\alpha$ -2b 3 MU three times a week. Multivariate analysis showed that only  $s\beta 2m$  was significantly predictive for survival ( $p = 0.0059$ ).

Finally, toxicity of IFN $\alpha$  resulted severe particularly in older patients in which a dose reduction or therapy discontinuation was frequently constrained.

Forty-three patients (82.7%) had side effects without any difference between the two groups. Seventeen patients (32.7%) discontinued therapy because of severe side effects without any difference between the two groups; the more frequent reasons for therapy discontinuation were anorexia and weight loss. Sixteen patients (30.8%) reduced IFN $\alpha$ -2b dose because of side effects without any difference between the two groups; major reasons of dosage reduction was grade II-III neutropenia. Our results showed that into low-risk MM category two

populations with very different prognosis should be considered. In our opinion, patients younger than 65 years and  $s\beta 2m > 3.0$  mg/L should be treated soon after diagnosis and considered for high-dose therapy because maintenance therapy with IFN $\alpha$  cannot substantially change their bad prognosis.

All the other patients probably could get advantage from the IFN $\alpha$  administration but only if this drugs will be administered 3 MU/day.

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### VINCRIStINE, CYCLOPHOSPHAMIDE, ESCALATING DOSE OF EPIRUBICIN, DEXAMETHASONE+rhG-CSF (VCED) FOR PBSC MOBILIZATION IN MULTIPLE MYELOMA

Vasta S, Morabito F,\* Pucci G,\* Marcenò R, Scimé R, Indovina A, Buscemi F, Pampinella M, Catania P, Fiandaca T, Majolino I

Dept. of Hematology and BMT unit, Ospedale Cervello, Palermo; Dept. of Hematology, BMT center, OORR, Reggio Calabria, Italy

In multiple myeloma (MM) PBSC mobilization regimens have included high-dose drugs as well as multiple-drug schedules±hemopoietic growth factors. In the present study escalating doses of epirubicin (EPI 2×60 mg/m<sup>2</sup>, 2×70 mg/m<sup>2</sup>, 2×80 mg/m<sup>2</sup>) in combination with VCR 2 mg, CTX 4×0.5 g/m<sup>2</sup>, DEXA 4×40 mg + rhG-CSF 5 mcg/kg/die was employed in 6 MM patients. EPI substitutes for adriamycin 2×50 mg/m<sup>2</sup> in the prior regimen VCAD because its minor cardiotoxicity. Three patients were Stage III, 2 Stage II and 1 Stage I. Their median age was 52 yrs (range 34-55). All patients were responsive after 1 line of therapy: 4 were in I PR, 2 were in I CR.

	days of PMN < 0.5	days of PLT < 50.0	Cells collected	
			CFU-GM x10 <sup>6</sup> /kg	CD34 x10 <sup>6</sup> /kg
EPI 2x60 n. pz. 2	1/3	0/0	35.9/78.8	NV/6.9
EPI 2x70 n. pz. 2	3/4	0/4	11.5/83.3	7.1/10.4
EPI 2x80 n. pz. 2	4/5	3/3	76.6/132.1	23.4/23.6
median	3.5	1.5	77.7	10.4

In all patients hematologic toxicity was WHO grade 4, with an increase of days of cytopenia proportionate to EPI dose. No cardiotoxicity was observed. The apheresis program (3 consecutive apheresis) started when WBC count increased to  $GR > 5.0 \times 10^9/L$ , at median day 13 (range 12-14) from chemotherapy. Escalating dosage of EPI increased days of cytopenia but better CD34 yields were obtained. With this scheme, also at higher dose of EPI, the length of cytopenia was comparable to that of VCAD but higher WBC count was observed the day of first apheresis (9.8 vs.  $3.5 \times 10^9/L$ ,  $p = 0.01$ ). In addition, better CFU-GM yields were obtained (69.7 vs.  $22.5 \times 10^6/kg$ ,  $p = 0.03$ ). The combination of EPI with VCR, CTX, DEXA+rhG-CSF is tolerable even for dose of 2×80 mg/m<sup>2</sup>. Mobilizing effect of prior scheme VCAD is increased by substitution of ADM by EPI.

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**ACTIVE IMMUNOTHERAPY IN MULTIPLE MYELOMA: PREPARATION OF ANTI-IDIOTYPIC VACCINES**

Battaglio S, Napoli P, Ciaiolo C, Bianchi A, Boccadoro M, Pileri A, Massaia M

*Divisione di Ematologia dell'Università di Torino, Dipartimento di Medicina e Oncologia Sperimentale, Azienda Ospedaliera S. Giovanni di Torino, Italy*

A number of experimental models have shown that T cells play a major role in antitumor responses. Unfortunately, the generation of appropriate responses *in vivo* is impaired by the low immunogenicity of tumor cells, the production of suppressive factors, and the possible activation of suppressor cells. To induce antitumor activity, it is necessary: (i) to modify tumor-host interactions so that T cells can recognize and be activated by tumor cells; (ii) to have a tumor-specific antigen available. In multiple myeloma (MM), antigenic determinants [idiotypes (Id)] of hypervariable regions derived from M proteins (MP) can be used as tumor-specific targets. It is necessary, however, to arrange an appropriate presentation to T cells. Experimental models have shown that Ids need to be conjugated to strong protein carriers such as KLH and emulsified in adjuvants to achieve an immunogenic presentation. Based on these data, we have set up a standard procedure to prepare clinical grade anti-idiotypic vaccines. They are prepared through a 3 steps procedure:

1. isolation and purification of MP from serum or urines;
2. conjugation to KLH;
3. achievement of safety and quality standards for clinical use (absence of bacteria, fungi, and mycoplasma contamination; endotoxin levels < 300 EU/mL). The vaccines have recently entered clinical trials and proved safe and devoided of side effects.

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**CIRCULATING PROGENITOR CELLS (CPC) MOBILIZATION CELLS AND SELECTION OF CD34+ CELLS FOR CLINICAL USE**

Cottafavi L, Carlo-Stella C, Mangoni L, Garau D, Dotti GP, Caramatti C, Almici C, Cocchi T,\* Sassi M,\* Talarico E,\* Rizzoli V

*Department of Hematology, Bone Marrow Transplantation Unit, University of Parma, Parma Italy; \*Blood Center, Parma, Italy*

The aim of this study is to report our data on the mobilization of circulating progenitor cells (CPC) induced by chemotherapy and/or growth factor and selection of CD34<sup>+</sup> cells by CEPRATE® SC Stem Cell Concentrator. Sixteen patients were included: 8 multiple myeloma (MM) and 8 non Hodgkin lymphoma (NHL). The median age was 49 years (range 41-64). Ten patients (Group 1) were mobilized with cyclophosphamide (7 gr/m<sup>2</sup>) and G-CSF (5 µg/kg/die s.c.) while 6 patients (Group 2) with G-CSF (10 µg/kg/die s.c.) alone. Eleven patients were pretreated with polichemotherapy regimens (median number of cycle = 8). Patients in Group 1 underwent a median of 3 apheresis

(range 2-4), and patients in Group 2 a median of 2 apheresis (range 1-3). 3.16×10<sup>8</sup>/kg (range: 1.6-5.7×10<sup>8</sup>/kg) nucleated cells and 6.4×10<sup>6</sup>/kg (range: 2-11.4×10<sup>6</sup>/kg) CD34<sup>+</sup> cells were harvested and cryopreserved in Group 1 patients, while 7.06×10<sup>8</sup>/kg (range: 2.8-10.5×10<sup>8</sup>/kg) nucleated cells and 6.9×10<sup>6</sup>/kg (range: 4-10.1×10<sup>6</sup>/kg) CD34<sup>+</sup> cells in Group 2 patients.

In 6 patients with multiple myeloma the apheresis product was submitted to CD34<sup>+</sup> cells selection by CEPRATE® SC Stem Cell Concentrator. Following incubation with a biotinylated IgM antibody anti-CD34 (clone 12.8), cell suspension was introduced into an immunoabsorption column filled with avidin-coated beads. After positive selection 1.98×10<sup>6</sup> cells/kg were recovered (cells recovery = 0,64%) with cell purity of 52%±27% and 20×10<sup>4</sup> CFU-GM/Kg (recovery 54%±28%). The enrichment of CD34<sup>+</sup> cells reduced the percentage of CD19<sup>+</sup> cells of 2-5 logarithms. In conclusion our data demonstrated: 1) the possibility to mobilize an adequate number of CPC in heavily treated patients, 2) the capacity of G-CSF to increase the number of circulating nucleated and CD34<sup>+</sup> cells without chemotherapy, 3) the significative reduction in lymphoid contamination after CD34 positive selection.

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**TUMORAL PLASMA CELLS EXPRESS SURFACE MOLECULES THAT CAN DIRECTLY MODULATE T CELL IMMUNITY***Dipartimento di Medicina ed Oncologia Sperimentale, Divisione di Ematologia dell'Università di Torino, Ospedale Molinette, e Dipartimento di Scienze Mediche, Laboratorio di Immunologia, Università di Torino, sede di Novara, Italy*

In multiple myeloma (MM) patients, various alterations of T cell immunity have been previously described. Among those, within both CD4<sup>+</sup> and CD8<sup>+</sup> populations there is an expansion of HLA-DR<sup>+</sup> and CD11b<sup>+</sup> cells. It has also been described that the potential anti-tumor activity of T cells in these patients is preceded by activation and expression of surface molecules such as CD25 that can indicate a recent functional activation, whereas HLA-DR expression alone is a marker of a *chronic activation* status. MM plasma cells (PC) can express surface molecules that can potentially interact with T cell immunity. Particularly, PC express CD40 which is a normal ligand of CD40L on activated T cells; they can also express CD28 that can compete with the same molecule on T cell surface, and CD56 that is normally expressed by NK cells and by a T cell subpopulation. We analyzed the median expression intensity of these molecules on PC of 20 MM patients (determined by the linear median of the cytofluorimetric signal). We simultaneously evaluated the percentage of T cells expressing HLA-DR, as a *chronic activation* marker, and CD25 as a *recent activation* marker. MM patients with low CD40 intensity on their PC had an increased percentage of T cell expressing CD25 compared to patients with high CD40 intensity (cut off = 60, range 2.3-107, CD4<sup>+</sup>: CD25+ 27% vs. 12.8%, CD8<sup>+</sup>: CD25 6.8% vs. 2.6%) but no difference was found in HLA-DR percentage. A similar result was obtained analyzing CD28 intensity (cut off = 10, range 1-72.3) where patients having a low PC CD28 intensity expressed a higher per-

centage of CD25<sup>+</sup> cells (CD4<sup>+</sup>: CD25<sup>+</sup> 24.1% vs. 12.2%, CD8<sup>+</sup>: CD25<sup>+</sup> 7.5% vs. 3.4%). On the contrary, CD56 intensity showed no relationship with T cell activation (cut off = 100, range 1-2158). These data suggest that MM PC can effectively modulate T cell immunity, possibly by providing inhibitory signals. These signals may act both by an excessive or a non-physiologic stimulation by the CD40 molecule, and through a competition for the CD28 ligands.

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#### DIAGNOSTIC APPROACH TO AND FOLLOW-UP OF DIFFICULT AL AMYLOIDOSIS CASES

Perfetti V, Merlini G, Garini P, Colli Vignarelli M, Marinone MG, Ascari E  
*Internal Medicine and Medical Oncology, University of Pavia, IRCCS Policlinico S. Matteo, Pavia, Italy*

Routine electrophoretic analysis fails to detect a monoclonal component (MC) in a considerable portion of AL amyloidosis patients. Demonstration of clonal excess in bone marrow plasma cells (BMPC) by calculating the intracytoplasmic light chain k/λ ratio may confirm diagnosis in these cases. We performed BMPC k/λ ratio analysis and immunofixation (IF) on high resolution agarose gel electrophoresis of serum and urine in 16 selected patients with no detectable MC at routine analysis, despite clinical features suggestive of primary amyloidosis. Abnormal k/λ ratios were found in 14 (sensitivity 87.5%), and a MC in 12 patients (sensitivity 75%). Combination of the two analyses confirmed diagnosis in all cases. In one patient changes in the size of the clone, monitored on serial bone marrow aspirates by a monoclonal anti-idiotypic antibody specific for the amyloidogenic immunoglobulin, paralleled variations of the k/λ ratio. In conclusion, this study demonstrates that the combined use of IF on agarose gel electrophoresis and the BMPC k/λ ratio is a powerful diagnostic tool in AL amyloidosis. In addition, the BMPC k/λ ratio should be considered for monitoring the amyloidogenic clone when serum or urine MC is not quantifiable.

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#### DISAGGREGATION OF AL AMYLOID FIBRILS WITH α<sub>1</sub>-ANTI-TRYPSIN

Merlini G, Janciauskiene S,\* Eriksson S\*  
*Laboratorio di Tecnologia Biomediche e Biotecnologie, IRCCS Policlinico S. Matteo, Pavia, Italy; \*Department of Medicine, Lund University, Malmö General Hospital, Malmö, Sweden*

Amyloidosis is characterized by deposition of fibrillar autologous proteins with a β-pleated sheet conformation determining organ damage and death. The β-structure is the basis for congophilia, the insolubility and the relative resistance of the amyloid fibrils to proteolytic digestion. Protease inhibitors, except α<sub>1</sub>-antitrypsin (AAT), appear to be intimately associated with light-chain-derived (AL)

amyloid fibrils. Previous studies have shown that the interaction between AAT and hydrophobic compounds, like cholesterol, is associated with a conformational transition of AAT from the stressed, active, to the relaxed, inactive, form with loss of its biological activity. These observations led us to study the putative interaction between AAT and the insoluble, hydrophobic, β-pleated sheet, AL fibrils. Amyloid fibrils were isolated from seven cases with I and four cases with k AL amyloidosis. The fibrils were incubated with electrophoretically pure AAT in the molar ratio 5:1 in 0.1 M Tris buffer pH 7.4 containing 0.15 M NaCl at room temperature for varying periods of time (2-236 h). The λ fibrils could be completely disaggregated, as shown by light and electron microscopy and Congo red uptake, by the addition of AAT, whereas k fibrils remained unaffected. The λ fibrils-AAT interaction determined characteristic changes of the physicochemical and biological properties of AAT apparent in an increased thermal stability and loss of elastase-inhibitory activity. These findings are compatible with a transition of AAT from native conformation to a relaxed, inactive, form. We propose that the interaction between amyloid fibrils and AAT can induce a reciprocal transformation of a β-pleated sheet into an α-helix, thus determining the disaggregation of amyloid fibrils. The ability of serpins to solubilize fibrils of the β-pleated sheet conformation may have implications in the development of new therapeutic strategies in amyloid diseases.

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#### ROLE OF BUSULFAN AND TOTAL BODY IRRADIATION ON GROWTH OF PRE-PUBERTAL CHILDREN GIVEN BMT AND RESULTS OF TREATMENT WITH RECOMBINANT HUMAN GROWTH HORMONE

Giorgiani G, Bozzola M, Locatelli F, Picco P,\* Carrà AM, Dallorso S,<sup>o</sup> Bonetti F, Dini G,<sup>o</sup> Borrone C,\* Giraldi E, Sayegh M, Severi F  
*Clinica Pediatrica, Università di Pavia, IRCCS Policlinico San Matteo, Pavia; \*Clinica Pediatrica II ed °Unità TMO, Istituto G. Gaslini, Genova; Italy*

Seventy-six prepubertal children given autologous or allogeneic BMT were enrolled in a prospective study on the impact of different pre-transplant preparative regimens on growth. Patients were divided into 3 groups: Group I, consisting of 37 children who had received total body irradiation (TBI) and cytotoxic drugs as preparative regimen; Group II, including 17 children given prophylactic cranial irradiation before being conditioned with TBI and cytotoxic drugs, and Group III composed of 22 patients transplanted after a busulfan (BU)-containing myeloablative therapy. All patients have a minimum follow-up of 2 years, while 48 and 34 patients have been studied until 3 and 4 years after transplant, respectively. Height and growth rate were expressed as standard deviation score (SDS). Growth hormone (GH) secretion in response to pharmacological stimuli was evaluated after documented growth failure. Patients with GH deficiency were treated with recombinant human GH, and response to therapy was evaluated. The main impairment of growth rate in patients belonging to Group II was observed in the

first year after TBI, (growth rate SDS changing from  $-0.12 \pm 0.23$  to  $-1.23 \pm 0.25$ ,  $p < 0.05$ ), with only slight loss in the following years, whereas in Group I children growth failure occurred in the third year after TBI ( $-1.36 \pm 0.28$  SDS in comparison to a pre-BMT one of  $0.10 \pm 0.15$ ,  $p < 0.01$ ). Therefore, growth velocity between these 2 groups differed significantly in the first 2 years ( $p < 0.005$ ), but subsequently equalized. On the contrary, all BU-treated children but two grew normally. GH deficiency was demonstrated in the vast majority of children with growth impairment. Twenty-three children treated with recombinant human GH are evaluable; a successful response was observed in all but one, mean growth rate increasing from  $-2.29 \pm 0.27$  before treatment to  $0.86 \pm 0.38$  and to  $1.66 \pm 0.56$  SDS, at one and two years after treatment, respectively ( $p < 0.0001$ ). In conclusion, growth rate impairment is common in patients receiving TBI, the speed of onset of both decreased growth velocity and GH deficiency depending mainly on the total dose of radiation. On the contrary, patients given BU did not experience significant problems in terms of growth velocity. Timely start of appropriate hormonal replacement therapy may ameliorate the final growth of children undergoing BMT.

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#### SECOND ALLOGENEIC BONE MARROW TRANSPLANTATION (BMT) IN ACUTE LEUKEMIA FOR PATIENTS (PTS) RELAPSED AFTER FIRST TRANSPLANT. A G.I.T.M.O. STUDY

Bosi A, Miniero R, Bacci S, Busca A, Laszlo D, Rondelli R, Longo G, Vassallo E, Locatelli F, Van Lint MT, Di Bartolomeo P, Amici A, Resegotti F, Dini G, Rossetti F, Andolina M, Sica S, Alessandrino EP

In order to assess the results of second BMT in pts with acute leukemia relapsed after first transplant we analyzed data from 6 pediatric (P) and 11 adult (A) allogeneic Italian centers referring 47 cases (27 A and 20 P) of second allogeneic BMT. In 9 cases the 1st BMT was autologous, in 1 case the 2nd was from a matched unrelated donor. In all instances the donor was an HLA identical sibling, the same of 1st BMT except in 4 cases.

**Patients and methods:** 30 male and 17 female, median age 22 years (2-48), 18 with an ALL diagnosis and 29 with ANLL; at the time of 2nd BMT 30 were in CR and 17 in relapse. The median time to relapse after 1st BMT was 14 months (2-80). Second conditioning regimen included TBI in 15 cases previously treated with chemotherapy only. Prophylaxis for aGvHD included CsA, MTX either alone or in association. In 7 pediatric cases aGvHD prophylaxis was not performed.

**Results:** All pts demonstrated prompt neutrophil recovery with a median time of 15 days (10-40) to an ANC  $> 0.5 \times 10^9/L$  and 32 days (13-51) to PLT  $> 50 \times 10^9/L$ . Grade III-IV aGvHD occurred in 6 pts after 2nd BMT, cGvHD in 13 (4 extensive). Hepatic VOD developed in 4 cases, interstitial pneumonia in 5. Leukemia recurred in 15 pts (31.9%) after 2nd BMT (8A and 7P). 28 pts died: 14 for relapse and 14 for TRM (29.8%). Early mortality (before +100) was related to aGvHD in 3, hepatic VOD in 2, CMV I.P. in 2, infection (fungal meningitis, fungal pneumonitis with cerebral localization) in 2, subarachnoid hemorrhage in 1 and neurotoxicity from conditioning regimen in 1. Three other pts died later: the cause

were pneumonitis in 2 (one in association with a *Salmonella enteritidis* sepsis) and pulmonary cGvHD in the other. At this time 18 (38.3%) pts are alive and disease free with a median of follow-up of 31 months (5-73). The 3-year probability of DFS was 52%; factors associated with DFS in univariate analysis were remission length after 1st BMT  $>$  or  $<$  6 months ( $p = 0.04$ ) and the interval time between the two transplants  $>$  or  $<$  12 months ( $p = 0.02$ ). There was no association between TRM and the conditioning regimen, GvHD prophylaxis, and 1st-2nd interval time; there was only a positive trend for pts transplanted in CR vs relapse ( $p = 0.09$ ). It is intriguing that in most cases a prolonged remission and an inversion (25 of 29 evaluable) of disease free time was obtained.

**Conclusions:** second allogeneic BMT for acute leukemia may be a therapy of 1st BMT relapse in pts in CR and with a relapse-time after 1st BMT  $>$  6 months.

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#### ALLOGENEIC BMT FOR MULTIPLE MYELOMA (MM). LONG-TERM CLINICAL OUTCOME AND PROGNOSTIC FACTORS ANALYSIS

Cavo M, Rosti G, Cirio TM, Benni M, Tosi P, Bonini A, Gozzetti A, Motta MR, Rizzi S, Fortuna A, Bandini G, Tura S

*Institute of Hematology "L. e A. Seràgnoli", University of Bologna, Italy*

Between January 1984 and March 1993 35 pts with MM (median age: 43 yrs; stage II-III: 91%; responsive to prior chemotherapy: 43%, refractory/relapsing: 57%) received in Bologna an allogeneic bone marrow graft from their HLA-matched, MLC unreactive, family members. Conditioning regimens included either a combination of TBI and chemotherapy ( $n = 14$ ) or chemotherapy alone with Bu (16 mg/kg) and Cy (200 mg/kg) ( $n = 21$ ). Complete remission (CR), as identified by the disappearance of M protein at immunofixation analysis, was recorded in 12 out of 22 (or 54.5%) assessable pts, the remaining 10 having either partial ( $n = 9$ ) or no response ( $n = 1$ ). At reporting, 24 pts had died (IP=6; GVHD=6; MM=6; other causes=6), while the remaining 11 were alive 1 to 10 yrs after BMT (projected 6- and 8-yr survival rates: 35% and 12%, respectively). Median progression-free survival duration was 33 mos for all responders to BMT and 51 mos for only those achieving CR. Pts receiving TBI as part of their program had greater freedom from disease progression than those treated with Bu-Cy (median: 51 vs. 18 mos, respectively). Univariate analysis of pre BMT variables affecting clinical outcome revealed that prior responsiveness to conventional chemotherapy conferred the highest complete remission rate (89%;  $p = 0.001$ ) as well as the longest progression free (median: 60 mos;  $p = 0.001$ ) and overall survival (median: 91 mos;  $p = 0.003$ ) durations. These results show that allogeneic BMT is of particular clinical benefit when employed as remission consolidation treatment. Among conditioning regimens, the addition of TBI to chemotherapy seems to offer an advantage over Bu-Cy in terms of durable control of MM. Based on this observation, since April 1993 we started a new protocol which included unfractionated TBI, Cy (120 mg/kg) and high-dose melphalan (70-90 mg/s.m.) as preparation to engraftment. Continued efforts to reduce

the morbidity and mortality of allogeneic BMT will improve the value of such a procedure for the treatment of MM in the next future.

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#### EVALUATION OF REGIMEN RELATED TOXICITY AFTER BUCY2 IN HEMATOLOGICAL MALIGNANCY: A SINGLE CENTER EXPERIENCE

Salutari P, Chiusolo P, Micciulli G, Di Mario A, Tommasi M, Sica S, Menichella G, Leone G

Istituto Semeiotica Medica Divisione di Ematologia-UCSC Rome, Italy

The combination of busulfan (16 mg/kg) and cyclophosphamide (120 mg/kg) has been found to be an effective preparative regimen for patients affected by hematological malignancies treated with bone marrow transplantation (BMT). At our institution, from February 1988 to April 1995 this preparative regimen was adopted in 83 patients: 18 autologous BMT (12 AML, 3 ALL, 2 NHL, 1 HD) median age 29, range 17-59; 29 allogeneic BMT (15 AML, 8 ALL, 1 NHL, 5 CML) median age 31, range 18-56; 36 peripheral blood progenitor cell transplantation (PBPCT) (25 NHL, 2 HD, 1 Ewing's S, 6 MM, 2 AML) median age 36, range 18-63. We report our experience in the evaluation of regimen related toxicity (RRT) using Bearman's criteria: the toxicity was scored from grade I to grade IV according to clinical and laboratory findings and was assessed on 8 systems (heart, bladder, lungs, CNS, mucosae, gut, kidney, liver). In the PBPCT groups, RRT grade I was 16.6% on mucosae; RRT grade II was 5.5% on the mucosae and RRT grade III 2.4% on the bladder; no grade IV was observed. Surprisingly, in the autologous group we observed only RRT grade I (11.1%) on the mucosae. No grade II, III or IV was observed on any system explored. In the allogeneic group we observed a larger involvement of systems. RRT grade I was 10.3% for the mucosae, 3.5% for the liver; RRT grade II was 6.9% for the mucosae, 13.8% for the bladder and RRT grade III was 6.9% for the CNS. No RRT grade IV was observed. Our opinion is that the BuCy2 regimen is highly effective, well tolerated also in patients heavily treated and over the age 45 (31% of in our series). All patients survived 100 days after transplant. One patient died at day +60 from BMT for acute intestinal GVHD. Patient age and performance status did not affect incidence or severity of RRT in any of the 8 systems. No hepatic VOD was observed despite the several reports about the association between BuCy regimen and hepatic toxicity. GVHD prophylaxis (cyclosporin and methotrexate) in the allogeneic group and antifungal treatment did not worsen the toxicity grade. It is noteworthy that no pulmonary or cardiac toxicity, acute or chronic, were observed with a maximum follow up of 6 years.

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#### TRANSFER OF ANTIGEN SPECIFIC IMMUNITY IN BMT RECIPIENTS: CLONAL DYNAMICS OF TETANUS SPECIFIC MEMORY T CELLS

Montagna D, Maccario R, Locatelli F, Moretta A, Comoli P, Maserati E, Vavassori M, Dellabona P, Casorati G

Clinica Pediatrica e Istituto di Genetica, Università di Pavia, IRCCS Policlinico San Matteo, Pavia; DIBIT, Istituto Scientifico San Raffaele, Milan, Italy

Tetanus-specific T cell response was evaluated in peripheral blood of 12 children who received allogeneic or autologous bone marrow transplantation (BMT). Before reimmunization, in the majority of patients (9 children) no bulk culture proliferation to Tetanus Toxoid (TT) could be obtained. However, a variable number (range 3-25) of TT-specific TCR  $\alpha\beta^+$ , CD3<sup>+</sup>, CD4<sup>+</sup> T cell clones were isolated from all patients. Cytogenetic analysis demonstrated that these clones were derived from the donor. Flow cytometric and molecular analysis of the TCR repertoire, performed by PCR cloning and sequencing on 4 patients, showed the presence of only 1 or 2 different TT-specific clones per patient, with a predominant TCR V $\beta$ 2 usage. Upon immunization, TT-specific immune response became widely polyclonal, as suggested by preliminary results obtained on *in vitro* generated T cells and clones. Moreover, one TCR clonotype, isolated from a patient before immunization, was identified within the newly generated TT-specific bulk line. This data indicate that: i) antigen-specific T cells can be transferred with BMT, ii) only few selected clones can be recovered from PBL of patients before reimmunization, and iii) TT-specific memory T-cells seem to persist with time, although each encounter with a given antigen might prime a new set of specific T-cells.

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#### DONOR LYMPHOCYTE TRANSFUSION FOR LEUKEMIC PATIENTS IN RELAPSE AFTER ALLOGENEIC BMT

Pinto RM, Iori AP, Mengarelli A, De Cuia RM, Rapanotti MC, Gozzer M, Arcese W

Hematology, Department of Human Biopathology, University "La Sapienza", Rome, Italy

Donor lymphocyte transfusion (DLT) has been used to treat 15 pts (9 CML, 3 AML, 3 ALL) who relapsed at a median time of 7 (1.5-93) months after allogeneic BMT.

	IFN	CR	GVHD	APLASIA	D	A	F-UP
							CR (months)
INDUCTION (n=6)							
CML: CYT	2	2	2	2	1	-	2 9-10
CP	3	3	3	3	1	1	2 20-25
AML:	1	-	1	-	-	-	1 3
SECOND LINE (n=3)							
CML: BC	2	2	-	-	-	2	-
ALL:	1	-	-	-	-	1	-
CONSOLIDATION (n=5)							
CML: BC	1	1	-	1	-	1	-
ALL:	2	2	-	2	-	2	2-17
AML:	2	1	-	2	-	2	8-14
TOTAL	14	11		10	2	5	9 10
		(79%)		(71%)	(14%)	(36%)	(64%) (2-25)

The median number of infused MNC $\times 10^8$ /Kg b.w. of the recipient was 2.5 (1.7-8.2). In 12 of 15 pts DLT was associated with  $\alpha$ -IFN administered at a median dose of 3.5 (1-5) $\times 10^6$ /sqm daily. DLT was given as first induction therapy to 6 patients (2 Cytog.-CML, 3 CP-CML, 1 AML), as

second line therapy to 3 pts (2 BC, 1 ALL) refractory to chemotherapy and as consolidation to 5 pts (1 BC, 2 ALL, 2 AML) in CR after chemotherapy. Results and outcome for 14 evaluable patients are summarized in the table: The causes of death were acute-GVHD in 1 CP and disease progression in 3 BC and 1 ALL. DLT was highly effective in inducing CR in CML patients in cytogenetic or chronic phase, but it was unsuccessful as second line therapy for refractory acute leukemia. Its role as consolidation of CR for ALL and AML has to be evaluated on a longer follow-up.

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#### PBSC COLLECTION IN HEALTHY DONORS AFTER MOBILIZATION WITH rhG-CSF

Scimé R, Majolino I, Vasta S, Buscemi F, Indovina A, Fiandaca T, Pampinella M, Catania P, Cavallaro AM, Caronia G, Santoro A  
*Department of Hematology and BMT Unit, Ospedale Cervello, Palermo, Italy*

The introduction of PBSC into allogeneic transplants implies problems involving both the patient and the donor. rhG-CSF shows a safe clinical profile and is therefore the drug of choice for PBSC mobilization in healthy donors. In order to assess its mobilizing capacity and side effects, we administered rhG-CSF to 11 sibling donors (10 HLA identical, 1 HLA partially matched) of patients with advanced/relapsed hematologic malignancies in view of allogeneic transplantation. All the donors were fully informed and gave their written consent. Their median age was 26 y (range 18-39). Five were males and six females. Five received rhG-CSF 16 µg/kg/day for 4 days, the other six received 10 mcg/kg/day for 5 days. Apheretic PBSC collections were regularly performed through the antecubital veins using the CS-3000 *plus* (Baxter) or the AS-104 (Fresenius) separator in 9 donors, the MCS-3p (Haemonetics) in 2 donors, starting the last day of rhG-CSF administration and processing 10 L of blood per run. Aphereses were 2 in all donors except two, who had 1 and 3 collections respectively. Treatment with rhG-CSF was well tolerated. Mild to moderate bone pain, fever, headache and fatigue were reported in most cases. Serum alkaline phosphatase and LDH increased, peaking the day after the last rhG-CSF dose. Thrombocytopenia developed in eight donors reaching a nadir of  $55 \times 10^9/L$  (range 41-87). CD34<sup>+</sup> cells peaked at  $16.3 \times 10^6/L$  (range 68.2-353), with an enrichment of 26.6 fold (range 5.4-118.7) the baseline values. Collection yields were the following: CFU-GM  $10,304 \times 10^4$  (range 3437-14516); BFU-E  $2,686 \times 10^4$  (range 417-7268); CFU-GEMM  $1752 \times 10^4$  (range 898-5,418); CD34<sup>+</sup> cells  $766 \times 10^6$  (range 364-2,599); CD3<sup>+</sup> cells  $283 \times 10^8$  (range 76-396); CD56<sup>+</sup>/CD3<sup>-</sup> cells  $21.3 \times 10^8$  (range 7.3-39.3). CD34<sup>+</sup> cell yields with the 1st apheresis were  $422 \times 10^6$  (range 173-1,158). Toxicity, cell mobilization and collection yields were comparable with the two rhG-CSF schedules. In healthy donors, treatment with rhG-CSF 10 to 16 µg/kg for 4-5 days followed by one-two leukaphereses will result in the collection of a progenitor cell number sufficient for safe allogeneic transplantation. A single apheretic procedure might be sufficient for safe engraftment in the majority of cases.

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#### CORD BLOOD BANK: DOES THE TYPE OF DELIVERY INFLUENCE UMBILICAL CORD BLOOD RECOVERY?

Picardi A, Screnci M, De Felice L, Carmini D, Valentini T, Ciliberti C, Arcese W  
*Hematology, Department of Human Biopathology, University "La Sapienza", Rome, Italy*

The interest in setting up *Cord Blood Banks* has been increasing since human umbilical cord blood (UCB) has been successfully used for allogeneic transplant in children. Several techniques for UCB collection have been proposed. The number of cells and progenitors recovered seems to be not related to the harvest method or the type of delivery. By using the same collection method, we have evaluated the recovery in 76 UCB samples collected after full-term vaginal or caesarean delivery. UCB was harvested in 34 cases after caesarean section and in 42 cases after vaginal delivery (34 while the placenta was still *in situ* and 8 after placenta expulsion). As shown in the Table the mean volume and number of nucleated and mononuclear cells recovered were significantly higher in UCB collections performed before placenta delivery than from caesarean section or after placenta delivery. Because of the wide range of *in vitro* growth and the small sample size, no significant difference was found for CFU-GM, BFU-E and CFU-GEMM.

	Caesarean section	Vaginal delivery		Total
		before placenta delivery	after placenta delivery	
		p	p	
N. samples	34		8	76
Volume (mL)	74.3±30.6	0.001	41.8±10.9	81.9±33.6
NC x 10 <sup>9</sup>	0.9±0.6	0.000	1.4±0.6	1.1±0.6
MNC x 10 <sup>8</sup>	4.2±2.6	0.006	5.8±2.1	4.7±2.5
CFU-GM x 10 <sup>5</sup>	6.3±9.6	NS	6.3±5.2	5.9±6.9
BFU-E x 10 <sup>5</sup>	4.5±3.7	NS	5.4±4.2	4.8±3.9
CFU-GEMM x 10 <sup>6</sup>	0.8±0.9	NS	0.8±0.7	0.8±0.8

In conclusion, our preliminary data show that the type of delivery and the timing of blood collection might significantly affect the UCB volume and cell number recovered. These results suggest that UCB harvest while the placenta is still *in utero* is recommended.

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#### G-CSF MOBILIZED PERIPHERAL BLOOD PROGENITOR CELLS FOR ALLOGENEIC TRANSPLANTATION: BIOLOGIC AND CLINICAL ASPECTS

Rambaldi A, Viero P, Bassan R, Buelli M, Bellavita P, Spinelli O, Micheletti M, Borleri GM, Cortelazzo S, Comotti B, Barbui T  
*Divisione di Ematologia e Centro Trasfusionale Ospedale Riuniti Bergamo, Italy*

Peripheral blood progenitor cells (PBPC) were mobilized by G-CSF (Filgrastim, 2×5 µg/kg/die sc) in normal HLA identical siblings and used for allogeneic transplantation in eight patients with refractory or relapsed acute leukemias. G-CSF administration was well tolerated and

no significant side effects were registered. The number of circulating WBC peaked at 5-6 days after G-CSF (range  $29.3-67.7 \times 10^9/L$ ) with a median of  $65/\mu L$  circulating CD34<sup>+</sup> cells (38-155). As a consequence of apheresis, platelets progressively decreased, reaching the nadir after the last procedure ( $84-205 \times 10^9/L$ ). A mean of 2 apheresis (1-3) were performed between day +4 and +7 during which 10 liters of blood were processed each time by a cell separator AS104 (Fresenius).

Conditioning regimens were: Ara-C (2 gr/m<sup>2</sup> × 2/die for 6 days) with TBI (1200 cGy fractionated in 6 doses) (5 patients); busulfan (4 mg/kg/die for 4 days) e melphalan (140 mg/m<sup>2</sup>) (2 patients, both relapsed after bone marrow transplantation and TBI); melphalan (140 mg/m<sup>2</sup>) with TBI (1 patient). At transplantation, a median of  $6.9 \times 10^6$  CD34<sup>+</sup> cells/kg (4.2-16.5) and  $279 \times 10^6$  CD3<sup>+</sup> cells/kg (161-786) were reinfused. Engraftment was rapid in all patients with ANC  $>1.5 \times 10^9/L$  and Plt  $>20 \times 10^9/L$  at day +18 (range: 11-20 and 10-26 respectively). Molecular analysis by PCR of hypervariable genomic regions (VNTR) allowed to demonstrate the status of donor chimera in all patients. Two patients died for interstitial pneumonitis at day +243 and +69, one patient died at day +62 for disseminated micosis. Five patients are alive between day +30 and +190 without clinical signs of severe GVHD. Our results suggest that mobilization of PBPCs by G-CSF in normal donors is well tolerated and the transplantation procedure is associated with a rapid hematologic recovery. Despite the infusion of such a large amount of mature CD3<sup>+</sup> lymphocytes, apparently GVHD (prophylactically treated in all patients by conventional CSA+MTX) is not worse than observed after transplantation of bone marrow progenitors.

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#### DONOR PERIPHERAL BLOOD STEM CELL (PBSC) INFUSION FOR HEMATOLOGIC MALIGNANCIES IN RELAPSE AFTER ALLOGENEIC TRANSPLANTATION

Vasta S, Santoro A, Pampinella M, Caronia G, Buscemi F, Scimé R, Indovina A, Catania P, Fiandaca T, Majolino I  
Department of Hematology and BMT Unit, Ospedale Cervello, Palermo, Italy

Transfusion of donor leukocytes is an effective treatment for hematologic malignancies relapsing after allogeneic transplantation, but is not devoided of severe complications, as marrow aplasia. Donor PBSC could both increase the GVL effect and reinforce the donor chimerism. In keeping with this hypothesis, 5 patients (2 CML in cytogenetic relapse, 1 MM with progressive disease, 1 APL and 1 biphenotypic AML in CR after chemotherapy) received rhG-CSF-mobilized PBSC from the original HLA-matched related marrow donors. A total of (median)  $2.6 \times 10^8/kg$  (2.4-4.9) CD3 and  $14.3 \times 10^6/kg$  (6.9-32.2) CD34 were infused to each patient without prior conditioning, in 1 or 2 occasions at 4-6 week intervals. No GVHD prophylaxis was administered. Characteristics of patients, response, GVHD occurrence and status at FU are shown in the Table.

Myelosuppression was never observed. Both the CML patients are in cytogenetic remission after donor PBSC infusion. Our data confirm the GVL effect of donor leukocytes in CML. With our protocol, no patient developed cytopenia even with GVHD. In patient #4 an isolat-

ed testicular relapse occurred, suggesting that GVL may fail to eliminate disease in immunologic sanctuary sites.

# disease	Status	N° Infusion	Response	GVHD Ac/Chr	Results and FU (months)
1, CML	Relapse	2	CR	0/0	CR (9)
2, CML	Relapse	1	CR	0/0	CR (5)
3, MM	Prog	2	NR	1/0	Prog (6)
4, AML	CR 3	1	CCR	11/Ext	Rel. testes (9)
5, APL	CR 2	1	CCR	0/Ext	CR (2.7)

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#### ALLOANTIGEN-INDUCED HUMAN LYMPHOCYTES RENDERED NON RESPONSIVE BY A COMBINATION OF ANTI-B7 MONOCLONAL ANTIBODY AND CYCLOSPORIN-A SUPPRESS MIXED LYMPHOCYTE REACTION IN VITRO

Maccario R, Comoli P, Montagna D, Moretta A, Carena I, Zecca M, Giorgiani G, Locatelli F  
Clinica Pediatrica dell'Università di Pavia, IRCCS Policlinico San Matteo, Pavia, Italy

Induction of a state of long-term alloantigen-specific T cell nonresponsiveness can have significant implications for human transplantation. It has been previously described that alloantigen-specific energy may be induced by addition of cyclosporin-A (Cs-A) together with anti-B7 mAb to a primary or secondary MLR. In the present study we endeavoured to verify whether alloantigen-induced PBL rendered anergic by the addition of a combination of anti-B7 mAb and Cs-A during a primary MLR had a suppressive effect when added to autologous lymphocytes activated in MLR with the same stimulators. We found that: i) the addition of cells rendered anergic by this procedure to a primary MLR suppress both proliferative and cytotoxic response of autologous responsive PBL to either the same or third-party stimulator cells; ii) the suppressive effect is limited to alloantigen-induced T cell activation, as addition of anergic cells does not influence mitogen- or antigen-induced proliferation of autologous responsive T cells; iii) nonresponsiveness of suppressed cells cannot be reversed by either subsequent restimulation with allogeneic cells (secondary MLR) or addition of exogenous IL-2 to the cultures; iv) the suppressive effect is not apparently due to secretion of anergic cell-derived soluble factors, but it seems to be dependent on cell to cell contact between anergic, responsive and stimulator cells. These data suggest that: a) competition between anergic and non-anergic lymphocytes for stimulator cell surface may be the mechanism responsible for the suppressive effect here described; b) anergic cells may propagate alloantigen-specific tolerance to potentially responsive autologous lymphocytes.

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#### PERSISTENCE OF MIXED CHIMERISM IN THALASSEMIC TRANSPLANTED PATIENTS. DONOR/RECIPIENT ORIGIN OF DIFFERENT HEMATOLOGICAL LINEAGES IN THREE LONG-TERM STABLE MIXED CHIMERA PATIENTS

Manna M, Nesci S, Andreani M, Tonucci P, Iliescu A, Talevi N, Agostinelli F, Lucarelli G

*Divisione di Ematologia e Centro Trapianti Midollo Osseo di Muraglia, Azienda Ospedaliera "Ospedale San Salvatore", Pesaro, Italy*

Fifty-five thalassemic patients with evidence of mixed chimerism were analyzed in order to study the evolution of the residual host cells (RHC) after bone marrow transplantation (BMT). Twenty patients showed an increasing of the RHC and rejected the transplant, 20 exhausted the amount of RHC showing full donor chimerism, while 15 patients revealed a status of persistent mixed chimerism with the graft functioning at a level enough to correct the anemia. In order to define the donor/recipient origin of the different hematological compartments, three patients with mixed chimerism persisting for 4, 5 and 7 years respectively, have been analyzed. The amount of RHC detected by the restriction fragment length polymorphism of variable number of tandem repeats (VNTR-RFLP) in the peripheral blood (PB) and in the bone marrow (BM) of these three patients, varied during the whole post transplant period studied with values of RHC ranging between 25% and 75%. Nevertheless a donor's pattern of  $\beta$ -globin chain synthesis with values of hemoglobin ranging between 12 and 14 gr/dL were constantly observed. In these three patients the polymerase chain reaction of the VNTR (PCR-VNTR) and the fluorescent *in situ* hybridization (FISH) analysis revealed the coexistence of donor and recipient cells in the different PB cell subpopulations (CD2<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup>, granulocytes), in the erythroid (Glycophorin-A<sup>+</sup>, BFU-E) and myeloid (CD33<sup>+</sup>, CFU-GM) precursors studied. In patients UPN 322 and UPN 572 donor and recipient cells were equally distributed in the different cell compartments analyzed. In patient UPN 688 predominant mixed chimerism was observed in the lymphoid cell subsets while in the marrow we detected predominant recipient mixed chimerism.

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#### LEUKEMIA RELAPSE FOLLOWING ALLOGENIC BONE MARROW TRANSPLANT

Fontana R, Rocco S, Attingenti E, Notaro R, Raiola AM, Luciano L, Camera A, Selleri C, Catalano L, Pezzullo L, Mattace Raso A, Parasole R, Rotoli B  
*Cattedra di Ematologia, Università Federico II, Naples, Italy*

From September 89 to May 95, 35 patients (median age 32, range 10-51) were allotransplanted in our institution from a HLA matched sibling (1 syngenic). Reasons for transplant were: AML (18), CML (12), ALL (2), AA (2), CMML (1). Five patients had advanced disease.

Conditioning was BU 16 mg + Cy 120 mg for all patients except AA (who received Cy 200 mg only). GvHD prophylaxis was CSA in 2 patients, CSA+MTX+short course in the others. Leukemia relapse occurred between 2 and 8 mo in 9/33 patients (27%): 6 AML, 2 CML, 1 ALL. Actuarial relapse risk at 4 years was 30%. Three AML patients had extramedullary relapse (2 CNS, 1 paravertebral granulocytic sarcoma). Acute GvHD  $\geq 2$  was observed in 3/9 relapsed vs 11/24 non relapsing patients; chronic GvHD occurred in 1/6 relapsed vs 8/23 non relapsing (p=n.s.). Leukemia relapse was treated by (a)

systemic CHT: 4 patients, 3 failures and 1 RC with stable allogeneic marrow reconstitution; (b) donor lymphocyte infusion (DLI) (total dose  $0.78 \times 10^8$ /kg): three patients (2 AML, 1 CML) with 2 stable CR complicated by controllable acute GvHD (skin, liver); (c)  $\alpha$ -IFN 3-6 MU/die in a case of relapsed CML, followed by complete cytogenetic remission after 3 mo of treatment. CNS involvement was treated by cranial radiotherapy and intrathecal CHT; 2/3 patients are in 2nd CR. 4/9 patients (44%) died; the other patients are alive after 8, 11, 12 and 32 mo from transplant (6-26 mo from relapse). Thus, it seems that about 50% of patients relapsing after alloBMT can be rescued using a variety of treatment (CHT, DLI, IFN), and may benefit of second remission of significant duration. In 2 patients transplanted for resistant leukemia, a transient allogeneic engraftment was followed by a rapid expansion of the leukemia clone; in these cases an early DLI infusion, possibly associated with a burst of PBSC could amplify the GVL effect and potentiate allogeneic engraftment.

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#### ROLE OF ALLOGENIC BMT FROM HLA-IDENTICAL SIBLING OR MATCHED UNRELATED DONOR IN THE TREATMENT OF CHILDREN WITH JUVENILE CHRONIC MYELOID LEUKEMIA

Zecca M, Pession A,\* Bonetti F, Prete L, Prete A,\* Santoro N,<sup>o</sup> De Stefano P, Severi F, Locatelli F

*Clinica Pediatrica, Università di Pavia, IRCCS Policlinico San Matteo, Pavia; \*Clinica Pediatrica, Università di Bologna, Ospedale Sant'Orsola Bologna; <sup>o</sup>Clinica Pediatrica, Università di Bari, Italy*

JCML is a rare malignant disorder of the hematopoietic multipotent stem cell. At present, allogeneic BMT appears to be the only reported curative strategy for the disease. We report on 7 children with JCML (3 males and 4 females, age range 1,8-8 years) given an allogeneic BMT from either an HLA-identical sibling (4 pts) or a matched unrelated donor (3 pts). In the 4 children given marrow transplantation from an HLA-identical sibling, conditioning regimen consisted of: busulfan (BU), cyclophosphamide (CY) and melphalan (L-PAM), three alkylating agents potentially capable of killing also dormant preleukemic stem cells. GVHD prophylaxis consisted of cyclosporine-A (Cs-A) administered intravenously at a dose of 1-3 mg/kg/day for the first 21 days and subsequently p.o. at a dose of 6 mg/kg/day for 6 months after transplant.

The preparative regimen was well tolerated and all patients engrafted promptly. One of them experienced grade II acute GVHD whereas none relapsed, all children being alive and well with an observation time of 4, 18, 19 and 30 months, respectively. One of the 3 children given marrow transplantation from an unrelated volunteer was younger than 2 years of age and she received the BU/CY/L-PAM conditioning regimen. In view of the increased risk of graft rejection described in patients transplanted with unrelated donors and of the more effective immunosuppression obtained using radiotherapy, we choose to prepare the other 2 patients with fractionated TBI (12 Gy in 6 fractions), thiotepa (TT) and CY. In this group of patients, Cs-A, *short term* methotrexate and Campath-1 G *in vivo* were employed to prevent the occurrence of GVHD. Graft fail-

ure with autologous reconstitution of hematopoiesis occurred in the child given the chemotherapy-based regimen who subsequently died of *Cytomegalovirus* interstitial pneumonitis. One of the two girls given TBI relapsed at 150 days after BMT, notwithstanding she had experienced both grade II acute and limited chronic GVHD. Therefore, only one of 3 patients who received transplants from matched unrelated donor survives in complete hematological remission 6 months after BMT. Our study confirms that allogeneic BMT from compatible sibling is the treatment of choice in pediatric patients with JMCL and suggests that the conditioning regimen we employed is a safe and effective means for eradicating the preleukemic malignant clone. In our experience, results using unrelated donors are less satisfactory and at present, even though these transplants are a potentially curative therapy, their application seems to be riskier and associated with lower success rate as compared to BMT from HLA-identical sibling.

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#### **PULMONARY FUNCTION CHANGES AND CHRONIC GVHD APPEARANCE IN ALLOGENEIC BMT: ANALYSIS OF 54 CASES**

Biaggi G, Alessandrino EP, Bernasconi P, Caldera D, Colombo A, Martinelli G, Nascimbene C,\* Vitulo P,\* Bernasconi C

*Istituto di Ematologia, Università di Pavia. Divisione di Ematologia, and \*Divisione di Pneumologia, IRCCS Policlinico San Matteo, Pavia, Italy*

Within a study aiming to evaluate possible factors predicting the chronic GvHD appearance, 54 patients, suffering from hematological malignancies and subjected to allo-BMT, have been submitted to periodical controls of pulmonary function before, 100 days and 1 year after bone marrow infusion. Before BMT, 52 patients presented normal pulmonary function, while 2 were showing restrictive respiratory syndrome (RRS) resulting from previous pneumonia. At day +100 evaluable patients were 44: 40 did not show any significant alterations compared to the beginning; 4/44 (9%) presented severe obstructive respiratory syndrome (ORS) (FEV-1 reduced of 48.5% and FVC reduced of 11.5%). One patient belonging to this last group, suffering from ORS, died of CMV pneumonitis showing chronic GvHD. After 1 year from transplantation evaluable patients were 31: 27 with pulmonary function tests unchanged; 4/31 (13%) presented severe ORS (FEV-1 reduced of 52.25%; FVC reduced of 15%). All the patients, presenting ORS after 100 days from BMT, developed chronic GvHD in a second time. In one patient ORS, that appeared after 1 year from BMT, was observed together with the diagnosis of chronic GvHD. On the base of this preliminary study, periodical pulmonary function monitoring seems useful in predicting the appearance of chronic GvHD.

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#### **FREQUENCY ANALYSIS OF DONOR-REACTIVE CYTOTOXIC T LYMPHOCYTE PRECURSORS (CTL-P) IN PEDIATRIC RECIPIENTS OF BMT FROM MATCHED UNRELATED DONOR**

Montagna D, Locatelli F, Prete L, Comoli P, Taibi RM, Borsellino A, Ambroselli F,\* Daielli C,\* Zecca M, Giraldi E, Bonetti F, Maccario R

*Clinica Pediatrica, Università di Pavia e \*Laboratorio di Tipizzazione HLA, Servizio Trasfusionale, IRCCS Policlinico San Matteo, Pavia, Italy*

BMT from matched unrelated donors is frequently associated with an increased incidence and severity of GVHD in comparison to HLA-identical sibling donors. In the last years, it has been suggested that the analysis of the CTL precursors (CTLp) frequency between unrelated HLA-matched donor/recipient pairs in the GVHD direction could be used as predictive test for development of severe acute GVHD after BMT. In this report, we summarize our experience regarding 20 pediatric patients affected by various hematological disorders, receiving allogeneic BMT from HLA A-B-DR matched unrelated donors. Molecular typing of HLA-class II antigens of potential donors was performed using PCR-SSP and PCR-fingerprinting techniques. CTLp values, estimated through limiting dilution analysis, before transplant were high (range 1:7.000-1:40.000) in 8 of 20 patients, while the other 12 children showed low or undetectable levels (< 1:100.000) of CTL precursors. The CTLp frequencies were then compared with the incidence and severity of GVHD observed in the patients after BMT. Statistical analysis was performed by means of Chi-square test. Our data demonstrate that the frequency analysis of donor CTLp does not statistically correlate with neither disparity for HLA-Class II molecular typing between donor and recipient nor with clinically significant acute GVHD. In particular, 3 out of the 12 evaluable patients with undetectable CTLp frequencies developed grade III, III and IV acute GVHD, respectively. Although the limited number of pairs studied does not allow us to draw any firm conclusion, our data seem to suggest a certain caution in considering this test suitable in the selection of potential donors.

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#### **ALLOGENEIC TRANSPLANTS USING CORD BLOOD STEM CELLS FOR CHILDREN WITH ACUTE LEUKEMIA**

Locatelli F, Bertolini F,\* Lirato L,<sup>o</sup> Zecca M, Giorgiani G, Maccario R, Moretta A, Canazzio A,<sup>o</sup> De Stefano P, Severi F, Sirchia G\*

*Clinica Pediatrica, Università di Pavia; \*Centro Trasfusionale e Immunologia dei Trapianti Ospedale Maggiore, IRCCS Policlinico, Milano; <sup>o</sup>Divisione di Pediatria Ematologica, Ospedale Pausillipon, Naples, Italy*

Cord blood represents an alternative source of hematopoietic stem cells, able to reconstitute the hematopoietic system of pediatric patients who had received myeloablative therapy. We report on two children with acute lymphoblastic leukemia (ALL) who were given cord blood

progenitors (CBP) transplantation from their HLA-identical siblings. Both patients were male and they were affected by ALL in 2nd remission (after a marrow relapse occurring during maintenance chemotherapy) and Ph+ ALL in 1st remission. Conditioning regimen consisted of TBI (12 Gy in 6 fractions) thiotepa (10 mg/kg) and cyclophosphamide (120 mg/kg). GVHD prophylaxis consisted of Cs-A given i.v. at the dosage of 1 mg/kg/day for the first 21 days and subsequently, p.o. for further 30 days. In the attempt to increase the number of hematopoietic progenitors infused, the child with ALL in 2nd remission was given also  $0.8 \times 10^6$ /kg bone marrow cells harvested from the 6-month-old sibling. In the patient given only CBP, ABO incompatible red blood cells were removed before cryopreservation through sedimentation using gelatine 3%. After thawing, CBP were washed with dextran 40 and albumin; recovery of CFU has been in the order of 86-91%. The number of CFU-GM and CD34<sup>+</sup> cells infused was  $40 \times 10^3$ /kg and  $63 \times 10^3$ /kg and of  $0.3 \times 10^6$ /kg and  $0.4 \times 10^6$ /kg, respectively. Donor hematopoietic progenitors engrafted in both patients; in the child who received both cord blood and marrow progenitors, time to achieve neutrophil and platelet engraftment were 12 and 26 days, respectively, whereas 18 and 42 days were needed in the patient given only CBP. The patient given both CBP and marrow progenitors experienced skin grade I acute GVHD, whereas the other child never developed GVHD; in both cases we have been able to discontinue Cs-A 2 months after transplant. An impressive increase in the percentage of fetal hemoglobin was observed in both patients (7% and 35% respectively) during the first 3 months after CBP infusion. Early immunological reconstitution did not differ significantly from that of marrow transplant recipients, even though neutrophil chemotaxis was slightly impaired in the first month after transplantation. Both patients are alive and in complete hematological remission 4 and 3 months after CBP infusion, respectively. Our experience confirms that CBP can be used to rescue pediatric patients after myeloablative therapy and that these transplants are an alternative tool for curing patients with malignant and non-malignant disorders. The low reactivity of cord blood immunocompetent cells against allogeneic targets may allow to reduce the dosage and/or the duration of immunosuppressive treatment and make the use of unrelated partially matched CBP transplants an attractive perspective for the future.

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#### **ALLOGENEIC BONE MARROW TRANSPLANTATION (ALLOBMT) VERSUS CHEMOTHERAPY (CHEMO) A TREATMENT FOR CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) IN SECOND REMISSION: EXPERIENCE OF GITMO AND AIEOP GROUPS**

Uderzo C, Valsecchi MG, Bacigalupo A, Meloni G, Messina C, Polchi F, Di Girolamo G, Dini G, Miniero R, Locatelli F, Colella R, Tamaro P, Lo Curto M, Di Tullio M, Masera G

*Participating Centers GITMO-AIEOP: Monza, Genova, Roma, Pesaro, Pescara, Torino, Pavia, Trieste, Palermo, Napoli. On behalf of GITMO and AIEOP-GROUPS*

Treatment of recurrent childhood ALL can cure 20 to 60% of affected children but these results are still controversial based on the current retrospective studies. In the last 10 years 287 consecutive children (230 treated with CHEMO and 57 with alloBMT) entered this study in GITMO-AIEOP centers. Statistical Cox analysis was properly made in order to retrospectively evaluate the effect of AlloBMT vs CHEMO. At a 6.2-year median follow-up the adjusted DFS curves for the alloBMT and CHEMO showed a probability of 41.1% (SE 6.6.) and 21.7% (SE 3.7), respectively. The significant advantage of alloBMT vs. CHEMO in early relapse patients (3 years DFS=33.4%, SE 8.6, vs. 16.1%, SE 4.5%) and the better result of AlloBMT vs. CHEMO in late relapse patients (3 year DFS= 54.7%, SE 9.2, vs. 39.6%, SE 5.9) underline the possibility of alloBMT as the best treatment in this setting. Prospective trials are needed to address the role of alloBMT vs. CHEMO in patients with ALL in 2nd CR and this is the future challenge for European groups involved in this field.

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#### **QUANTITATION OF HUMAN CYTOMEGALOVIRUS DNA IN BONE MARROW TRANSPLANT RECIPIENTS**

Comoli P,\* Gerna G, Furione M, Baldanti F, Percivalle E, Locatelli F\*

*Viral Diagnostic Service, and \*Department of Pediatrics, University of Pavia, IRCCS S. Matteo, Pavia, Italy*

Viral DNA was retrospectively quantitated in the early post-transplant period in 36 pediatric bone marrow transplant (BMT) recipients prospectively monitored for human cytomegalovirus (HCMV) infection on the basis of the antigenemia and viremia assays. Viral DNA was quantitated in peripheral blood leukocytes (PBL) by PCR using an internal control of amplification and a series of external standards. Densitometric analysis of hybridization results obtained on PCR products allowed construction of a standard curve from which DNA amounts of clinical samples, expressed in terms of genome equivalents (GE), were interpolated. Of the 36 BMT recipients, 3 had clinically symptomatic HCMV infection with mean peak levels of viral DNA >5000 GE (antigenemia and viremia mean peak levels were 873 and 35, respectively), while 19 with HCMV reactivation were asymptomatic (5 of them had abortive HCMV infection) showing mean peak DNA levels of 131 GE (and of 6.8 and 1.3 for antigenemia and viremia, respectively) ( $p \leq 0.01$ ). Single or multiple courses of preemptive therapy with ganciclovir or foscarnet were given to 14 out of the 19 asymptomatic children in whom antigenemia levels were >2 or lower yet persisting. Overall, in the 14 asymptomatic treated patients the mean antigenemia level was 9.3 (range 1-22), while the mean DNA level was 184.6 (range 20-710) GE. Antiviral drugs were also administered to the 3 symptomatic patients, who due to late diagnosis of HCMV infection, escaped preemptive therapy. Antiviral treatment caused marked decrease or disappearance of antigenemia and viremia, and quantitative mean reduction in viral DNA from 9,077 to 208 GE in the 3 symptomatic patients, and from 184 to < 15 GE in the 14 treated HCMV-asymptomatic patients.

In conclusion our study suggests that: i) although quantitation of HCMV DNA does not seem to be clearly superior to the quantitative antigenemia assay, it could represent a fine tool for monitoring of HCMV infections and antiviral treatment in BMT recipients, providing indications largely superior to those of qualitative PCR; ii) in our group of patients, starting therapy in the presence of a mean antigenemia level of 9.3 (range 1-22) corresponding to a mean DNA level of 184.6 (range 20-710) GE avoided occurrence of any major HCMV-related clinical complication; iii) clinical symptoms were associated with antigenemia levels > 100 and DNA levels > 1000 GE.

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#### SMALL BOWEL INFARCTS BY ASPERGILLOSIS

Catalano L, Picardi M, Anzivino D, Insabato L, Formato A, Lambertini M, di Grazia C, Mazzeo M, Notaro R, Rotoli B

*Cattedre di Ematologia, Microbiologia, Anatomia Patologica e Chirurgia Generale, Università Federico II, Napoli*

Aspergillosis is a frequent and severe cause of morbidity and mortality in neutropenic patients. Most frequently involved species are *A. fumigatus* and *A. flavus*. In a majority of cases, the fungus is localized in the respiratory tract, from where it can be disseminated. Its peculiar angiotropism may cause infarcts due to septic thromboembolism. Disseminated aspergillosis is a difficult diagnosis *in vivo*, since blood cultures are usually negative; histology and microbiology positive results are often obtained from necropsy specimens. We have diagnosed small bowel ischemic necrosis due to aspergillosis.

A 58-year-old patient with AML-M4Eo was treated by the EORTC-GIMEMA protocol LANL93 (DNR arm) in Nov 94. At the end of induction, highly remittent fever started, that was unresponsive to antibiotics and fluconazole. After a few days, recurrent gut bleeding episodes occurred; microbiological work up was negative. On day +25 an urgent laparotomy was required for acute abdomen. Enteric liquid was found in the peritoneal cavity, with markedly dilated small bowel. The fourth duodenal segment and the first two jejunal bends were necrotic, with multiple lacerations involving the whole wall. A duodeno-jejunal resection was performed, with anastomosis on the right of the superior mesenteric artery. Histology revealed embolic occlusion of mesenteric vessels by mycetes; bowel fragment culture showed *A. fumigatus* colonies. Since the patient was in renal failure, intensive treatment with liposomal amph B was started. While hematological reconstitution was apparent, further gut bleeding occurred and the patient died a week after surgery. This patient succumbed to disseminated aspergillosis while going into CR from a good prognosis AML form. Although documented *in vivo*, the unusual localization in the duodeno-jejunal wall, leading to hemorrhage and multiple perforations, could not be controlled by antimycotic treatment. Early usage of amph B for febrile aplastic patients has to be considered.

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#### CEREBRAL ASPERGILLOSIS IN PATIENTS WITH ACUTE LEUKEMIA: REPORT OF 13 CASES

Pagano L, Ricci P, Montillo M, Nosari AM, Tonso A, Cenacchi A, Cudillo L, Savignano C, Chierichini A, Buelli M, Melillo L, Sica S, Bonini A, Del Favero A.\* and the GIMEMA-Infection Program

*Objective.* We evaluated in a retrospective study the characteristics of patients (pts) with acute leukemia and aspergillus infection with colonization of central nervous system (CNS).

*Patients.* Between 1987 and 1992 we observed in 14 centres 100 cases of aspergillus infection in 1969 pts with acute leukemia. In 50 patients the infection was fatal.

Here we report 13 pts with CNS colonization that resulted the main cause of death. They were 3 ALL and 10 AML, m/f 10/3, median age 48 (21-66). At the time of infection 8 pts were in post-chemotherapeutic induction phase, 1 in complete remission, 3 after BMT and 1 after aBMT.

*Results.* The diagnosis was made on the basis of neurological symptoms, microbiological findings, cerebral CT scan and autopsy. The aspergillus isolated were: not characterized (7), *flavus* (4), *fumigatus* (1), *nidulans* (1). The primary site of infection was identified in 11 cases (6 lung, 4 CNS, 1 sinus). All pts presented a median previous neutropenia of 16 days (range 12-54). At the time of primary infection 9 pts presented a neutrophil count <0.1 x 10<sup>9</sup>/L, 3 out of 4 non-neutropenic pts were on cyclosporin treatment for GvHD post-BMT. The last patient has been treated with high doses of corticosteroids. Nine pts in total received corticosteroids. Oral antimycotic prophylaxis was administered to all pts (fluconazole 6, amphotericin B 5, nystatin 1, ketoconazole 1) and 10 pts were treated with amphotericin B (median dose 920 mg, 90-3400). All pts died within a median of 5 days (1-34) from the onset of neurological signs.

*Conclusions.* Cerebral aspergillosis 1) is an index of systemic infection; 2) resulted unresponsive to antifungal treatment also when the treatment was started early in the course of the disease; 3) was always fatal; 4) seems related to prolonged severe neutropenia and to the immunosuppressive therapy (corticosteroids and cyclosporin).

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#### CEREBRAL NOCARDIOSIS AFTER BONE MARROW TRANSPLANT: A CASE REPORT

Attingenti E, Fontana R, Rocco S, Catalano L, Raiola AM, Cappabianca P,\* Del Basso De Caro ML,^ Belfiore G,^ Anzivino D,° Notaro R, Luciano L, Rotoli B.

*Cattedre di Ematologia, \*Neurochirurgia, °Microbiologia e Istituti di ^Anatomia Patologica e #Radiologia, Università Federico II, Naples, Italy*

A 47 year old male patient suffering from CML in chronic phase received an allo-BMT from his HLA-matched brother in January 1994. Allogeneic marrow reconstitution was rapidly achieved, but several complica-

tions occurred: acute skin and gut GvHD, hemorrhagic colitis and cytopenia by CMV, chronic skin and liver GvHD requiring prednisone and CS-A. Ten months after transplant, a high grade fever episode was followed by fits, left hemiparesis and dysarthria. A TC scan showed multiple nodular lesions in the brain, that were enhanced by contrastography and had a clear perilesionar oedema; the right lung showed an irregular infiltrate. Cerebro-spinal fluid was clear, with normal pressure, no cell increase, negative chemical and microbiological examination. Echocardiography did not show endocardic vegetations. No filamentous fungi were isolated from nose and spit. CMV antigenemia was negative. The patient was pancytopenic, transfusion dependent; the Ph chromosome was absent. Empiric treatment by itraconazole, cotrimoxazole, gancyclovir, imipemen, teicoplanin and parenteral nutrition was ineffective. In December 1994 a brain biopsy through craniotomy was performed: multiple colliquative lesions were found in both hemispheres, with a jelly appearance. Histology showed microabscesses with necrosis and granulocyte infiltration at the periphery. Grocott staining was positive for *Nocardia*. Cotrimoxazole treatment was potentiated. In February 1995 the performance status deteriorated (KS 20%) and a multi-organ failure (MOF) appeared with encefalopathy, coryoretinitis, severe pancytopenia, acute liver failure; the terminal event was abrainhemorrhage. However, a TC scan revealed regression of the brain nodular lesions.

This report stresses the unfavorable prognostic significance of brain infection in transplanted patients, and the relevance of invasive diagnostic procedures with an interdisciplinary approach. Unfortunately, a correct diagnosis may not be resolute: a MOF due to multiple damaging mechanisms may shorten patient survival.

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#### HIGH DOSE CHEMOTHERAPY AND AUTOGRAFT WITH PERIPHERAL PROGENITORS AS FIRST LINE TREATMENT IN MULTIPLE MYELOMA: HIGH EFFICACY IN PATIENTS WITH HIGH PLASMACELL PROLIFERATIVE ACTIVITY

Tarella C, Boccadoro M, Corradini P, Caracciolo D, Palumbo A, Argentino C, Lombardi F,\* Bregni M,\* Siena S,\* Gianni AM,\* Pileri A

*Dipartimento di Medicina e Oncologia Sperimentale, Divisione Universitaria di Ematologia-Azienda Ospedaliera S. Giovanni, Torino e \*Unità Trapianto Midollo, Istituto Nazionale Tumori, Milan, Italy*

Since 1989 we carried out pilot studies to evaluate feasibility and efficacy of intensive chemotherapy as first line treatment in patients with multiple myeloma (MM). Three different schemes have been subsequently employed, as a result of a progressive protocol evolution: initially we employed the original HDS protocol, including sequential administration of CY (cyclophosphamide), Mtx (methotrexate), VP16 (etoposide) and finally TBI+L-PAM followed by autograft with peripheral blood progenitor cells (PBPC); subsequently Mitoxantrone went to replace Mtx; the most recent protocol includes now double Cy administration and double PBPC autograft (following L-PAM and, 3-4 months later, following L-

PAM+mitoxantrone). Overall we treated 39 patients; median age was 49 years (38-58); plasma cell proliferative activity (LI) was determined in 37 cases, and a high LI (>1,2%) was found in 17 patients. Albeit in the absence of acute letal toxicity, the high dose treatment could not be completed in 9 patients (23%), due to insufficient PBPC harvests (3), extraematological toxicity (2), no response or progression (4). An objective response (50% or more disease regression) was observed in 34 patients (87%), and complete remission (CR) was achieved in 18 patients (46%); patients with high LI showed a better response with 11 out of 17 patients obtaining CR (64%), compared to those with low LI (6/20, 30%). Twenty-five patients are presently alive at a median follow up of 28 months, with a projected median survival at 49 months. Survival curves in both groups are almost overlapping, with median survivals projected at 47 and 59 months for patients with low or high LI, respectively. In conclusion, high dose chemotherapy overall improves life expectancy in patients with MM, especially in those with high LI. Actually this intensive program allows suppression of the survival difference observed in the two groups when treated with a conventional approach.

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#### KINETIC OF IMMUNE RECOVERY IN 40 PATIENTS AUTO-TRANSPLANTED WITH BLOOD PROGENITOR CELLS

Brunori M, Olivieri A, Offidani M, Ciniero L, Ombrosi L, Scalari P,\* Montroni M,\* Leoni P

*Clinica di Ematologia, \*Servizio di Immunologia Clinica e Tipizzazione Tissutale Università di Ancona, Italy*

The delay of immune recovery (IR) after bone marrow transplantation can play a critical role not only in the incidence of severe infections, but also for the probability of relapse and for the arising of second neoplasia. Although the immunologic reconstitution after allogeneic bone marrow transplantation has been widely studied, only few experiences has been reported concerning the kinetic of IR after blood progenitors cells (BPC) autotransplantation. We studied 40 patients with median follow up of 6 months (range 1-24) autotransplanted (30 with BPC alone, 10 with BPC together with bone marrow), affected by hematologic malignancies, with the aim to evaluate the kinetics of lymphoid subsets and to individuate the parameters which can influence their behaviour. Total lymphocyte and T lymphocyte count (CD3<sup>+</sup>) recovered very quickly; the CD8<sup>+</sup> (suppressor/cytotoxic) subset showed a rapid reconstitution with mean count (1237±166/μL) above the normal range until 3<sup>th</sup> month. The CD4<sup>+</sup> (helper/inducer) lymphocyte count, on the contrary, showed an important depression in all patients (<500/μL) during the first 15 months and under normal range after 24 months; consequently the CD4/CD8 ratio resulted strongly inverted for a long period, reaching a normal value only after 24 months. The CD3-/CD16<sup>+</sup> (NK) cell absolute count was slightly reduced until 24th month, while the CD8<sup>+</sup>/CD57<sup>+</sup> subset (cells with cytotoxic activity) showed an impressive increase during the first 6 months. The B lymphocyte count (CD21<sup>+</sup>) increased with a complete normalization at 15th month. We did not

observe any severe opportunistic infection after transplantation in our 40 transplanted patients. Choosing a CD4<sup>+</sup> threshold >200/μL (significant for the incidence of opportunistic infections in AIDS patients) the probability of reaching this cut-off, performed by Kaplan-Meier method, was 75% at the first month and 100% at the 12<sup>th</sup> months post transplantation; on the other hands, choosing a CD4<sup>+</sup> cut-off of 400/μL, the median time for reconstitution was 6 months with a 90% probability at the 12<sup>th</sup> month. As regard the CD21<sup>+</sup> subset the probability of normalization was 20% at the first months, 50% at the 3<sup>th</sup> month and 95% at the 15<sup>th</sup> month. A univariate and multivariate analysis have been performed considering as dependent variable the time employed to reach respectively 200 CD4<sup>+</sup>/μL and a normal value of CD21; the independent variables were age, sex, diagnosis, previous chemotherapy, pretransplant status, kind of priming, conditioning regimen, source of stem cells (BPC alone or BPC plus bone marrow), sepsis incidence and the number of MNC/kg, CFU-GM/kg and CD34<sup>+</sup>/kg reinfused; none of the above factors significantly influence the rate of IR. Our result demonstrate that the kinetic of IR after BPC auto-transplantation is independent from the clinical features and from the harvest characteristics but is probably correlated to the ontogenesis of immune system. In any case a threshold of CD4<sup>+</sup> >200/μL is probably enough to avoid severe opportunistic infections and so the antibody production after the first year after transplant. Finally, even if the cytotoxic subset is early expanded after transplantation, the NK compartment result significantly depressed and therefore the role of immunotherapy after BPC transplant should be explored.

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#### **AUTOGRAFT WITH SELECTED CD34<sup>+</sup> PBSC IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)**

Buscemi F, Felice R, Santoro A, Pampinella M, Catania P, Fiandaca T, Scimè R, Indovina A, Vasta S, Rizzo A, Majolino I

*Department of Hematology and BMT Unit-Ospedale Cervello, Palermo, Italy*

In CLL, standard treatment, either with single or multiple-agent chemotherapy, induce few durable complete remissions and seldom produce the eradication of disease. More recent experience with new agents as fludarabine is more encouraging with an increase in overall response rate ranging 50-70%, but long term results are still unknown. In young patients a major response to fludarabine might represent an indication for high-dose therapy followed by autologous stem cell transplantation, using purging procedures to reduce contaminating tumor cells. In this study we are treating selected B-CLL, stage B and C patients, aged less 60 years, with fludarabine and autologous transplant with peripheral blood CD34<sup>+</sup> selected cells. PBSC are harvested after mobilization with cyclophosphamide 4 gr/m<sup>2</sup> and rhG-CSF 5 mcg/kg/d. Apheresis are started at hematologic recovery, when the WBC exceeds 5×10<sup>9</sup>/L. The products of 2 leukapheresis are processed by the CEPRATE SC column (CellPRO). As autograft myeloablative regimen, the combination CVB-3 is employed. After

CD34<sup>+</sup> cell infusion, rhG-CSF, 5 mcg/kg/d, is given until a steady count of ANC >1.0×10<sup>9</sup>/L. Until now three previously treated patients have been enrolled in the study. Patients are male aged 50, 55, 58 years, in Binet stage B disease. They received 6 courses of fludarabine (25 mg/m<sup>2</sup>/d × 5 days every 4 weeks) which resulted in CR in two patients and in PR in one. CD34<sup>+</sup> cells collected by apheresis were 12.7, 3.3 and 0.89×10<sup>6</sup>/kg respectively. After processing with the column, the CD34<sup>+</sup> cells were 4.38, 0.88, and 0.28×10<sup>6</sup>/kg with a recovery of 34%, 27% and 31% respectively. With respect to the total adsorbed cells, the CD34<sup>+</sup> population was 70%, 51% and 7%. In all processed samples, we observed 2.2-3 log reduction of CD19<sup>+</sup> CD5<sup>+</sup> cells and T lymph as determined by immunofluorescence analysis. CD19 antigen was not detected on CD34<sup>+</sup> cells. One patient has been autografted, receiving 4.38×10<sup>6</sup>/kg CD34<sup>+</sup> cells and 11.5×10<sup>6</sup>/kg CFU-GM. He had a prompt granulocyte engraftment with 0.5 PMN on day +12 and 50 PLT on day +19.

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#### **AUTOGRAFTING CHRONIC MYELOID LEUKEMIA WITH MAFOSFAMIDE PURGED MARROW: *IN VIVO* AND *IN VITRO* STUDIES**

Carlo-Stella C, Mangoni L, Dotti G, Almici C, Caramatti C, Rizzoli V

*Bone Marrow Transplantation Unit, University of Parma, Italy*

To improve the efficacy of autografting for chronic myeloid leukemia (CML) either purging of the leukemic stem cells or selecting for the non leukemic stem cells are required. The aims of the present study were: (a) to investigate the cytogenetic status of progenitors generated by CD34<sup>+</sup> stroma-adherent (Stro<sup>+</sup>) cells; (b) to update our experience with mafosfamide purging for CML.

CD34<sup>+</sup>Stro<sup>+</sup> cells were isolated through an immunoadsorption technique followed by incubation on confluent stromal layers. CD34<sup>+</sup> cells generated 22±10% (mean±SEM) Ph-neg CFU-GM. A significant increase of Ph-neg clones could be obtained by combining CD34 selection with either stroma-adherence (38±14%, p≤.025) or mafosfamide incubation (46±18%, p≤.05). The three-step purging (CD34 selection + mafosfamide + stroma adherence) failed to significantly improve selection of Ph-neg clones as compared to the two-step purging (CD34 selection + mafosfamide).

Twelve patients ineligible for an allogeneic BMT were autografted with mafosfamide-purged marrow. Patients were conditioned with: busulfan (BU, 4 mg/kg/d × 4d), cyclophosphamide (CY, 60 mg/kg/d × 2d) and melphalan (90 mg/sqm/d × 1d) (eight cases); BU and CY (two patients); BU, CY and VP16213 (30 mg/kg × 1d) (two cases). One patient died of transplant related toxicity by day 19. The median days to achieve 500 neutrophils/μL and 20,000 platelets/μL were 32 (range, 17 to 72) and 37 (range, 21 to 151), respectively. At ABMT, standard cytogenetic analysis revealed 100% Ph-pos metaphases in all patients. After transplant, 100% Ph-neg metaphases were detected in 7/11 patients with a median duration of Ph-neg hematopoiesis of 6.5 months (range, 2 to 30). Four

patients reinfused with a mixture of Ph-pos and Ph-neg Stro<sup>+</sup> progenitors engrafted Ph-pos whereas 7 patients reinfused with 100% Ph-neg Stro<sup>+</sup> progenitors engrafted Ph-neg. Currently, 3 patients in cytogenetic relapse are under interferon-alpha (3-6×10<sup>6</sup> U weekly) therapy and two achieved a complete disappearance of leukemic hematopoiesis lasting 3 and 13 months, respectively. The overall median survival from diagnosis and ABMT is 62 and 20 months, respectively. Six patients died of blast crisis and 3 from other causes. Our results demonstrate that: (a) *in vivo* non-leukemic hematopoiesis occurs only in patients reinfused with ≥95% Ph-neg Stro<sup>+</sup> progenitors; (b) post-graft administration of interferon-α might restore non-leukemic hematopoiesis; (c) a purging approach combining CD34 selection and mafosfamide treatment should be explored in pilot studies.

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#### **BUSULFAN-INDUCED APOPTOSIS: IN VITRO EFFECTS AND POSSIBLE USE AS MONITORING OF TOXICITY RELATED TO AUTOLOGOUS BONE MARROW TRANSPLANTATION CONDITIONING REGIMEN**

Tosi P, Visani G, Ottaviani E, Gherlinzoni F, Salvucci M, Barassi M, Manfroi S, Tura S  
*Institute of Hematology "L. e A. Seràgnoli", University of Bologna, Italy*

Methods designed to study the pharmacokinetics of busulfan (BUS) are difficult to be performed and this is further accomplished by the uneven absorption of the drug after oral administration. As BUS is known to cause DNA damage, in this study we aimed to verify whether this effect could be attributed to apoptosis. This phenomenon has been initially studied in 3 cell lines, K562 (chronic myeloid leukemia in blastic crisis), Raji (B-lymphoid) and HL-60 (acute myeloid leukemia). The IC<sub>50</sub> of BUS was 12.7 mM, 11.4 mM and 8.8 mM in K562, Raji and HL-60 cell line, respectively. In all the cell lines, incubation with BUS caused apoptosis in a dose-dependent manner, with a percent increase, compared to control, of 201% (K562), 98% (Raji) and 180% (K562) at 2 mM, as determined by a cytofluorimetric method. BUS-induced apoptosis was also evaluated *in vivo*, on mononuclear peripheral blood cells of patients submitted to autologous bone marrow transplantation, during conditioning with BUS 16mg/kg (day -7→-4), prior to cyclophosphamide administration. Preliminary results obtained so far in 5 patients, indicate:

- 1) possibility of *in vivo* quantification of apoptotic cells
- 2) progressive increase in the number of apoptotic cells from the first to the fourth day of BUS therapy.

Supported in part by MURST 40%-60%

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#### **HIGH-DOSE THERAPY FOR AGGRESSIVE LYMPHOMA WITH PERSISTENT BONE MARROW INVOLVEMENT.**

Santini G, Congiu AM, Chisesi T, Figari O, Benvenuto F, Piaggio G, Nati S, Pierluigi D, Damasio E\*

\*Department of Hematology, S.Martino Hospital, Genova, Italy

The study was carried out on 33 patients with diffuse, intermediate or high-grade NHL, pre treated with a median of two combination chemotherapy lines (range 1-4), but not in CR because of a persistent BM involvement (median 15%). Pts. underwent HD-CY (7 gr/mq, single dose) and G-CSF infusion (5 ug/kg/day) to reduce tumor burden and to collect PPC. The median age of pts. was 43 yrs. (range 19-54); 19 males and 14 females; 29 all so presented nodal disease. Initial collection was a median of about 1.000/mcl WBC. Median number of apheresis was 5 (range 3-12); median collected cells were 7.5×10<sup>8</sup>/kg (range 2.6-19); median of CFU-GM was 11×10<sup>4</sup>/kg (range 0.2-133) and of CD34<sup>+</sup> was 4.8×10<sup>6</sup>/kg (range 0.2-59). Phenotypic analysis of the collected cells showed a low level of B-cells (median = 0.9%). In two patients, peripheral and BM progression occurred during collection. Two pts. did not undergo PPC rescue because of a low number of CFU-GM. After a conditioning regimen (melphalan 120 mg/sm + TBI, or BEAM), 26 pts. received PPC rescue (3 pts. are in treatment). According to intention to treat, 24 out of 30 evaluable pts. (80%) obtained BM and nodal CR. Overall short-time procedure was well tolerated. However, 5/24 pts. (21%) died in CR after rescue (2 of infection; 1 of lung fibrosis; 2 of BM aplasia at 3 and 7 months). Four patients relapsed. Up to now 14/30 pts. (47%) are in CR 4 to 33 mos. (median 17) after PPC rescue. A first interim analysis shows a 3-year probability of Survival and PFS of 57% and 44% respectively. These results seem to be superior to those proposed by conventional treatment in this poor category of patients.

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#### **SALVAGE CHEMOTHERAPY AND AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELLS TRANSPLANTATION IN RESISTANT LYMPHOMA**

Sica S, Salutari P, Chiusolo P, Di Mario A, Teofili L, Marra R, Micciulli G, Ortu La Barbera E, Leone G  
*Istituto Semeiotica Medica, Divisione Ematologia -UCSC Rome, Italy*

From 1991 to April 1995 in our institution we enrolled 38 patients, 22 males and 16 females, median age 38 yo (range 17-63), affected by resistant lymphoproliferative disease (NHL 30 and HL 8) previously treated with conventional therapy in a two step protocol including salvage chemotherapy followed by peripheral blood progenitor cells transplantation (PBPC).

The state of disease at enrollment was: progressive disease (PD) 5 patients (13%), partial remission (PR) 12 patients (32%), resistant disease (RD) 7 patients (18%) and relapse (Rel) 14 patients (37%). Salvage chemotherapy was mitoxantrone 10 mg/sm at day 1, carboplatinum 100 mg/sm day 1 to 4, Aracytin 2000 mg/sm at day 5, methylprednisolone 500 mg/sm day 1 to 5 (MiCMA) and followed by rhG-CSF 5 ug/kg. All patients were submitted to 1-4 chemotherapy cycles. The response to MiCMA was: 9 PD (23.7%), 23 PR (60.5%), 6 complete remission (CR) (15.8%).

Leukapheretic procedures (median number 3, range 2-4) were started when WBC count was  $>1.0 \times 10^9/L$ . Twenty-seven (71%) of patients were submitted to PBPC transplantation after BuCy2 conditioning regimen. Eleven patients (29%) did not complete the protocol. Reasons for protocol interruption were: progressive disease in 5 patients, eligibility for involved field irradiation therapy in 2 patients in PR and refusal in 4 patients in CR. Seven of these patients are alive, 6 in CR with a maximum follow-up of 3 years. All but one patients in PD have already died from their disease. For 27 patients completing the protocol, the response to transplant was: 17 CR (63%), 5 PD (18.5%), 2 PR (7.4%). Three patients transplanted recently are still not evaluable. Six patients relapsed (22%). The median time to relapse after PBPC transplant was 10 months (range 7-15). Eighteen out of 27 (66.7%) are currently alive with a follow up of 24 months (3-38): 11 CR (61.1%), 2 PR (11%), 1 Rel (5.5%), 1 PD (5.56%) and for the remaining 3 patients evaluation is ongoing. Nine patients (33.3%) died after a median time of 12 months (range 7-22), 4 because of progression disease, 5 for relapse of disease. Salvage chemotherapy and subsequent transplantation is feasible given the high percentage of patients completing the protocol and well tolerated with no early deaths related to the treatment. Patients over the age of 50 and submitted to transplantation did not develop additional toxicity. Further follow-up is warranted in term of disease free-survival.

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#### **PBSCT VERSUS ABMT IN HODGKIN'S (HD) AND NON-HODGKIN'S (NHL) LYMPHOMAS: A NEW MATCHED-PAIR ANALYSIS OF THE EBMT REGISTRY DATA**

Majolino I,<sup>o</sup> Pearce R,\* Taghipour G,\* Goldstone AH,\* for the Lymphoma Working Party of the EBMT  
\*University College Hospital, London, UK, and <sup>o</sup>BMT Unit, Ospedale Cervello, Palermo, Italy

In order to compare the results of autologous bone marrow (ABMT) and peripheral blood stem cell (PBSCT) transplantation we have reviewed the registry data of 3324 patients with lymphoma, 2859 undergoing ABMT and 465 PBSCT. In multivariate analysis the relevant prognostic factors for PFS were status at transplant for NHL; sex, size of largest mass at transplant, status at transplant and conditioning regimen for HD. NHL and HD patients were matched separately by their prognostic factors. Additionally, NHL were matched for histology, while both HD and NHL patients were matched for date of transplant as closely as possible. With this method 578 patients were matched in the NHL group, 352 in the HD group. In the HD group the PFS was unexpectedly better for ABMT patients (51% vs. 40% for PBSCT at 3 y;  $p=0.0191$ ). There was no difference in PFS in the NHL group. In HD the overall relapse or progression rate at 3 y for was lower after ABMT (34.2% vs. 57.5% after PBSCT;  $p=0.0105$ ); in NHL there was no difference. Transplant related mortality (<90 days) was lower, but not significantly, with PBSCT. Leukocyte, neutrophil and platelet recovery occurred faster with PBSCT, irrespective of disease. The matched-pair analysis confirms the advantage of PBSCT in terms of hematopoietic reconstitution, but fails to show any superiority in the long-term. The

worse results observed in HD receiving PBSCT may be explained by the fact that HD patients with chemoresistant disease showed a significant difference in PFS (60% at 1 y for ABMT vs. 38% for PBSCT;  $p=0.0065$ ) while those with chemosensitive or untested disease did not (53% at 3 y for ABMT vs. 47% for PBSCT;  $p=0.0745$ ). These findings need to be confirmed in randomized studies.

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#### **KINETICS OF HEMATOLOGICAL RECOVERY AFTER ABMT IN NON HODGKIN'S LYMPHOMAS USING DIFFERENT GROWTH FACTORS**

Miggiano MC, Gherlinzoni F, Rosti G, Lemoli RM, \*Mazza P, Ricci P, Salvucci M, Motta MR, Mangianti S, Rizzi S, Zinzani P.L, Tura S  
*Institute of Hematology "L. e A. Seràgnoli", S. Orsola Hospital, Bologna University; \*SS. Annunziata Hospital, Taranto, Italy*

We evaluated the kinetics of hematological recovery after autologous bone marrow transplantation (ABMT) for aggressive non-Hodgkin's lymphomas (NHL) in 58 patients (pts) all conditioned with the same chemotherapeutic regimen BAVC (BCNU, ARA-C, VP-16, cyclophosphamide). Twenty-four pts did not receive any growth factor (GF) after marrow reinfusion: 12 were autografted at diagnosis (Dia) and 12 were pretreated (at least 1 line of conventional chemotherapy - NoGFs); 10 pts received GM-CSF 10  $\mu\text{g}/\text{kg}/\text{die}$  iv; 14 pts received 5  $\text{mg}/\text{kg}/\text{die}$  sc; 10 pts received a combination of G-CSF 5  $\mu\text{g}/\text{kg}/\text{die}$  sc + IL-3 10  $\text{mg}/\text{kg}/\text{die}$  sc. All GFs were administered until the achievement of  $> 0.5 \times 10^9/L$  ANC for three consecutive days. Patients transplanted at diagnosis received the highest median number of mononuclear cells and CFU-GM, compared with the other four groups ( $p < 0.05$ ). One G-CSF + IL-3-patient died on day + 14 from ABMT because of pulmonary complication and is not evaluable for platelet recovery. Granulocytic recovery was significantly hastened in G-CSF and G-CSF + IL-3-patients compared to the other three groups (Dia, NoGFs, GM-CSF) (day with  $> 0.5 \times 10^9/L$  PMN + 12 and + 11.5 vs 15.5, 15.5 and 16, respectively;  $p = 0.01$ ). Patients transplanted at Dia exhibited the shortest platelet recovery, statistically significant in comparison to NoGFs, GM-CSF and G-CSF-patients (day with  $> 20 \times 10^9/L$  PLT + 13 vs + 18, + 17 and + 17.5, respectively -  $p < 0.003$ ) while G-CSF + IL-3 accelerate platelet recovery without a statistically significance (day with  $> 20 \times 10^9/L$  PLT + 14). These data confirm that marrow collection before any chemotherapy is a crucial factor influencing the kinetics of hematopoietic reconstitution after ABMT, probably due to the higher number and better quality of reinfused stem cells; as regards pretreated pts, the association of G-CSF and IL-3 seems to be the most effective both for granulocytic and platelet recovery.

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#### **AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR ACUTE MYELOID LEUKEMIA IN CHILDREN USING TOTAL BODY IRRADIATION AND MELPHALAN AS CONDITIONING REGIMEN**

Bonetti F, Montagna D, Pession A,<sup>o</sup> Messina C,<sup>#</sup> Santoro N,<sup>§</sup> Porta F,<sup>\*</sup> Taibi RM, Favre C,<sup>@</sup> Locatelli F  
*Clinica Pediatrica, Università di Pavia, IRCCS Policlinico San Matteo, Pavia; \*Clinica Pediatrica, °Clinica Pediatrica, Università di Bologna, Ospedale S.Orsola, Bologna; °Clinica Pediatrica, Università di Padova; §Clinica Pediatrica, Università di Bari; Università di Brescia, Spedali Civili, Brescia; @Clinica Pediatrica, Università di Pisa, Italy*

We have conducted a prospective study on 19 children with *de novo* AML in first complete remission (CR) treated with autologous bone marrow transplantation (ABMT), evaluating the combination of total body irradiation (TBI, 12 Gy in 6 divided fractions) and high-dose melphalan (140 mg/m<sup>2</sup> in single dose) in an attempt to improve antitumour efficacy of conditioning regimen. FAB classification for our group of patients was as follows: 3 patients were affected by M0, 1 by M1, 4 by M2, 2 by M3, 1 by M4, 7 by M5 and 1 child had M6. Seventeen patients received cryopreserved and *in vitro* purged (mafosfamide at a dose of 100 mg/mL) bone marrow, whereas in the remaining 2 patients marrow purging was not performed. The median time from 1st CR to ABMT was 5 months (range 2-25) with 14 patients having been transplanted within the first 6 months from diagnosis. All patients engrafted and the median time to achieve a sustained granulocyte count > 0.5×10<sup>9</sup>/L was 16 days (range 13 to 25 days), whereas the median time to obtain self-sustaining platelet levels higher than 20×10<sup>9</sup>/L was 34 days (range 25 to 50 days). Overall, extramedullary toxicity was mild, and largely restricted to oral and gastrointestinal mucositis. Otherwise the aplastic period was well tolerated, without significant complications and none of the 19 patients died of transplant-related complications. Two patients relapsed at 4 and 6 months after marrow transplant, respectively and this determined a relapse rate of 12%. Eighty-eight percent of all patients are projected to be alive and disease-free at 6 years (median follow-up 17 months). All surviving patients had a Karnofsky score of 100% at the time of the last clinical evaluation. Although the limited number of patients does not allow us to draw definitive conclusions, the association of TBI and melphalan proved to be safe in children given ABMT for AML. The limited incidence of relapse and the good EFS suggest that this myeloablative therapy together with *in vitro* marrow purging can increase the probability of patients with AML in 1st CR to be maintained in prolonged hematological remission.

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#### **AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELLS (PBPC) TRANSPLANT WITH IMMUNOSELECTED CD34<sup>+</sup> CELLS IN LYMPROLIFERATIVE DISEASES**

Di Mario A, Sica S, Teofili L, Menichella G, Rumi C, Salutati P, Rutella S, Lai M, Micciulli G, Storti S, Laurenti L, Leone G  
*Divisione di Ematologia-Istituto di Semeiotica Medica-UCSC Rome, Italy*

From December 1994 to March 1995, 4 patients, affected by lymphoproliferative disease with bone marrow

involvement (3 NHL-HG and 1 NHL-LG), were submitted to autologous transplantation with peripheral blood immunoselected CD34<sup>+</sup> cells. Two males and 2 females, mean age 42.5 years (range 25-43), refractory to conventional treatment, received salvage chemotherapy (HD-CTX or MiCMA) and rhG-CSF 5 mg/kg bw/day administration, followed by apheresis procedures to collect PBPC. The collected cells were positively selected using the CEPRATE SC stem cell concentrator (CellPro, Inc. Bothell, WA). A mean of 4.9×10<sup>6</sup>/kg bw (range 1.2-6.6) CD34<sup>+</sup> cells were transfused after myeloablative chemotherapy (3 BuCy2 and 1 BuMel). rhG-CSF 5 mg/kg bw/day was administered subcutaneously by day +1 until stable granulocyte recovery (neutrophils >0.5×10<sup>9</sup>/L).

Mean days to recovery 0.5×10<sup>9</sup>/L neutrophils and 50×10<sup>9</sup>/L platelets were 12 (range 11-15) and 43 (range 18-71) respectively. Despite this prompt engraftment we observed a mean of 5 days of fever >38°C, but a total of 7 documented infections (5 sepsis, 4 bacterial and 1 fungal; 2 viral infections, 1 CMV and 1 adenovirus). The length of iv broad spectrum antimicrobial therapy was 18 days (9-26). Three out of 4 patients also required antifungal treatment for persistent fever. We also observed one case of transfusional GvHD despite all patients received irradiated blood components (15 Gy). Transfusional support consisted of a mean of 4 erythrocyte concentrates and 2 single donor platelet units.

In this small group of patients we observed an increased number of documented infections in comparison with only 5 microbiologically documented sepsis in a historical group of 42 patients receiving transplant of unfractionated PBPC. This difference could be explained by the absence in immunoselected PBPC of lymphocytes, determining prolonged lymphopenia with very low levels of CD4<sup>+</sup> cells found after transplant, favouring opportunistic and viral infections.

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#### **CLINICAL FEATURES, RESPONSE TO THERAPY AND SURVIVAL IN A SMALL SERIES OF ANAPLASTIC LARGE CELL LYMPHOMA (ALCL)**

Cerno M, Fanin R, Geromin A, Silvestri F, Savignano C, Trani G, Rinaldi C, Damiani D, Russo D, Biffoni F, Baccarani M

*Division of Hematology and Blood Bank, Department of Bone Marrow Transplantation, Udine University and Hospital, Udine, Italy*

*ALCL is a distinct clinicopathologic entity among high-grade non Hodgkin's lymphomas. Its features are now well recognized and the frequency of its diagnosis is increasing. From February '90 through June '94, 16 consecutive unselected patients (pts) with ALCL were first seen in our Institution. The median age was 35 years (range 15-45), the male:female ratio was 10:6. Ten were common type and 6 were Hodgkin-related. Immunophenotype was T-cell in 6 cases, B-cell in 5 cases and null in 5 cases. Nine pts (56%) had stage II-IV disease; bulky adenopathy was present in 14 pts (87%) with mediastinal involvement in 9 pts. B*

symptoms were observed in 11 pts (69%). LDH level was elevated in 7 cases. Prognostic groups, according to the age-adjusted International Index were as follows: low 6 pts, low-intermediate 5 pts, high-intermediate 2 pts, high 3 pts. All pts were initially treated with 6 courses of chemotherapy (CHT) according to the F-MACHOP protocol. Residual mediastinal mass required radiotherapy (RT) in 6 cases. After first line therapy (CHT or CHT + RT), 8/16 pts (50%) were in CR and 8 were in PR. Between 1 and 4 months (median 2.5 mo.) after the end of therapy the marrow was harvested and autologous bone marrow transplantation (ABMT) was performed 1 to 8 months (median 2.7 mo) after the harvest. The BAVC regimen was used prior to the ABMT. No transplant-related death was observed. At present all the pts are alive with a median follow-up of 31 months (range 3-55) from ABMT and of 42 months (range 12-63) from diagnosis. Thirteen of the patients are in CR (81%), 3 have stable radiologic findings of a minimal residual disease. No progression or relapse were documented. These data suggest that most cases of ALCL can be successfully managed with sequential intensive chemotherapy and autologous stem cell rescue.

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#### **AUTOLOGOUS BONE MARROW TRANSPLANTATION (ABMT) FOR AGGRESSIVE NON HODGKIN'S LYMPHOMA (NHL)**

Geromin A, Fanin R, Cerno M, Silvestri F, Savignano C, Grimaz S, Rinaldi C, Damiani D, Russo D, Biffoni F, Baccarani M

*Division of Hematology and Blood Bank, Department of Bone Marrow Transplantation, Udine University and Hospital, Udine, Italy*

ABMT is currently widely applied for the treatment of aggressive NHL. Evidence that in these tumors dose intensity is a critical factor for remission and probably for disease free survival has already been accumulated. The results that were reported so far in the patients (pts), who were transplanted in complete or partial remission, suggest that ABMT can improve the cure rate when it is performed in cases with treatment-sensitive, low residual burden disease.

Here we report the data of a single center non-randomized trial, where ABMT was employed after first-line chemotherapy (CHT) in a group of 30 consecutive pts with aggressive NHL (M/F 18/12; median age 38 years, range 15-49). They were affected by anaplastic large cell lymphoma (16 pts), centroblastic l. (10 pts), T-lymphoblastic l. (2 pts), immunoblastic l. (1 pt) and T peripheral l. (1). Out of 30 pts, 19 were in stage III and IV, 23 had a bulky disease and 18 had general symptoms. Twenty-three pts were treated with the third generation regimen F-MACHOP for a total of 6 courses. The other 7 patients were treated with a similar regimen. After this induction CHT 17/30 (57%) pts were in CR and 13/30 (43%) pts in PR. Between 1 and 9 months (median 3 mo.) after the end of CHT the marrow was harvested and ABMT was performed 0.5 to 10 mo. after the harvest (median 3 mo.). The BAVC regimen was used prior to the ABMT. All the patients received G-CSF after the ABMT, for a median of

11 days. No transplant related deaths were observed. Median time to  $0.5 \times 10^9$  g/L PMN was 13 days (11-21) and to  $50 \times 10^9$ /L PLT was 17 days (20-38). Median time of hospitalization was 26 days (20-38). At present, with a median follow up of 33 months (11-76) from diagnosis, 26 of 30 pts (87%) are in CR and 4 are alive and well with small and stable residual disease. No progression or relapse were observed. In conclusion our data confirm that in pts with aggressive NHL, responding to CHT, consolidation with ABMT is a safe procedure, that is associated with optimal disease free survival.

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#### **GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF) MOBILIZED PERIPHERAL BLOOD PROGENITOR CELLS FOR AUTOLOGOUS TRANSPLANTATION**

Rinaldi C, Savignano C, Damiani D, Silvestri F, Fanin R, Cemo M, Grimaz S, Trani G, Geromin A, Baccarani M, Biffoni F

*Blood Bank and Division of Hematology, Department of Bone Marrow Transplantation, Udine S.M.M. and University Hospital, Udine, Italy*

In the field of peripheral blood stem cell transplantation (PBSCT) granulocyte colony-stimulating factor (G-CSF) has been employed in association with chemotherapy for the mobilization of stem cells into the peripheral blood. More recently G-CSF has been used as a single mobilizing agent both in the autologous and allogeneic PBSCT settings. Since May, 1994, at the Bone Marrow Transplantation Department of Udine, 10 patients eligible for PBSCT have been mobilized with G-CSF, employed at the dose of 16  $\mu\text{g}/\text{kg}/\text{die}$  as a single subcutaneous injection for 5-6 consecutive days. Five patients were affected by NHL, 2 by HD and 3 by MM. Median age was 43 years (range 23-55); male / female ratio was 6/4. The aphereses procedures, performed with the cell separator Fenwall CS 3000 Plus, were started on day +4; median aphereses/patient were 3 (3-4), with a volume of processed blood/apheresis of 9L (range 5-10). In all patients, baseline PB CD34<sup>+</sup> cells were calculated before starting the therapy and daily afterwards. PB CD34<sup>+</sup> cells peak was obtained on day +4 in 3 patients, on day +5 in 4 patients, while in the remaining 3 cases (2MM and 1NHL) a significant increase was not observed. Overall, median number of mononuclear cells/kg b.w. harvested was  $5.4 \times 10^8$  (2.0-7.9); median number of CD34<sup>+</sup> cells/Kg B.W. was  $4.3 \times 10^6$  (1.1-12.7). Six patients (4 NHL, 1 HD and 1 MM) have already been transplanted; all engrafted promptly, reaching PMN  $>500/\mu\text{L}$  and PLT  $>20000/\mu\text{L}$  at day +11 and +12 respectively. The results of this study indicate that a short course of G-CSF at the dose of 16  $\mu\text{g}/\text{kg}/\text{die}$  can result in the mobilization of significant number of MNC and CD34<sup>+</sup> cells that ensure prompt hematopoietic recovery after marrow ablation.

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#### **FEASIBILITY AND TOXICITY OF HIGH DOSE CHEMOTHERAPY WITH GROWTH FACTOR AND CIRCULATING PROGENITOR**

### SUPPORT: THE EXPERIENCE IN 111 PATIENTS WITH LYMPHOPROLIFERATIVE DISORDERS

Tarella C, Caracciolo D, Gavarotti P, Corradini P, Castellino C, Cherasco C, Bianchi A, Massaia M, Palumbo A, Boccadoro M, Pileri A

*Dipartimento di Medicina e Oncologia Sperimentale, Divisione Universitaria di Ematologia-Azienda Ospedaliera S. Giovanni, Torino, Italy*

Growth factor availability allowed novel chemotherapy modalities along with dose intensification. In particular the high dose sequential chemotherapy model (HDS) has been proposed, which includes a preliminar high-dose phase with cyclophosphamide, methotrexate and etoposide; an intensification phase ensues thereafter with peripheral blood progenitor cells (PBPC) autograft, mobilized following chemotherapy and growth factor. We report here feasibility and toxicity data in 111 patients treated in our Division. The program is mainly based on the original HDS scheme, and the autograft phase consisted of either TBI+L-PAM (45 patients) or L-PAM±mitoxantrone (38 patients). Lately the scheme was partially modified and tailored to different clinical situations. We treated 66 patients with non Hodgkin's lymphoma (42 at onset), 40 with multiple myeloma (39 at onset) and 5 with relapsed Hodgkin's disease. All but 14 patients received growth factor support (G-CSF in 80 and GM-CSF in 17 patients). The program was carried out in ordinary wards; protected rooms were only needed for the final autograft phase. The high dose phase was completed in 101 patients (90%), consolidation with PBPC autograft was performed in 83 patients (75%). Disease progression (13 cases) or poor PBPC harvests (6 cases) were the most common causes for not going through autograft. There were 5 acute fatal events (4,5%) occurring in the high dose phase (4 cases) or after autograft (1 case). Severe hematological toxicity was of short duration during the whole program. Severe extrahematological toxicity occurred in 15 patients (13,5%), precluding program completion in only 3 patients. In conclusion the HDS approach turned out to be feasible and well tolerated. These data, along with the high therapeutic efficacy make this strategy of potentially wide applicability in most chemosensitive tumors.

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### ENRICHMENT OF LEUKAPHERESIS HEMOPOIETIC PROGENITORS BY LOW DENSITY GRADIENT CENTRIFUGATION

Ferrero D, Cherasco C, Bondesan P, Caracciolo D, Omedè P, Giaretta F, Tarella C

*Dipartimento di Medicina ed Oncologia Sperimentale, Divisione Universitaria di Ematologia, Azienda Ospedaliera S. Giovanni, Torino, Italy*

Circulating hemopoietic progenitors, collected by leukapheresis after chemotherapy and growth factor stimulation, are widely employed for transplantation purpose. The application to this procedure of immunological purging techniques used for autologous bone marrow (BM) transplantation is quite cumbersome and expensive because of the large amount of cells involved (usually 2-4 leukapheresis containing a total cell number at least three

fold higher than average BM explant cellularity). Therefore, a pre-enrichment of hemopoietic progenitors could be quite valuable. However, in our experience, the classical Ficoll/metrizoate density gradient (1,077 g/L) was not so useful for that purpose as in case of BM explants, probably because of monocyte and granulopoietic precursor abundance in leukapheresis collections. Therefore, we have reduced the gradient density by adding to the commercial Ficoll/metrizoate solution (Lymphoprep, Nycomed Pharma, 1,077 g/L) 0.9% saline (NaCl) solution up to a final concentration of 15-16% Vo/Vo (final density 1,066-1,067g/L). Cells from 12 leukapheresis (median cellularity  $19,5 \times 10^9$ , range: 2.8-49.6), collected from 6 patients with non-Hodgkin lymphoma (4) or multiple myeloma (2) after chemotherapy and G-CSF stimulation, were centrifuged at 500 g on the above described density gradient for 30' at 20°C in 50 mL tubes ( $7-8 \times 10^8$  cells/tube). Cells at interface were collected. Cell depletion reached a median value of 80.5% (53-86), whereas 88% (49-97) of CD34<sup>+</sup> cells and 72% (51-100) of CFU-GM were collected. In 4 leukapheresis collections, from 3 patients, residual neoplastic cells were detected by immunofluorescence: 91.5-99.7% of them were removed by the gradient centrifugation. Therefore, low density gradient separation can greatly reduce the cellularity of leukapheresis collections (and possibly contaminating neoplastic cells of B lineage too), with a minimal loss of hematopoietic progenitors. It should be regarded as a useful pretreatment for immunological purging of leukapheresis cells.

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### HIGH-DOSE THERAPY AND AUTOLOGOUS PERIPHERAL BLOOD (PBSC) OR BONE MARROW (BM) STEM CELL TRANSPLANTATION FOR MULTIPLE MYELOMA: A GITMO RETROSPECTIVE STUDY

Majolino I, Vignetti M, De Laurenzi A, Meloni G, Fioritoni G, Leone G, Sajeve MR, Alessandrino EP, Carlo-Stella C, Coppetelli U, Tribalto M, Mandelli F.

*Gruppo Italiano Trapianti di Midollo Osseo (GITMO), Autologous Transplant Registry*

In order to analyse the long-term results and assess the impact of previous characteristics and transplant modalities on survival of myeloma patients, we have retrospectively reviewed the data of 87 patients autografted between 1988 and 1994 and reported to the *Autologous Transplant Registry* of GITMO. 51 were males and 36 females. Their median age at autograft was 51 y (range 33-61). At diagnosis 8 were stage I, 24 stage II and 49 stage III. 51 patients had IgG, 17 IgA, 1 IgM and 1 IgD disease. 12 were in CR1 at autograft, while 82 were chemosensitive but more advanced, 6 refractory and 2 in relapse. As transplant high-dose regimen, 19 received a combination including TBI, and 68 a chemotherapy combination. 73 patients were autografted with PBSC alone, while 6 received BM cells and 5 both PBSC and BM. Of the 57 patients evaluable for response, 33 (57%) entered CR, 16 (28%) PR, and 8 (14%) were non responders after the autograft. At the time of report, 65 patients are alive at a median of 18 months (range 1-60) from autograft. The overall survival for the entire group is 67% and progres-

sion-free survival 26% at 5 years.

We have analysed the impact on overall survival of the following factors: sex, age, Ig class, stage, response to previous treatment(s), time from diagnosis to transplant, BM plasma-cells and  $\beta$ -2 level at autograft, conditioning regimen (TBI vs non-TBI), response to autograft, use of growth factors and interferon after the autograft. In univariate analysis only initial stage, response to previous treatment(s), non-TBI conditioning and use of interferon appeared to positively correlate with survival. We confirm that high-dose therapy followed by autologous transplantation is able to induce remission in the majority of myeloma patients. However, most of them will eventually relapse. The factors influencing the outcome will be further studied in multi-variate analysis.

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#### **BLOOD PROGENITOR CELL AUTOTRANSPLANTATION: ANALYSIS OF FACTORS AFFECTING HEMOPOIETIC RECONSTITUTION IN 50 PATIENTS**

Olivieri A, Offidani M, Ciniero L, Brunori M, Centurioni R,\* Cantori I, Masia MC,\* Leoni P  
*Clinica di Ematologia, \*Istituto di Clinica Medica Università di Ancona, Italy*

Blood progenitor cells (BPC) autotransplantation can hasten the hematologic engraftment only if the amount of CD34<sup>+</sup> cells or CFU-GM reinfused is enough; but there is not a general agreement about the threshold dose required for these two parameters and about which factors can influence the speed of engraftment.

We studied 50 patients transplanted with BPC mobilized by high-dose chemotherapy followed by G-CSF or G-CSF plus erythropoietin. A mean of  $2.6 (\pm 0.3) \times 10^6/\text{kg}$  MNC,  $56.6 (\pm 10.7) \times 10^4/\text{kg}$  CFU-GM and  $9.8 (\pm 1.3) \times 10^6/\text{kg}$  CD34<sup>+</sup> cells have been infused.

Patients reached PMN  $> 0.5 \times 10^9/\text{L}$  on day +10 (range 8-14) and transfusion independence (platelets  $> 20 \times 10^9/\text{L}$ ) on day +12 (range 9-30). Two patients did not reach the  $50 \times 10^9/\text{L}$  platelets: one for the onset of an autoimmune thrombocytopenia and the other one because of the very low amount of CD34<sup>+</sup> cells reinfused ( $0.5 \times 10^6/\text{kg}$ ). The Cox univariate analysis included the following variables: age, sex, diagnosis, bone marrow involvement, previous chemo-radiotherapy, priming chemotherapy, growth factors priming, myeloablative regimens, sepsis, MNC/kg, CFU-GM/Kg and CD34<sup>+</sup> cells/kg; among these parameters, only CFU-GM/kg and CD34<sup>+</sup> cells/kg significantly influenced the rate of engraftment. Stepwise Cox analysis selected CD34<sup>+</sup> cells/kg as the only significant factor affecting the time for hemopoietic reconstitution. Patients reinfused with more than  $5 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells reached platelet transfusion independence more quickly than those reinfused with a lower dose (11 vs 15 days;  $p < 0.0001$ ). This threshold proved to be significant also for reaching the  $50 \times 10^9/\text{L}$  platelets (14 vs 17 days;  $p = 0.0013$ ) and  $150 \times 10^9/\text{L}$  platelets (19 vs 23 days;  $p = 0.0008$ ). Only one among the 40 patients (2.5%) given more than  $5 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells did not engraft completely vs 4/10 (40%) patients transplanted with a lower dose. Moreover both platelet transfusions (2.5 vs 4

apheresis units;  $p = 0.0044$ ) and hospitalization days (13 vs 16;  $p = 0.0003$ ) proved to be significantly reduced in patients transplanted with more than  $5 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells; on the contrary, even if in the latter group the duration of neutropenia (PMN  $< 0.5 \times 10^9/\text{L}$ ) was lower, both sepsis incidence and antibiotic requirement were similar. The probability of hemopoietic reconstitution, according to Kaplan-Meier method, showed that  $5 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells represent the most predictive cut-off, particularly for the platelet engraftment, surely better than 2.5 or  $8.0 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells.

In conclusion the CD34<sup>+</sup> cell/kg number can be used to carefully predict the rate of engraftment regardless all the other patient characteristics; the majority of patients transplanted with  $5 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells get the transfusion independence within 2 weeks and the complete and stable hemopoietic reconstitution within 4 weeks with a very low blood product transfusion requirement.

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#### **G-CSF 5 vs 10 mcg/kg GIVEN AFTER CYCLOPHOSPHAMIDE 4 G/M<sup>2</sup> FOR MOBILIZATION OF PBSC**

Indovina A, Buscemi F, Vasta S, Scimè R, Malleo C, Felice R, Pampinella M, Catania P, Fiandaca T, Santoro A, Majolino I

*Department of Hematology and BMT Unit, Ospedale Cervello, Palermo, Italy*

To assess the best PBSC collection scheme, we randomized 18 patients (median age 42.5 years, range 15-56) with malignant lymphomas (16 NHL, 2 HD) to receive 2 different doses (5 vs. 10  $\mu\text{g}/\text{kg}/\text{day}$ ) of G-CSF after cyclophosphamide (CY) 4g/m<sup>2</sup>. G-CSF was given starting the day after CY and until the end of collections. Age, chemotherapeutic data, status and stage at mobilization were similar in both groups. After CY, short duration neutropenia (PMN count  $< 0.1 \times 10^9/\text{L}$ ) was observed in 4 patients. In no case a platelet count  $< 20 \times 10^9/\text{L}$  was found. The PMN and platelet nadir was reached after a median of 7 days (range 5-9 and 5-8 days respectively). No transfusional support was required and only 1 patient needed hospitalization due to fever without isolations. No significant difference was noticed between the 2 groups in terms of hematologic toxicity. CD34<sup>+</sup> cells and CFU-GM peaked at a median of 9 days (range 7-14 and 7-11 respectively). CD34<sup>+</sup> cells increased 36 fold (range 10-191) and CFU-GM 22 fold (1.8-197.2) above baseline values, without difference between the 2 mobilization schedules. 2-4 (median 2.5) collections were performed with a target of 3 procedures or  $7 \times 10^6/\text{kg}$  CD34<sup>+</sup> cells collected. Median yield was  $7.3 (1-29.7) \times 10^6/\text{kg}$  CD34<sup>+</sup> cells and  $29.8 (8-105.3) \times 10^4/\text{kg}$  CFU-GM without significant difference among the 2 groups. In conclusion, this mobilization protocol is avoided of toxicity and suitable for an outpatient collection program. After Cy 4g/m<sup>2</sup>, PBSC mobilization is not influenced by G-CSF dose.

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#### **HIGH-DOSE (HDS) CHEMOTHERAPY AND AUTOGRAFTING IN**

### LOW/INTERMEDIATE GRADE B-CELL NON-HODGKIN'S LYMPHOMAS: ANALYSIS OF MINIMAL RESIDUAL DISEASE

Corradini P, Astolfi M, Ladetto M, Caracciolo D, Bondesan P, Cherasco C, Campana S, Pileri A, Tarella C

*Dipartimento di Medicina ed Oncologia Sperimentale, Divisione Universitaria di Ematologia, Az. Ospedaliera S. Giovanni, Torino, Italy*

Twenty-four patients with low/intermediate grade B-cell non-Hodgkin's lymphomas (NHL) (B through G subtypes, according to the Working Formulation) were enrolled in a HDS program including a final autografting phase. The presence of residual lymphoma cells has been evaluated in peripheral blood (PBPC), bone marrow (BM) cell harvests, and after autografting. Residual disease was assessed by PCR, using the bcl-2 oncogene or immunoglobulin heavy-chain (IgH) gene rearrangements as tumor cell markers. Rearranged variable regions (VDJ) were amplified using sense primers derived from the leader, first or third framework (FR) regions, and an anti-sense primer derived from joining regions (JH). Amplified VDJs have been directly sequenced or cloned, and the second and third complementarity determining regions (CDR2 and CDR3) identified. Minimal residual disease was assessed using CDR2 and JH primers. PCR products were then hybridized to a CDR3 probe. In 20 of 24 patients (83%) a molecular marker was available (9 based on bcl-2 translocations, and 11 on IgH rearrangements). Fifty-two PBPC and 19 BM harvests have been tested for the presence of residual lymphoma cells. In 10 of 20 patients, lymphoma cells were not detected in PBPC or BM cell harvests. Among patients with PCR-negative cell harvests, 6 had a morphologically evident BM infiltration at diagnosis, and 4 were PCR-positive. It is noteworthy that after autografting, PCR-negative patients had BM samples negative, with a median follow up of 25 months (range 3-46 months). In conclusion, HDS regimen is able to provide clinical and molecular remission in 50% of patients. A larger panel of cases and a longer follow-up is required to verify if PCR-negativity corresponds to a longer disease free survival.

*Supported by AIRC.*

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### HUMAN HERPESVIRUS-8 (HHV-8) IN AIDS-RELATED KAPOSI'S SARCOMA (KS) AND NON-HODGKIN'S LYMPHOMA (NHL)

Torelli G, Luppi M, \*Maiorana A, \*Collina G, Barozzi P, Marasca R

*Department of Medical Sciences, Section of Hematology and \*Department of Pathology, University of Modena, Italy*

Very recently, Chang *et al.* (*Science* 1994; 266:1865), identified novel and specific DNA sequences in KS tissues from patients with AIDS. The presence of these sequences, which showed homology to two genes of the Epstein-Barr virus (EBV), argued in favour of the existence of a new human herpesvirus called HHV-8, which was thus proposed to be the major etiologic infectious agent of AIDS-

related KS. First, we used polymerase chain reaction (PCR) to identify specific HHV-8 sequences, derived from the putative minor capsid gene, in 8 of 10 KS tissue specimens obtained from AIDS patients, confirming the results recently reported. We also reasoned that if this novel putative virus does really cause KS, it should be also found in classic KS patients, the elderly Mediterranean men not infected with HIV and without iatrogenic immunosuppression. In fact, using the same PCR technique we could identify viral sequences in KS skin biopsies of 16 out of 20 Italian elderly men not infected with HIV. Of interest, HHV-8 sequences were identifiable in tissue samples representative of all the three different histologic lesions, namely the patch (2 of 3), the plaque (4 of 6) as well as the nodular (10 of 11) stages of the disease. The putative lymphotropism of this newly identified herpesvirus as well as the recent observation that HHV-8 sequences could be detected in non KS tissues from AIDS patients, prompted us to investigate the presence of these viral sequences also in a well characterized series of Hodgkin's disease (HD) and NHL patients, AIDS related or not. Of interest, we could identify HHV-8 sequences in the pathologic lymph node biopsies obtained from 4 out of 18 AIDS-related B-cell NHL patients, while we did not identify viral sequences in any of the 20 HD and 15 B- and T- cell NHL patients not infected with HIV. Moreover, all the peripheral blood samples from 13 healthy donors resulted negative. In conclusion, the occurrence of HHV-8 sequences represents a phenomenon specific of KS, certainly not common to healthy population nor to HIV negative patients with lymphoid diseases. However, the identification of HHV-8 sequences in a percentage of AIDS-related NHL suggests a possible role of this newly identified herpesvirus in the multistep process of AIDS-associated lymphomagenesis, as it has been already proposed for EBV, which is another member of the same  $\gamma$ -herpesvirus family.

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### EXPRESSION OF DIPEPTIDYLAMINOPEPTIDASE IV-CD26 IN CIRCULATING LYMPHOCYTES OF HEMOPHILIC SUBJECTS

Poli L, Iannone AM, Fiamenghi C, Giusto M, Mosconi E, Comolli G,\* Bernuzzi S, Invernizzi R, Gamba G

*Dipartimento di medicina Interna, Medicina Interna ed Oncologia medica; Dipartimento di malattie Infettive, IRCCS Policlinico S. matteo e Università di Pavia, Italy*

CD26 antigen, a 110KD membrane glycoprotein with hexopeptidase activity (DAPIV), represents an activation marker of T lymphocytes that is preferentially expressed on CD4<sup>+</sup> memory cells. CD26 antigen is involved in T-cell proliferation and IL-2 production after antigenic stimulation and may constitute a co-receptor for HIV. We employed cytochemical and immunocytochemical techniques, using a monoclonal antibody (cl-BA5), to study DAPIV-CD26 expression in circulating lymphocytes from 43 hemophilic subjects in order to identify possible immunological-related alterations. In fact, it is well known that hemophilic patients chronically treated with coagulation factors show an imbalance among lymphocyte subpopulations as a result of both exposure to viral agents and antigenic stimulation from transfusional thera-

py. Among the group of HIV-negative hemophiliacs (38 cases), DAPIV-CD26 expression was not significantly different from that in normal controls, independently of the quantity of blood transfused and possible previous exposure to hepatitis viruses. In the 5 HIV-positive hemophilic patients, all asymptomatic for AIDS (stages CDII-CDIII), both the cytochemical and immunocytochemical procedure, revealed a statistically significant reduction in the absolute number of reactive lymphocytes with respect to both normal controls and the HIV-negative hemophiliacs. In 3 cases of the 5 cases this deficit was associated with CD4<sup>+</sup> cell depletion; on the other hand, a progressive reduction in the number of CD4<sup>+</sup> lymphocytes was only detectable through sequential studies in the other 2 HIV-positive hemophilic patients.

In conclusion, evaluation of DAPIV-CD26, which seems to decline sooner than CD4<sup>+</sup> in the course of HIV infection, might represent a useful option for the immunological monitoring of all patients, hemophilic or non-hemophilic, at risk for AIDS.

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#### COMBINATION AZT + METHOTREXATE IN HIV-RELATED HIGH-GRADE NON HODGKIN'S LYMPHOMAS (NHL)

Gherlinzoni F, Tosi P, Coronado O,\* Mazza P,<sup>#</sup> Costigliola P,\* Miggiano MC, Visani G, Chiodo F,\* Tura S.

*Institute of Hematology "L. & A. Seràgnoli", \*Institute of Infectious Disease, S. Orsola Hospital - Bologna University; <sup>#</sup>SS. Annunziata Hospital, Taranto, Italy*

AZT is a thymidine analogue useful in ARC-AIDS treatment. We have previously demonstrated that this compound possesses a significant antineoplastic activity when combined with drugs that inhibit *de novo* thymidylate synthesis, such as fluorouracil or methotrexate (MTX). In the present study we have tested the combination AZT + MTX in patients with high grade HIV-related NHL.

We have so far treated 22 patients, 16 males and 6 females, the mean age was 34.5 years. Mean CD4<sup>+</sup> lymphocytes at diagnosis were 82.6/ $\mu$ L, in 18 cases they were less than 150/ $\mu$ L; mean p24 antigen was 60.5 pg/mL. A previous diagnosis of AIDS was made in 12 patients: in 10 cases for infection and in 2 cases for Kaposi sarcoma. According to REAL classification, histological diagnoses were: Burkitt lymphoma (5 cases); diffuse large cell B lymphoma (12 cases); lymphoblastic lymphoma (1 case); anaplastic large cell lymphoma CD-30-positive (1 case); peripheral T cell lymphoma (1 case); high grade inclassifiable lymphoma (2 cases). Stage IV was present in 14 patients at diagnosis; extranodal involvement was fairly common (bone marrow in 8 cases; Waldeyer's ring in 3 cases; liver CNS and skin in 2 cases; spleen, cervix, gut, pleura and soft tissues in 1 case); bulky disease was present in 9 cases. The chemotherapy protocol consisted of MTX 1 g/sqm/day (day 1, 8 and 15) with leucovorin rescue and AZT at increasing doses: 2 g/sqm (days 1 to 3), 4 g/sqm (days 8 to 10) and 6 g/sqm (days 15 to 17). In case of Burkitt lymphoma or CNS involvement, intrathecal MTX + Dexametazone were administered. At the end of the third course, restaging procedures were performed and, in case of complete or partial remission, 3 more

courses with AZT at the maximum dose were administered. Out of 18 evaluable patients, 9 (50%) obtained a complete remission and 6 (33.3%) a partial response. Mean duration of the response was 8.7 months. Tolerance to therapy was good; grade III-IV neutropenia was observed after 17/81 courses, grade III-IV anemia after 10/81 courses, thrombocytopenia was never observed. Granulocyte-colony stimulating-factor (G-CSF) was administered after 46 courses in order to avoid neutropenia. In no case was it observed an increased incidence of infections. In conclusion, the combination AZT + MTX was effective and well tolerated in patients with HIV-related NHL.

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#### AMPHOTERICIN B AND GRANULOCYTE TRANSFUSION FOR AN APLASTIC PATIENT WITH PULMONARY ASPERGILLOSIS TO BE TRANSPLANTED

Fontana R, Catalano L, Scarpato N,\* Notaro R, Picardi M, Attingenti E, Rocco S, Parasole R, PEtrillo E, Cacciapuoti C,\* Formisano S,\* Rotoli B

*Cattedre di Ematologia, \*Immunoematologia and Microbiologia, Università Federico II, Naples, Italy*

A 19-year-old man had very severe aplastic anemia in February 1995 (PMN 0, PLTS 3000). Soon after diagnosis, he developed rhinosinusitis and bilateral pneumonia by *Aspergillus fumigatus*. A HLA matched brother was available. In order to proceed with the transplant, an intensive antifungal treatment was adopted: amphotericin B 1.5 mg/kg i.v./day associated with alternate day transfusion of white cells, which were obtained by granulocyte-apheresis from related donors who had been pretreated with G-CSF 300 ug s.c. x 4 days. Conditioning was Cy 200 mg/kg;  $3.63 \times 10^8$ /kg bone marrow nucleated cells were infused, and GvHD prophylaxis was CS-A+MTX. GM- and G-CSF were given at day +1.

**Results.** Nine apheresis from three donors were performed by processing 4,400 mL blood volume with a CS 3000 (program 2), yielding a mean of  $187 \times 10^6$  PMN cells ( $2.67 \times 10^8$  patients b.w.). At 12h from the infusion, *in vivo* WBC were >500 after 7 infusions and unchanged after 2. Within 12h from the first infusion, the fever disappeared, cough was reduced and a marked clinical improvement was observed. Hematological reconstitution was PMN >500 on day +23 and PLTS >25,000 on day +40. Irradiated blood products given during transplant: 14 platelet apheresis and 14 RBC concentrates. Amph B total dose was 3.5 g. A second course of amph B was started on day +46 because of recurrence of fever and cough and evidence of *Aspergillus* in the spit; a complete recovery followed.

**Comments.** *Aspergillus* pneumonitis is an emergency in bone marrow transplant. Granulocyte-apheresis from G-CSF treated donors is a safe and well tolerated procedure that may be highly effective in selected patients in the peri-transplant period.

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#### LOW INCIDENCE OF PLATELET REFRACTORINESS IN

**PATIENTS UNDERGOING AUTOLOGOUS BONE MARROW TRANSPLANTATION (ABMT) TRANSFUSED WITH NOT FILTERED BLOOD COMPONENTS**

Rinaldi S, Amaddii G, Gentilini I, Casini M, Graziotin B, Prinoth O, Coser P

*Hematology and Blood Transfusion Center, Ospedale Generale Regionale, Bolzano, Italy*

From January 1988 to December 1993 we performed a study on platelet refractoriness in 89 patients undergoing ABMT for hematologic malignancies. Only 18% of transfusions were filtered, because, lacking CMV negative donors, CMV positive blood components had to be transfused to some CMV negative patients.

71% of platelet transfusions derived from single donor aphereses, random platelets being transfused only in case of emergency. All blood components transfused had been irradiated with 20 Gy of  $\gamma$ -rays to prevent the transfusional GVHD. Platelet refractoriness has been evaluated by the *corrected count increment* (CCI) and patients have been

considered to be refractory, if CCI was  $<7,500$  24 hours after platelet transfusion for two consecutive transfusions, in the absence of fever  $>38^{\circ}\text{C}$ , diffuse intravascular coagulation, sepsis, splenomegaly and therapy with Amphotericin-B. 6 out of 89 patients, 3 males and 3 females (6.7%), developed platelet refractoriness. In such refractory patients transfusion requirements of erythrocytes and platelets were higher than those of not refractory patients, with an average number of 51.8 versus 33.4 units transfused. These results are in agreement with the percentage of refractoriness reported in literature, using filtered blood components (*Brand A et al, Vox Sang 1988; 54: 160-6. Van Marwyk Kooy et al, Blood 1991; 77: 201-5*). We believe that this might depend on three factors: 1) chemotherapy dependent immunodeficit; 2) administration of less antigenic diversities through the use of single donor platelet aphereses (even if in literature a different incidence of sensitization using random platelets has not been reported); 3) irradiation of blood components with  $\gamma$ -rays (*Mincheff MS et al, Vox Sang 1993; 65: 18-24*), which could modify

the antigenicity of the product, through the alteration of the function of the APC (*antigen presenting cells*), as demonstrated with UV irradiation.

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