established in 1920

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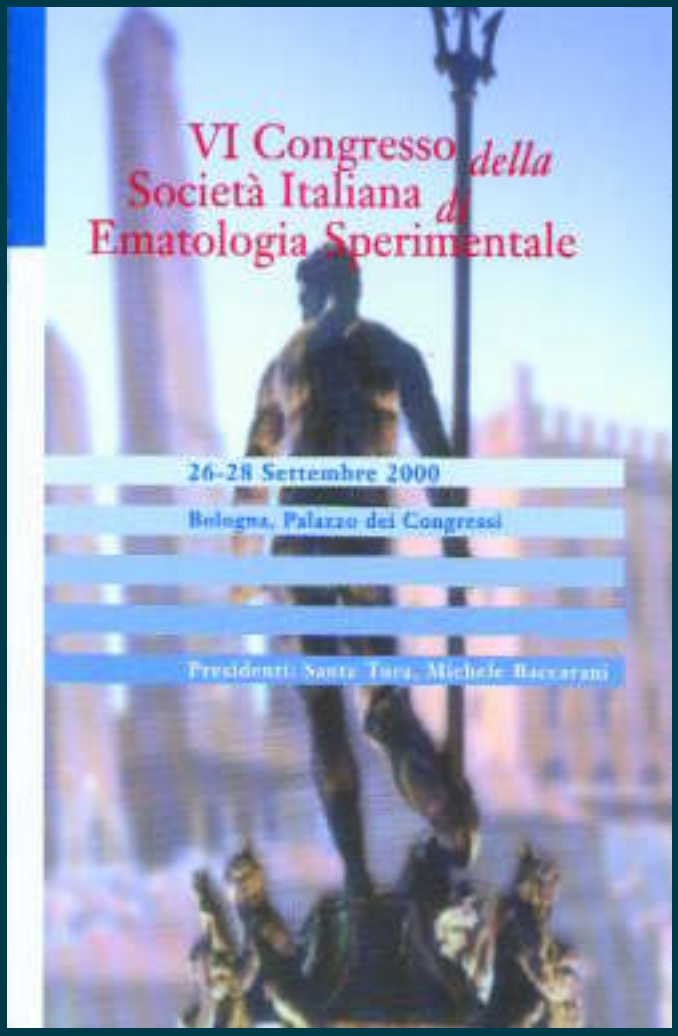

Haematologica

Journal of Hematology
founded in 1920 by Adolfo Ferrata

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Editorial policy, subscriptions and advertisements

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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. p. 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. p. 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. p. 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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Nervi C, Ferrara FF, Fazi F, Bianchini A, Padula F, Gelmetti V, Pelicci PG, Lo Coco F

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Acute promyelocytic leukemia (APL) is a prototype of successful differentiating therapy with retinoic acid (RA) and is characterized by alteration of the RA signaling pathway.In fact, *in vitro* studies have shown that APL fusion protein(s) are able to aberrantly recruit a transcriptional repressive complex containing histone deacetylase activities (HDACs) to the response elements of RA target genes. Such molecular features, which account for the differentiation block underlying APL pathogenesis seem not restricted to APL, having been recently described also in other AMLs. In fact, a complex containing HDAC activity is recruited by AML1/ETO, the oncoprotein resulting from the t(8;21) chromosomal translocation associated with AML-M2. Furthermore, RA and nuclear hormone receptors transcriptional co-regulators with putative HAT activities (such as p300, CBP, MOZ and TIF2), are present in chromosomal rearrangements associated with some AML-M4 and M5 cases. In addition, a number of recent pieces evidences from several laboratories indicate a pivotal role for the RA receptor signaling pathway in regulating normal myelopoiesis. Together, these findings suggest that modification of HDAC activities resulting in altered chromatin structure at the level of RA target promoters and genes might represent a general mechanism associated with non-M3 AML pathogenesis. We therefore analyzed, in AML cell lines and cytogenetically characterized primary cells from 23 M2 or M4 AML patients, the effect of RA and HDAC inhibitors, tricostatin A (TSA) and sodium phenylbutyrate as single agents or in combination. In all cases, TSA treatment was able to restore or potentiate the effect of RA on granulocytic differentiation. This event occurred irrespectively of the presence or absence of known genetic lesions through the modification of the acetylation status of H3 and H4 histones. This in turn, resulted in the restoration of the RA-dependent transcriptional activation of specific RA-responsive promoter activities and target gene expression. In addition, we found that the AML1/ETO, the commonest AML-associated fusion protein, acts as an HDAC-dependent repressor of RA-signaling. These findings relate alteration of the RA-pathway to myeloid leukemogenesis and underscore the potential of transcriptional /differentiation therapy in AML.

05
OLIGOMERIZATION OF TRANSCRIPTION FACTORS IN ACUTE MYELOID LEUKEMIAS

Minucci S, Pelicci PG

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RAR and AML1 transcription factors are found in acute myeloid leukemias (AMLs) as fusion proteins with PML and ETO, respectively. Association of PML-RAR and AML1-ETO with the nuclear co-repressor (NCoR)/histone deacetylase (HDAC) complex is required to block hematopoietic differentiation. We show that PML-RAR exists *in vivo* within high molecular weight (HMW) nuclear complexes, reflecting its oligomeric state. Oligomerization requires the coiled coil region of PML, and is responsible for abnormal recruitment of NCoR, transcriptional repression, and impaired differentiation of primary hematopoietic precursors. Fusion of RAR to an heterologous oligomerization domain recapitulated the properties of PML-RAR, indicating that oligomerization *per se* is sufficient to achieve transforming potential. Other chromosomal translocation partners of RAR in AML (PLZF, NPM, NuMA, STAT5) are also found as oligomers, and they are found to contribute their oligomerization domain to the corresponding fusion proteins (PLZF-RAR, NPM-RAR, NuMA-RAR, and STAT5-RAR, respectively). Strikingly, oligomerization of AML1 through a self-association domain of ETO is also required for abnormal transcriptional regulation, and impaired differentiation of primary hematopoietic precursors. These results show that oligomerization of a transcription factor, imposing an altered interaction with transcriptional co-regulators, represents a novel mechanism of oncogenic activation.

06
IN VIVO RESISTANCE OF HUMAN BCR/ABL+ LEUKEMIC CELLS TO THE ABL INHIBITOR STI571 MEDIATED BY ALPHA 1 ACIDIC GLYCOPROTEIN

Gambacorti-Passerini C, Barni R, leCoutre P, Zucchetti M, Cabrita G, Cleris L, Rossi F, Gianazza E, Pioltelli P, Pogliani E, Corneo GM, Formelli F, D'Incalci M

Dept. of Experimental Oncology, Istituto Nazionale Tumori, Milano, Italy; Mario Negri Institute for Pharmacological Research, Milano, Italy; University of Milano, Department of Pharmacological Sciences, Milano, Italy; Section of Hematology, University of Milano Bicocca, S.Gerardo Hospital, Monza, Italy

STI571 (formerly known as CGP57148B) is a potent inhibitor of bcr/abl, an oncogenic fusion protein that causes chronic myeloid leukemia (CML). We previously showed that STI571 can cure mice injected with human BCR/ABL+ leukemic cells, if continuous inhibition of the kinase activity of bcr/abl is maintained. This model was used to study the possible development of resistance to STI571. Nude mice were injected with the Bcr/Abl-positive human leukemic line KU812. Tumor-bearing mice were treated orally with STI571 according to three different time schedules (1, 8 and 15 days after the s.c. injection of $50x10^6$ KU812 cells). Leukemic cells were recovered from relapsing animals and used for *in vitro* experiments. Tumor reduction was observed in all animals; however cure rates decreased in the three groups from 100% to 0%. Relapsed animals did not respond to further treatment, and the bcr/abl kinase activity was not inhibited by STI571 administration in these mice, although plasma concentrations as high as 10 µM were obtained. Tumors from relapsed, resistant animals, showed *in vitro* IC_{50} (0.1-0.3 µM) not significantly different from that of the parental KU812 line. These results were compatible with the presence of a binding factor in the plasma of relapsed animals. A number of proteins were tested *in vitro* for their ability to inhibit the biological activity of STI571. While albumin did not substantially influence STI571 activity, 1 acidic glycoprotein (AGP) did at physiologic concentrations, increasing the IC_{50} for STI571 up to 90 fold. AGP also inhibited the effect of STI571 on bcr/abl phosphorylation *in vitro*. The association constant (Ka) for specific binding to STI571 was calculated and found to be 21 times higher for AGP than for albumin. AGP levels were measured in mice by an immunoassay: a strong correlation was found between tumor load and AGP concentrations. In addition, pretreatment with STI571 *in vivo* also increased AGP plasma levels. These results suggest that rising AGP levels, induced either by the tumor or by the treatment itself, were responsible for the development of resistance to STI571. Several compounds able to competitively bind AGP were tested. Erythromycin (at 5-30 µM) reverted the blockage of STI571 activity operated by AGP *in vitro* (proliferation and phosphorylation assays), and had no activity by itself. Erythromycin was therefore co-administered (350 mg/kg p.o.) with STI571 (160 mg/kg p.o.) every 8 hours for 21 days to animals bearing large tumors. The tumors in all animals initially shrunk; however the animals receiving the combined treatment experienced a faster and greater tumor reduction, a higher percent of animals with disappearance of tumors (14/15 vs. 5/15) and a larger fraction of mice obtaining long-term tumor-free survival (10/12 vs. 1/13 at day 180, $p < 0.001$). AGP binds STI571 and can significantly inhibit its biological activity both *in vitro* and *in vivo*; erythromycin competes with STI571 for AGP binding and increases its biological activity both *in vitro* and *in vivo*. Data from CML patients treated with STI571, which are compatible with the above reported model, will also be presented. In addition, results obtained *in vitro* will be presented and discussed.

07
CELL CYCLE INHIBITORS

G. Draetta

SIES-GITMO Symposium EXPERIMENTAL ASPECTS OF ALLOGENEIC STEM CELLS TRANSPLANTATION

Chairmen: J. Ferrara, M.F. Martelli

08
PLASTICITY OF ADULT STEM CELLS: NEW EXPERIMENTAL STRATEGIES AND CLINICAL APPLICATIONS

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Somatic tissue-derived stem cells of mammalian adults have been traditionally viewed as pluripotent precursors capable of life-long maintenance of cellular compartments typical of the tissue in which they reside. However, in recent years, *in vitro* cultures and *in vivo* transplantation assays have indicated that adult somatic stem cells of various species are capable of undergoing multiple fates. Bone marrow cells can give rise to a wide array of phenotypes, including blood, endothelial, bone, cartilage, fat, tendon, lung, liver, muscle, marrow stroma and even brain cells. Conversely, neural stem cells as well as muscle progenitors may contribute to blood cell production, indicating that adult stem cells present in numerous tissues can generate multiple cell types even of different dermal origin. Therefore, the developmental potential of adult somatic stem cells should be reassessed, although the mechanisms that ultimately lead to cell fate determination are not completely defined. Homeobox-containing genes, which encode DNA-binding proteins, appear to be strong candidate genes to regulate a number of developmental processes, including neurogenesis and hematopoiesis. Several findings point to the therapeutic potential of somatic adult stem cells and a major challenge will be to develop new strategies to exploit their versatility and self-renewal ability. First, the successful culturing and long-term expansion of versatile stem cells provides a great means to gain further insight into stem cell biology and may provide a source of renewable cells that can be transplanted. Second, it is now conceivable to direct *in vitro* adult pluripotent cells along defined lineages and generate cell type-specific somatic precursors that might represent ideal targets for gene ther-

apy. Third, much evidence indicates that stem cells derived from different sources and systemically introduced into the recipient can migrate via the circulation, reach the appropriate target tissue and acquire the phenotype of the cell compartments typical of that tissue. Furthermore, an important point is that genes introduced in the stem cells are then expressed in a tissue-specific manner. Fourth, it appears that stem cells can contribute to the regeneration of multiple tissues without preconditioning of the recipients. Therefore, these observations, mostly obtained in the murine system, lead to future hope of therapeutic stem cell use in a wide spectrum of diseases and disorders of several human tissues.

09
MARROW STROMAL CELL TRANSPLANTABILITY

Carlo-Stella C

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In addition to hematopoietic stem cells (HSCs) which can differentiate to produce progenitors committed to terminal maturation, bone marrow also contains stem cells of non-hematopoietic tissues which are currently referred to as *mesenchymal stem cells* or *marrow stromal cells* (MSCs). Homing of reinfused stromal cells to the marrow microenvirnoment is a crucial prerequisite for the therapeutic use of MSCs. In animal models, reinfused MSCs migrate to and become incorporated into several tissues of the recipients where they are capable of eliciting tissue-specific differentiation programs. In humans, conflicting results so far reported can be explained by either methodological differences in detecting donor-derived MSCs, or differences in the doses of reinfused stromal cells. The heterogeneity of conditioning regimens, post-transplant immunosuppression, graft manipulation and patient-related biological differences might account for such conflicting results. Changes of stem cell transplantation (SCT) methodology, including the introduction of highly immunosuppressive and myeloablative conditioning regimens or the use of blood-derived mega stem cell doses, might play a role in MSC transplantability. Indeed, data in NOD/SCID mice suggest that transplantation of mobilized blood progenitors can also regenerate the marrow microenvironment. Recently, we investigated the transplantability of MSCs in patients who had received a sex-mismatched, T-cell depleted allograft from an HLA-matched or HLA-mismatched family donor. Polymerase chain reaction (PCR) analysis of human androgen receptor (HUMARA) or amelogenin genes was used to detect donor-derived MSCs. Sixty-eight marrow samples from 41 consenting patients were inoculated in long-term culture but only 14/41 patients (34%) evaluated at a median of 17 months (range, 1 to 82) after allografting, generated a marrow stromal layer adequate for PCR analysis. To prevent the risk of false positive detection of donor cells caused by monocyte-macrophage contamination of marrow stromal layers, cultures were repeatedly trypsinized and treated with the anti-lysosomal compound leu-leu methyl ester. Under these experimental conditions, monocyte-macrophage contamination was below the levels of sensitivity of HUMARA and amelogenin assays (5% and 3%, respectively). Twelve patients allografted with female donors were analyzed by means of the HUMARA assay and in 5/12 cases a partial female origin of stromal cells was demonstrated. Two patients allografted with male donors were analyzed by amplifying the amelogenin gene and in both cases a partial male origin of stromal cells was shown. Fluorescent *in situ* hybridization analysis using a Y probe confirmed the results of PCR analysis and demonstrated in two cases the existence of mixed chimerism at the stromal cell level. In conclusion, MSCs reinfused in patients receiving a T-cell depleted allograft have a limited capacity to reconstitute marrow mesenchymal cells. Such a limited functional capacity strongly suggests that conventional hematopoietic grafts should be supplemented with *ex vivo* generated mesenchymal cells capable of long-term functional capacity in order to improve marrow stromal reconstitution following hematopoietic SCT.

10
CHARACTERIZATION OF NON-IMMUNOGENIC CD34+ PROGENITOR CELLS

Rondelli D

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Professional antigen presenting cells (APC) activate T-cells by delivering a first signal through the HLA:T-cell receptor binding and a second signal through co-stimulatory molecules such as B7-1 (CD80) and B7-2 (CD86), or CD40. Highly purified CD34+ blood cells are on average 95±2% HLA-DR+, 65±15% CD18+, 1.9±1.2% CD86+, but do not express CD80. Irradiated CD34+ cells that express CD18, the beta-chain of a leukointegrin family, induce a high proliferative response of allogeneic purified CD4+ and CD8+ T-cells in primary mixed leukocyte culture (MLC), while CD34+CD18- cells, that are enriched in early progenitors, stimulate allogeneic T-cells poorly. Moreover, the immunogenic activity of CD34+CD18+ cells is likely to be predominantly mediated by B7 co-stimulatory molecules since both CD80 and CD86 are upregulated in 7-12% of these cells after 24-30 hours of liquid culture with autologous or allogeneic mononuclear cells and anti-CD80 plus anti-CD86 blocking monoclonal antibodies induce > 80% inhibition of CD34+ cell APC activity. Another important co-stimulatory molecule, CD40, is constitutively expressed on 3.2±4.5% CD34+ blood cells. However, after 24 h in liquid culture with medium alone, or with tumor necrosis factor-α (TNF-α), or with allogeneic mononuclear cells, on average 10±3%, 75±15% and 53±17% CD34+ blood cells, respectively, are CD40+. TNF-α-primed CD34+CD40+ blood cells express myeloid markers, such as CD13 and CD33, but not monocytic (CD14), dendritic (CD1a), or lymphoid (CD3 and CD19) antigens, and contain <5% CD86+ and CD80+ cells. Also, after short priming with TNF-α CD34+ blood cells show a 2-3 fold greater alloantigen presenting function than CD34+ cells primed with GM-CSF or unprimed ones. In fact, highly purified TNF-α-primed CD34+CD40+ blood cells, which are enriched in both granulocytic and monocytic/dendritic precursors, stimulate a potent alloreactive T-cell response in MLC. In contrast, CD34+CD40- blood cells, which are enriched in granulocytic and erythroid precursors and in LTC-IC, are unable to stimulate HLA mismatched T-cells *in-vitro*. Furthermore, in the presence of an anti-CD40L antibody, CD34+ cells have a reduced capacity to activate primary CD4+ T-cell proliferative responses, but still stimulate efficient cytotoxic

responses. Current studies are evaluating whether selection of non-immunogenic CD34+ cells, or use of blocking antibodies in combination with CD34+ cells may result in an antigen specific T-cell unresponsiveness that might allow new clinical strategies aimed at overcoming HLA barriers in hematopoietic and solid organ allogeneic transplantation.

11
ASSESSMENT OF HUMAN STEM CELL EXPANSION IN THE NOD/SCID MOUSE XENOGENIC TRANSPLANT MODEL

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Identification of culture conditions that support expansion or even long-term maintenance of *in vivo* repopulating human hematopoietic stem cells is a major challenge. It has been reported that $CD34^+$ cord blood cells can be expanded *in vitro*, for several months, in serum containing culture conditions. The use of combinations of recombinant 'early acting' growth factors and the absence of stroma was essential in determining this phenomenon. Recently a new approach has been developed to establish an *in vivo* model for human primitive hematopoietic precursors by transplanting human hematopoietic cells into sublethally irradiated *non-obese diabetic severe combined immunodeficient* (NOD/SCID) mice. We examined the expansion of cells, $CD34^+$ and $CD34^+38^-$ subpopulations, colony-forming cells (CFCs), long-term culture initiating cells (LTC-ICs) and the maintenance or the expansion of SCID-repopulating cells (SRCs) during stroma-free suspension cultures of human $CD34^+$ and CD34+ CD38- cord blood cells for up to 12 weeks. To FLT3 ligand (FL); thrombopoietin (TPO); stem cell factor (SCF), interleukin 6 (IL6) or interleukin 3 (IL3) were added. Groups of sublethally irradiated NOD/SCID mice were injected with either unmanipulated CD34+ cord blood cells at start of cultures, or their progeny after stroma-free suspension cultures for up to 12 weeks with a combination of 'early acting' recombinant growth factors (FL, TPO, SCF) with the additional presence of IL6 or IL3. Mice that had been injected with 50,000 or 100,000 uncultured CD34+ CB cells showed engraftment. Mice injected with the cells that had been generated by the same number of initial CD34+ CB cells for up to 12 weeks of expansion cultures engrafted the vast majority of NOD/SCID mice. The level of engraftment, well above that usually observed when the same number of uncultured cells were injected into similar recipients suggested that primitive hemopoietic cells were maintained for up to 12 weeks of culture and, probably, expanded (as suggested by dilution experiments). By contrast, CD34+ cells cultured in the same conditions, but with the additional presence of IL3 could engraft NOD/SCID mice for no longer than 4 weeks. These results support and extend our previous findings that CD34+ CB stem cells (identified as long term colony-initiating cells, LTC-IC) can indeed be grown and expanded *in vitro* for an extremely long period of time only in the presence of some, but not all the growth factors employed in this study.

12
MINI MISMATCHED TRANSPLANTATION IN THE MOUSE USING ALLOREACTIVE NATURAL KILLER CELLS FOR CONDITIONING

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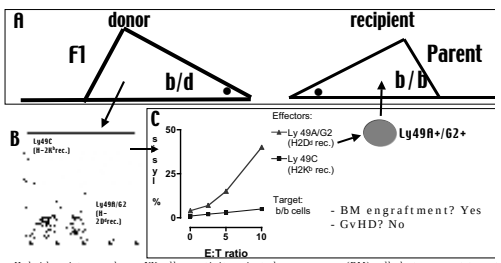
Recipients of HLA haplotype-mismatched hematopoietic stem cell transplants (Aversa *et al.*, N Engl J Med, 1998; 339:1186) often do not express class I alleles which block natural killer donor (NK) cells. Thus, some donor pre-transplant natural killer (NK) clones kill recipient target cells. After transplant, stem cells regenerate such alloreactive NK clones which kill pre-transplant cryopreserved host lymphocytes and leukemia cells and which are blocked by targets expressing the missing class I allele (Ruggeri *et al.*, Blood 1999; 94:333). This phenomenon is associated with low rejection and relapse rates and no GvHD in heavily conditioned high-risk leukemia patients. This study determined whether, in non-lethally-conditioned hosts, infusing donor alloreactive NK cells before transplant promotes sufficient immune suppression for engraftment. Hybrid resistance shows NK alloreactivity rejects bone marrow (BM) cells but may not target tissues as it does not reject organ grafts. We reversed the hybrid resistance transplantation partners. Irradiation below 8.5 Gy is non-lethal, mice reject BM and recover uneventfully. After 5 to 7 Gy irradiation, the infusion of hybrid H-2d/b mouse Ly49A+/G2+, H-2b/b reactive, NK cells into H-2b/b hosts killed the mice (spleen and BM counts were greatly reduced). Strikingly, adding donor BM rescued all mice which displayed full donor chimerism in their BM and spleens, without GvHD. The minimum effective Ly49A+/G2+ cell dose was 100,000 cells/mouse (the equivalent of 4 million cells/Kg body weight, a feasible dose with human NK clones). Eight-fold higher NK doses were needed to overcome resistance to engraftment in recipients exhibiting anti-donor NK alloreactivity (d/d donor into b/b recipient). Third-party (d/b) NK cells conditioned the host without interfering with BM engraftment when host (b/b) and donor BM (d/d) were susceptible to third-party NK lysis (a situation which reflects any mismatched, matched or autologous human transplant in which recipient and graft can be targeted by third-party alloreactive NK cells).



Hybrid resistance shows NK alloreactivity rejects bone marrow (BM) cells but may not target tissues as it does not reject organ grafts. We reversed the hybrid resistance transplantation partners (Panel A). Ly49A+/G2+, H-2$^{b/b}$ reactive, NK cells (panels B and C) were isolated from H 2$^{d/b}$ donor mice and infused into non-lethally-conditioned H-2$^{b/b}$ recipients. Goal of the experiments was to test whether alloreactive NK cells would promote sufficient immune suppression for BM engraftment in non-lethally-conditioned hosts, without causing GvHD. The results show this is indeed the case.

In conclusion, anti-host NK cells effectively promote BM engraftment, without GvHD, in sublethally-conditioned MHC disparate hosts. In the future, the infusion of anti-host alloreactive NK cells, given as part of a non-lethal conditioning regimen, may be used to promote engraftment (in addition to exerting anti-leukemic effects) and, thus, help reduce toxicity of conditioning regimens and ultimately extend hematopoietic transplantation to patients for whom and diseases for which the hazards of the current conditioning regimens are not justified.

Lunch meeting
ACUTE MYELOID LEUKEMIA IN THE ELDERLY: WHY IS IT SO HARD TO CURE?

Chairmen: M. Baccarani, F. Mandelli

13
ACUTE MYELOID LEUKEMIA IN THE ELDERLY: RESULTS OBTAINED WITH CONVENTIONAL CHEMOTHERAPY

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Acute myeloid leukemia (AML) occurs predominantly in adults, and its incidence increases with age. Many elderly AML patients are not entered into clinical trials. Thus, controlled clinical studies in the elderly most probably reflect a selection of cases. Anyway, a comparison of remission rates achieved in multicenter trials that include patients above and below the age of 60 years consistently indicates that older age is associated with a poorer response to initial chemotherapy and shorter disease-free survival and overall survival. Complete remissions are frequently obtained in less than half of older patients. The different clinical behavior of AML at older age raises questions about the most appropriate clinical management. The potential benefits of antileukemic therapy have been questioned repeatedly. However, the majority of reported trials show a more favorable outcome after specific antileukemic therapy, with no advantage from a palliative strategy. Chemotherapy is then the treatment of choice and may offer a chance of longer survival. At what intensity should this approach be followed and should elderly patients be treated in a similar way as younger patients? A series of clinical trials have addressed these questions, by adding etoposide to the combination of cytarabine (AraC) and daunorubicin (DNR); by using different doses of AraC within an AraC/DNR regimen; by applying low-dose AraC versus a full- dose protocol of AraC; by evaluating the oral combination of etoposide plus thioguanine (6TG) and idarubicin versus a DNR/AraC/6TG regimen. The majority of the studies are in favor of more intensive therapy, demonstrating a higher initial response rate and/or an improved long-term outcome. On the other hand, a reduced tolerance to intensified treatment is frequently observed. In particular, CALGB showed a significant increase in long-lasting remissions with high-dose AraC only in patients younger than 60 years of age, most probably because roughly 1/3 of patients older than 60 years of age could tolerate high-dose AraC, compared with 2/3 of younger patients. Patients older than 60 years of age have, thus, a high risk of dying during the course of induction therapy, mainly due to uncontrolled infections. The currently available data support the prophylactic administration of hematopoietic growth factors after intensive cytoreductive chemotherapy; no reports indicate an increased proportion of patients with persistent leukemia or early relapses. The higher frequency of unfavorable biolog ical and prognostic factors, anyway, is, other than age, one of the major determinants of poorer prognosis for elderly patients. The development of more effective therapies for bad-prognosis subgroups and the improvement of supportive measures are aimed to improve the outcome.

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14
BIOLOGIC PROFILE OF ACUTE MYELOGENOUS LEUKEMIA IN OLDER ADULTS

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Acute myelogenous leukemia (AML) in older adults is the most frequent form of AML. When compared to younger patients, AML in the elderly is frequently highly resistant to conventional chemotherapy and accordingly, its clinical response remains very poor. Many data suggest that AML in the elderly may be biologically different, based on the following evidence: a) Above the age of 55, up to 30% of AML patients show complex chromosomal aberrations or karyotypic abnormalities typically associated with a poor prognosis such as -5/del(5q) and -7/del(7q). Also, striking cytogenetic similarities have been noted between AML arising *de novo* in elderly individuals and AML and myelodysplastic syndrome developing after chemotherapy with alkylating agents or particular occupational exposure. Moreover, cytogenetic abnormalities associated with a good prognosis in *de novo* AML of younger patients such as inv(16) and t(8;21), are remarkably less frequent. b) In more than 70% of elderly AML patients, leukemic blasts show an intrinsic resistance to chemotherapeutic agents as determined by phenotypic expression of the multidrug resistance glycoprotein 1 (MDR1) or by functional assays quantifying the drug efflux from leukemic cells. c) AML in the elderly seems to arise from an earlier level of pluripotent hematopoietic cells which might affect more than one hematopoietic cell lineage thus determining trilineage dysplasia and delayed recovery of neutrophils and platelets after standard chemotherapy. In summary, AML in the elderly seems to be a biologically distinct disease and the aforementioned factors all contribute, independently, to poor outcomes. The identification of biological predictors of the clinical response may help to identify those patients for whom alternative treatments are urgently needed.

15
LIPOSOME ENCAPSULATED DAUNORUBICIN (DAUNO XOME) FOR TREATMENT OF ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Patients aged more than 60 years affected by acute myeloid leukemia (AML) usually have a poor prognosis. The majority of elderly patients who do not respond to standard treatment have a secondary leukemia, specific or complex cytogenetic abnormalities and high expression of several proteins, such as the P-glycoprotein (Pgp), the multidrug related protein (MRP), or the lung resistance-related protein (LRP), that interfere with drug sensitivity.[1,2] For these cases standard treatment is more toxic than useful and there is very little evidence that treatment intensification can improve the results. On the other hand, a significant proportion of elderly patients cannot be treated with aggressive chemotherapy because of their performance status, presence of concomitant diseases or organ failure. Therefore, the main problem in the managment of elderly AML patients is to reduce the host-toxicity of chemotherapeutic agents without affecting the antitumor efficacy. Liposomal anthracyclines have been recently developed with the aim of optimizing the delivery and selectivity of free drug to tumors and of reducing the toxicity in normal tissues. Daunoxome (DNX, Nexstar) is a preparation of daunorubicin (DNR) that is encapsulated into small liposomes with remarkable physical stability and is registered for the treatment of AIDS-related Kaposi's sarcoma.[3,4] Preclinical observations have pointed out that DNX can be at least as effective as free DNR against leukemic cells,[5] that it is likely to be less toxic than free DNR in normal tissues,[6] and that DNX partially escapes the mechanisms of multidrug resistance related to the pump function of several transport proteins, such as Pgp, MRP, and LRP.[7] Based on these preclinical observations, the effects of DNX either alone or in combination with cytosine-arabinoside (AC) were investigated in a phase II clinical trials, including either patients with resistant or relapsed AML, or elderly patients with *de novo* AML.[8,9] In these patients, the rate of complete remission (CR) was about 50%, the treatment-induced cytopenia lasted about 3 weeks and the toxic profile of this liposomal-encapsulated anthracycline was relatively low. The preclinical observations strongly suggest that DNX is an attractive drug for the treatment of acute leukemia of the elder in whom Pgp is usually overexpressed and low non-hematologic toxicity is required. More studies are required to assess the advantage of DNX over DNR.

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16
NOVEL THERAPEUTIC APPROACHES IN ELDERLY ACUTE MYELOID LEUKEMIA PATIENTS: TOPOTECAN AND CMA-676

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Acute myeloid leukemia (AML) is the most frequent form of acute leukemia in elderly patients; the incidence of the disease progressively increase with age, as does the disease-related mortality. The optimal management of elderly AML remains controversial and achievement of complete remission (CR) after conventional chemotherapy progressively decreases in the older patients who are characterized by several unfavorable prognostic factors. In fact, the biological features of AML cells in elderly patients are frequently characterized by trilineage myelodysplasia, unfavorable karyotype and overexpression of the multidrug resistance (mdr1) phenotype. In addition, the clinical characteristics of these patients show a limited tolerance to intensive chemotherapy. Inclusion of growth factors and mdr1-modulators in the treatment of elderly AML patients, aimed at ameliorating treatment-associated mortality and morbidity and overcoming drug resistance, respectively, has not significantly improved the duration of CR and survival, which remain poor. Efforts to increase the rate of response and disease-free survival are currently underway in several areas, including the development of novel active chemotherapeutics agents characterized by a lower chemoresistance and a more targeted antileukemic effects. Topotecan is a novel less toxic semisynthetic analog of the alkaloid camptothecin which acts as a specific inhibitor of topoisomerase-I. Inhibition of this enzyme involved in DNA replication and repair results in lethal DNA damage. Topotecan is active against a number of solid tumors and is characterized by a lack of cross resistance with other chemotherapeutic agents. The promising antileukemic effect of this drug, used as a single agent in continuous infusions over 24 hours for five days every three to four weeks in refractory AML and in myelodysplastic syndromes (MDS), supports the use of topotecan in combination with Ara-C, etoposide or cyclophosphamide. Data generated at the M.D.

Anderson suggest that the combined use of topotecan plus Ara-C in high risk MDS patients, especially those with unfavorable cytogenetics, resulted in a better response compared to Ara-C alone. Based on these data, a comparative multicenter randomized trial between topotecan and idarubicin, both in combination with Ara-C and G-CSF, is currently underway in patients with high risk MDS and in AML evolved from MDS. The risk/benefit and toxicity of these regimens are awaited. The availability of antibodies reactive against antigens expressed only by hematopoietic cells has provided clinical investigators with new tools to be used in the management of hematologic malignancies. Studies performed to date have investigated the use of such antibodies in an unmodified state, or combined with toxic molecules as immunotoxins or radionuclides. Among several antigens, CD33 is characterized by its expression on normal maturating myeloid cells and on more than 90% of AML cells, while being absent on normal hematopoietic stem cells. Initial work with the non-conjugated humanized anti-CD33 MoAb (HuM195) has shown only some degree of activity in animal models, not confirmed in AML patients with large tumor burden. Since AML is a radiation-sensitive disease, radiolabeled antibodies (131_I-labeled murine anti CD33 MoAb) have also been used; these have, however, resulted in prolonged and severe pancytopenia due to the physical properties of the beta-particle emitter which can destroy other cells around the target area, including normal hematopoietic progenitors. The most promising approach in terms of antibody therapy is represented by the humanized anti-CD33 MoAb linked to a potent cytotoxic antibiotic calicheamicin, which induces double-stranded DNA breaks only when introduced into the target cell. *In vitro* studies have confirmed the effectiveness of this molecule in inhibiting tumor growth in AML cells and phase I and II studies have confirmed in AML patients in first relapse, treated with 9 mg/m2 i.v. every 14 days for two doses, the antileukemic activity of CMA-676 used as a single agent. In contrast to conventional chemotherapy, the absence of hair loss and a significant reduction of systemic toxicity, has been reported. Studies are now starting in different AML settings, elderly – relapsed – pre-transplant, and the recent FDA approval of CMA-676 for the treatment of CD33+ AML patients in first relapse > 60 years, will allow further evaluation of this new treatment modality for patient with AML.

Lecture

17
IMMUNOLOCALIZATION OF FUSION PROTEINS WITH MONOCLONAL ANTIBODIES: NEW APPROACH IN THE STUDY OF LEUKEMIAS AND LYMPHOMAS

Falini B

Symposium
MECHANISMS OF LEUKEMOGENESIS AND LYMPHOMAGENESIS

Chairmen: C. Croce, G. Saglio

18
ACQUIRED CHROMOSOME 11q DELETION INVOLVING THE ATAXIA TELEANGIECTASIA LOCUS IN LYMPHOID NEOPLASMS

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The purpose of this two-phase study was to assess the incidence of 11q deletion involving the ataxia-teleangiectasia locus (ATM+/-) in lymphoid neoplasms, in order i) to define its clinicobiologic significance better in B-cell non-Hodgkin's lymphomas (NHL) and, ii) to establish whether it represents a primary or a secondary change in NHL and B-cell chronic lymphocytic leukemia (CLL). We assessed 135 NHL lymphomas at diagnosis; 95 B-CLL were analyzed at diagnosis and in 1-2 occasions at 1-2 year intervals. All cases were submitted to conventional cytogenetic analysis and to fluorescence *in situ* hybridization (FISH) using an 11q22-23 probe recognizing ATM sequences.

Point i) A hemizygous ATM deletion was seen in 44-88% of the interphase cells in 15/135 NHL (11.1%): 4 patients had an indolent lymphoma (follicle center cell lymphoma), 11 patients had an aggressive lymphoma (5 mantle cell lymphomas and 6 diffuse large cell lymphomas). Ten out of 15 ATM+/- patients had a complex karyotype, 11 out of 15 had more than 90% abnormal metaphases (AA karyotype status); +12, 13q14 deletion or 17p13 deletion were seen in 7, 4 and 5 cases, respectively. Patients with ATM+/- more frequently had a complex karyotype (p=0.01) and the AA karyotype (p=0.04) as than patients without ATM+/-. With the exception of a poor performance status (p=0.001) no correlation was found between ATM+/-, initial clinical variables and complete remission rate, whereas a highly significant association was found with shorter survival (p<0.0001). This cytogenetic lesion maintained its prognostic importance in multivariate analysis (p=0.0004), along with performance status (p=0.0006), serum LDH level (p=0.03), splenomegaly (p=0.01) and histologic grade (p=0.03). When analyzing separately indolent lymphomas and aggressive lymphomas, ATM+/- maintained its prognostic importance as an independent variable in both histologic groups (p=0.0001 and p=0.016, respectively).

Point ii) Dual color hybridization studies showed ATM deletion to be a secondary aberration with respect to BCL1 rearrangement in 3 cases with mantle cell lymphoma. One patient with CLL developed ATM+/- during the course of the disease; six patients who underwent transformation into prolymphocytic leukemia (PLL) were found to have acquired the ATM deletion in the PLL phase. We arrived at the following conclusions: i) the ATM+/- status has clinicobiological importance in NHL, possibly representing a major cytogenetic determinant of outcome; ii) whereas 11q-/ATM

deletion is usually an early event in typical CLL, it may represent a secondary change in NHL and in CLL undergoing transformation into PLL.

19
PROMISCUITY AND LEUKEMOGENESIS. TWO NOVEL SITES OF RECOMBINATION FOR NUP98/11p15 AND PDGFBR/5q33 GENES IN MYELOID MALIGNANCIES

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The term "promiscuity" in leukemia was first coined for the MLL/11q23 gene which undergoes a number of translocations producing new transcripts from fusions with a specific gene of the partner chromosome. Later on multiple translocations were also shown for other genes, such as ETV6/12p13 or BCL6/3q27. Here we discuss the identification of two novel sites of recombination for NUP98/11p15 and PBGFBR/5q33 in myeloid malignancies. The first case was a 65-year old man with AML-M1 FAB and a 46,XY,t(8;11)(p11;p15) karyotype. FISH studies were performed with BACs 118H17 and 290A12 for NUP98 (kindly provided by M Negrini, University of Ferrara), and yacs 770C2 and 176C9 for FGFR1 and MOZ genes, respectively (kindly provided by M Chaffanet, University of Marseille). The mix of BACs for NUP98 gave two signals on normal 11 and derived 11p15, and one signal on 8p11, showing the involvement of NUP98 in the breakpoint of the translocation. The partner was identified by splitting of the yac 770C2 (FGFR1 gene) between 8p11 and 11p15. The second case was a 49-year old man with atypical chronic myeloid leukemia and a 46,XY,t(5;10)(q33;q22). FISH for 5q33 showed the splitting of a cosmid probe, cosB, containing PDGFBR gene. Chromosome 10q was involved proximally to yac 781F5 (10q21.3) and yac 876H2 (10q22.1), so that breakpoint on 10q was more precisely assigned to 10q21. The 10q gene was identified by a PCR approach as the H4 gene (D10S170) (G. Gilliland et al., Harward Medical School, Boston). FISH with PAC 29F6, encompassing the 5' portion of the H4 gene, further documented the fusion between H4 and PDGFBR on chromosome 5. The AIRC (Associazione Italiana per la Ricerca sul Cancro) is kindly acknowledged.

20
ALTERATIONS IN SIGNAL TRANSDUCTION IN ACUTE LEUKEMIAS

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Granulocyte colony-stimulating factor (G-CSF) induces proliferation and maturation of myeloid progenitor cells. The Jak-Stat pathway and Shc-ras pathway are activated by G-CSF stimulation. Distinct cytoplasmic regions of human G-CSF receptor (G-CSF-R)have been identified as responsible for proliferation and maturation signaling. Mutated forms of G-CSF-R have been identified in acute myeloid leukemias. Lyn and Syk kinases have been reported to form a three-component complex with G-CSF-R. We investigated whether Lyn and Syk kinases were tyrosine phosphorylated after G-CSF stimulation, and whether Shc protein was activated as a consequence. The activity of wild type (WT) human G-CSF-R was compared to that of mutants in which carboxyterminal tyrosines (704Y, 729Y, 744Y and 764Y) were substituted for phenylalanine in 32d myeloid cells transfectants. Kinases such as Shc, Syk and Lyn were independently tyrosine phosphorylated after G-CSF stimulation of humanWT/G-CSF-R 32d cells. Lyn kinase was constitutively co-immunoprecipitated with Syk, but it was activated upon G-CSF binding to the receptor. We showed that tyrosines 764 and 729 of G-CSF-R cytoplasmic domain are crucial for activation of Shc and Syk, respectively. In Y729F G-CSF-R mutants anti phosphotyrosine immunoblots did not indicate Syk as an activated substrate, whereas Y764F mutants did not succeed in phosphorylating Shc. Tyrosine 729 is localized in the putative ITAM (immunoreceptor tyrosine-based activation motif)- like motif of G-CSF-R cytoplasmic region. We can affirm that Shc phosphorylation after G-CSF stimulation is independent of Syk activation. On the other hand, the presence of activated Syk is not sufficient to provoke Shc phosphorylation when Y764 is mutated. Syk tyrosine kinase is essential for signal transduction events in myeloid cells, but its role in granulocytic precursor proliferation and maturation has not been completely clarified. We thus analyzed the pattern of tyrosine phosphorylation and phagocytosis of sheep red blood cells and of latex beads of 32d myeloid cells WT/G-CSF-R transfectants, and DA/G-CSF-R naturally occurring truncated mutant (d715, lacking the ITAM-like motif and 729Y), after G-CSF stimulation. DA/G-CSF-R mutant has been cloned in acute myeloid leukemia cases arising in SCN patients. DA/G-CSF-R mutant did not show Syk phosphorylation. WT/G-CSF-R transfectants had a baseline phagocytosis of 10 ± 2 % (300 cells scored), but after G-CSF stimulation 44 ± 5 % of cells were phagocytic. The truncated DA mutant had only rare cells showing incomplete phagocytosis, and no increase was obtained after stimulation with G-CSF. From our evidence, G-CSF-R signals phagocytosis through a specific region. As we have demonstrated that the same region of the receptor is responsible for Syk activation, we concluded that the phagocytic activity stimulated by G-CSF in 32d WT/G-CSF-R transfectants is modulated by the carboxy-terminal region of the receptor via Syk in analogy to that observed in FcγR signaling in monocytes.

21
ALL1/MLL GENE IN ACUTE LEUKEMIA: ITS PUTATIVE ROLE IN LEUKEMOGENESIS

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ALL1/MLL gene spans approximately 90 kb of DNA, encodes for a major transcript of approximately 15 kb, and consists of 36 exons, ranging in size from 65 bp to 4249. The protein product consists of more than 3,910 amino acids containing three regions homologous to sequences of the Drosophila Trx gene, including cysteine rich regions that can fold into six zinc finger-like domains and a highly conserved 200 amino acid SET domain located at the carboxyl-terminal end. As occurs in Drosophila, also in mice ALL1/MLL is a positive regulator of Hox genes. The ALL1/MLL gene prod-

uct possesses two other regions, which would be directly or indirectly involved in the control of gene transcription, by favoring conformational DNA changes. These are: 1) the AT hook of high-mobility-group-I; and 2) a cysteine-rich region (CRR) homologous to the mammalian DNA methyltransferase double helix. To date, at least 16 different fusion partner genes involved in chromosomal translocation with ALL1/MLL have been characterized. Additionally, internal duplications within the amino-terminal part of ALL1/MLL and specific deletions of exon 8 have been detected in leukemic blast cells of some leukemic patients. The active functional contribution of partner genes in determining the oncogenic capacity of the resulting hybrid gene is presently strongly suggested by several observations. However, it is still unclear how fusion products participate in leukemogenesis. As far as concerns interaction of ALL1/MLL fusion proteins with their target genes, it is interesting to note the potential role of the ALL1/MLL AT hook region, which is believed to be important in targeting and regulating transcriptional units of genes, for normal hematopoietic growth and differentation, in conjunction with the loss of SET domain occurring when the amino-terminal ALL1/MLL and carboxyl-terminal partner residues fuse to form ALL1/MLL chimeric protein. In particular, the loss of this domain may explain the downregulation of some target genes, for example the ARP1. Finally, another very recent approach to studying gene expression is the high-density microarray technology, which allows large numbers of genes to be screed to see whether or not they are active under various conditions. This technology has begun to be used to study acute leukemic cells, and data on the ALL1/MLL positive acute leukemia setting will soon be available.

22
MOLECULAR PATHOGENESIS OF MULTIPLE MYELOMA: THE ROLE OF CHROMOSOMAL TRANSLOCATIONS INVOLVING THE IG LOCI

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In the recent past several advances in the molecular biology of multiple myeloma (MM) have provide new insights into the pathogenesis of this disease. In particular, we and others have demonstrated that, despite the apparently low incidence by cytogenetic analysis, translocations involving the immunoglobulin (Ig) loci, mainly the heavy chain locus (IGH) at 14q32, are a very frequent event associated with MM. Translocations to the IgH locus usually occur within the switch regions and involve a large array of chromosome loci, mostly the 11q13, 4p16.3, 16q23 and 6p25 where putative target genes are located. We and others have identified the novel, karyotypically not detectable, t(4;14)(p16.3;q32) chromosomal translocation in MM. The 4p16.3 breakpoints occur 50 to 100 kb centromeric to the FGFR3 gene and within the 5' regions of the novel WHSC1/MMSET gene. These genes are overexpressed in the translocated cases and interestingly, activating FGFR3 gene mutations have been found in a limited number of MMs with t(4;14). We performed a double-color FISH assay using IgH and 4p16.3 specific probes showing that this lesion occurs in approximately 20% of MM patients without apparent association with the clinical stage. It has been previously demonstrated in MM cell lines with t(4;14) that the translocation results in the formation of IgH-MMSET hybrid transcripts; we found that the presence of these transcripts correlates with the translocation in primary tumors further confirming that they represent a specific marker of the translocation. The t(11;14)(q13;q32) chromosomal translocation, the hallmark of mantle cell lymphomas (MCL), is found in less than 5% of MM by conventional cytogenetics; however, rearrangements of the BCL-1/cyclin D1 regions involved in MCL are not detected in MM by Southern blotting. The molecular cloning of 11q13 breakpoints in a limited number of MMs (mostly cell lines) with the t(11;14) indicated that they are highly scattered over a relatively large area encompassing the BCL-1/cyclin D1 loci, but the fact that cyclin D1 was found to be overexpressed in these cases strongly suggests that it is deregulated in MM as a result of the translocation. We investigated primary MMs for structural evidence of BCL-1/cyclin D1 locus involvement by double-color FISH, and for cyclin D1 expression by immunohistochemistry (IHC). We found that the t(11;14) occurs in approximately 20% of cases and it is strictly associated with cyclin D1 overexpression, suggesting that IHC may represent a reliable means of identifying this lesion in MM. Interestingly, we found that cyclin D1 expression significantly correlated with the degree of bone marrow involvement, advanced clinical stage and the presence of clinical symptoms. Further studies are required to demonstrate whether cyclin D1 expression can be considered a prognostic marker in MM.

23
PATHOGENETIC AND HISTOGENETIC HETEROGENEITY OF B-LINEAGE DIFFUSE LARGE CELL LYMPHOMA

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B-lineage diffuse large cell lymphoma (B-DLCL) is the most common type of lymphoma in immunocompetent as well as immunodeficient hosts. The extreme clinical heterogeneity of B-DLCL is thought to reflect a marked degree of heterogeneity in the pathogenesis and histogenesis of this lymphoma. Studies of B-DLCL pathogenesis have suggested the existence of at least three distinct molecular pathways leading to the disease in immunocompetent hosts. The first of these molecular pathways is identified by rearrangements of BCL-6, a zinc finger transcription factor mapping to chromosomal band 3q27, and clusters with B-DLCL developing de novo in the absence of a previous follicular phase. The second molecular pathway is identified by the combination of rearrangement of BCL-2 and inactivation of p16 and/or p53 and clusters with B-DLCL transformed from a previous follicular phase. The third molecular pathway associates with de novo B-DLCL devoid of BCL-6 rearrangements and is thought to associate with currently unidentified genetic lesions. The molecular pathogenesis of immunodeficiency related B-DLCL differs substantially from that of B-DLCL

of immunocompetent hosts and frequently involves tumor clone infection by Epstein-Barr virus in the absence of other known genetic alterations. Recently, the role of gene inactivation through promoter methylation has been implicated in B-DLCL pathogenesis, as exemplified by the case of 06-methylguanine-DNA-methyltransferase (MGMT) and death-associated protein kinase (DAP-kinase). MGMT encodes a DNA repair protein that removes alkyls from the O6 position of guanine and its loss of expression in MGMT–/– knockout mice favors lymphomagenesis. In B-DLCL of both immunocompetent and immunodeficient individuals, inactivation of MGMT through promoter methylation occurs in approximately 40% of cases. Immunohistochemical studies have shown that MGMT promoter methylation associates with absent MGMT expression by B-DLCL cells. Intriguingly, MGMT inactivation in B-DLCL appears to be an independent factor of prognosis which correlates with prolonged disease free survival and overall survival after therapy with standard regimens. Because MGMT inactivation abolishes the cellular capacity to repair DNA damage induced by alkylating agents, it is thought that cyclophosphamide may exert a more profound genotoxic and, consequently, more effective therapeutic action in B-DLCL lacking MGMT expression than in cases expressing the enzyme. DAP-kinase is a serine/threonine kinase required for apoptosis induced by interferon-gamma, TNFalpha and FAS. Expression of DAP-kinase in human tumors may be reduced or absent as a consequence of promoter hypermethylation, thus increasing the pro-apoptotic threshold of the tumor. Among B-DLCL, inactivation of DAP-kinase through promoter hypermethylation is detected in 62% cases, including all clinico-pathologic variants of the disease. Beside displaying pathogenetic heterogeneity, B-DLCL also show a certain degree of histogenetic heterogeneity. Virtually all B-DLCL in both immunocompetent and immunodeficient individuals are thought to derive from germinal center related B-cells at various stages of differentiation. This fact is documented by the association of B-DLCL with well established histogenetic markers denoting transit through the germinal center, such as somatically acquired point mutations of the hypervariable regions of immunoglobulin genes and mutations of the BCL-6 gene regulatory regions. These studies have shown that also extranodal B-DLCL, including cases primarily localized to the central nervous system (i.e. primary central nervous system lymphoma), are consistently related to germinal center B-cells. Intriguingly, B-DLCL carrying mutations of BCL-6 appear to associate with improved disease free survival, suggesting that these mutations may influence prognosis through a currently unidentified mechanisms. Despite their common origin from germinal center related B-cells, B-DLCL are histogenetically heterogeneous since they may reflect either classical germinal center cells (i.e. centroblasts and centrocytes) or post-germinal center B cells (i.e. immunoblasts). This notion is well established in the case of B-DLCL of immunodeficient hosts, which have proven to be a valuable model for the understanding of B-DLCL histogenesis. In this setting, in fact, the expression pattern of the histogenetic markers BCL-6 and CD138/syndecan-1 allows the distinction of B-DLCL reflecting a germinal center phenotype from B-DLCL reflecting a post-germinal center stage of differentiation. The identification of histogenetic subsets of B-DLCL may be of great clinical relevance, since growing evidence in this and other diseases indicate that histogenesis may influence prognosis.

Symposium
ANTI-FACTOR VIII AND ANTI-FACTOR IX ANTIBODIES (INHIBITORS) IN HEMOPHILIA

Chairmen: G. Mariani, V. Vicente

024
GENETIC BASIS OF FACTOR VIII INHIBITORS

Bernardi F

025
CELLULAR AND SEROLOGICAL ASPECTS OF FACTOR VIII INHIBITORS

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Factor VIII (FVIII) inhibitors, i.e. antibodies specific to FVIII and able to inhibit FVIII pro-coagulant activity, represent an interesting challenge not only because of the requirement for a form of therapy that can eliminate the formation of such detrimental antibodies, but also in terms of immunology and mechanisms of tolerance to self. FVIII inhibitors are indeed present not only in patients with severe hemophilia A and infused with FVIII, but also as part of an auto-immune response in some auto-immune diseases or spontaneously after surgery or pregnancy. Such antibodies are also present in the natural repertoire of healthy people in whom their inhibitory activity is neutralized by anti-idiotypic antibodies. FVIII is made of more than 2,000 amino acids, thus representing a very complex molecule, the immunogenicity of which is hardly understood. In an attempt to solve a number of issues related to its immunogenicity, we have started a systematic evaluation at clonal level of both the humoral and cellular arms of the response.

Thus, B-cell clones have been generated from the B-memory cell repertoire of hemophilia A patients with inhibitors, from which human monoclonal antibodies (hu-mAbs) have been obtained. These hu-mAbs are being used to: (1) define with precision the FVIII epitope to which they bind; (2) evaluate at the molecular level the mechanism by which such antibodies inhibit the procoagulant activity of FVIII; (3) identify the origin of anti-FVIII antibodies by comparing variable part sequences between different hemophilia A and auto-immune patients; (4) provide a support for evaluating idiotype-anti-idiotype interactions.

T-cell clones have also been derived from the peripheral blood of hemophilia A patients. These are being analyzed: (1) to determine the mechanisms by which they are activated; (2) the profile of cytokines secreted; (3) the major T-cell epitopes of FVIII; (4) the preferential usage of MHC-class II determinants, which could provide a means of identifying patient-related risk factors.

Thanks to the availability of different animal models, it is hoped that such strategy will help to identify ways of therapeutically controlling the anti – FVIII immune response in patients.

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TREATMENT OF INHIBITORS IN HEMOPHILIA

Mariani G

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The appearance of an inhibitor is the severest complication that may arise in hemophilia treatment. The treatment strategy for bleeding in patients with an inhibitor requires (i). knowledge of the actual inhibitor titer, (ii). information on the type of anamnesis (high or low responder) and, (iii). a thorough evaluation of the bleeding episode. Ideally, the optimal treatment of a bleeding complication should be based on obtaining hemostatically effective levels of the missing factor. This is possible only in low responders (max. historical inhibitor titer < 10 U), or when the inhibitor titer is low. However, this treatment modality brings about a brisk anamnesis and therefore, clashes with the widely accepted belief of keeping the inhibitor titer as low as possible. Thus, in general, concentrates of the missing factor should be administered in low responders, only or in the case of high responders with a low titer, only in emergencies when it is difficult to treat with other means. At any rate, when concentrates have to be administered, the dose must be assessed on the grounds of the current inhibitor titer.

When bleeding occurs and the inhibitor titer is high, either porcine FVIII or the bypassing agents (FEIBA™ or Novoseven™) can be used, depending on the anti-porcine FVIII titer and the clinical setting. Bypassing agents are effective in 70-90% of the cases, and Novoseven is apparently more efficacious. Inhibitors to FIX are much rarer than those to FVI-II but up to 50% of the patients have allergic reactions which may lead to a nephrotic syndrome.

As the quality of life of a hemophilic patient with an inhibitor is generally poor anyway, it is always important to consider an immune tolerance regime, the only treatment modality which may modify the natural history of an inhibitor. This treatment procedure is becoming very popular, notwithstanding the costs. It is based on the administration of high doses of FVIII (50-300 IU/Kg b.w./day), which, after a variable interval of time (6 months to 3 years), may lead to the disappearance of the inhibitor and the suppression of the immune reaction to FVIII. The International Registry of Immune tolerance protocols (comprising 315 patients) has indicated the variables associated with success (in order of significance): the FVIII dose (high vs. low), the inhibitor titer before treatment (low vs. high) and the time between inhibitor diagnosis and beginning of treatment. Such variables, in clusters, enabled the identification of patients with favorable prognostic indicators in whom the response is rapid and the response rate high: children, treated soon after inhibitor appearance and with high or intermediate dosages of FVIII (> 100 IU/Kg b.w./day). Furthermore, treatment, when carried out in this subset of patients, becomes more affordable.

Lunch Meeting
OSTEOGENESIS AND OSTEOLYSIS IN HEMATOLOGY

Chairmen: A. Pileri, S. Tura

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BONE PHYSIOPATHOLOGY

Angeli A

28
MARROW MICROENVIRONMENT AND HEMATOPOIESIS

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Microenvironmental marrow components can modify the proliferative and differentiative behavior of hematopoietic stem cells (HSCs) by means of (i) cell-to-cell interactions, (ii) interactions of cells with extracellular matrix (ECM) molecules, and (iii) interactions of cells with soluble growth regulatory molecules. Marrow stromal cells (MSCs) provide the physical framework within which hematopoiesis occurs, play a role in directing the processes by synthesizing, sequestering or presenting growth-stimulatory and growth-inhibitory factors, and also produce numerous extracellular matrix proteins and express a broad repertoire of cell adhesion molecules (CAM) that serve to mediate specific interactions with hematopoietic stem/progenitor cells of both myeloid and lymphoid origin. Despite their regulatory role, it seems improbable that hematopoiesis is only regulated by a random mix of growth factors and responsive cells. Rather, localization phenomena within marrow stroma are required to sustain and regulate hematopoiesis. Although it is commonly accepted that stem cells are capable of homing to the marrow and docking at specific sites, the exact role of MSCs, CAMs, and ECM proteins in regulating the localization and spatial organization of HSCs in the marrow remains a matter of hypothesis. Studies in animals demonstrated that stem and progenitor cells have a different distribution across the femoral marrow cavity of the mice, thus suggesting that marrow stroma is organized into functionally discrete environments, such as *primary microenvironment* and *secondary microenvironment* areas, allowing distinct differentiation patterns of hematopoietic stem cells. The *stem cell niche* hypothesis suggested that certain microenvironmental cells of the marrow stroma could maintain the stem cells in a primitive, quiescent state. Another mechanism supporting the concept of specialized microenvironmental areas is represented by stroma-mediated, compartimentalized growth factor production. Growth factor produced locally by stromal cells may bind to the extracellular matrix and be presented to immobilized target cells which recognize each growth factor through specific receptors. This mechanism may provide the opportunity for localizing distinct growth factors at relatively high concentrations to discrete sites. A growing body of evidence suggests that marrow stroma is not only involved in regulating myeloid cell growth but also in T- and B-cell lym-

phopoietic development. Distinct adhesion molecules and cytokines are known to regulate stroma-dependent T- and B- lymphopoiesis, suggesting that marrow stroma may function as a site of T- as well as B-cell lymphopoiesis. In conclusion, the crosstalk between receptors and counter-receptors broadly distributed on HSCs and MSCs represents an attractive target for therapeutic intervention aimed at modulating cytokine production, hematopoietic cell homing or stromal cell function.

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METABOLIC AND NEOPLASTIC OSTEOPATHY

R. Bartl

30
BONE AND MULTIPLE MYELOMA

Cavo M

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Multiple myeloma (MM) is commonly associated with bone lesions causing bone pain, fractures, spinal cord compression, disturbances of calcium metabolism and renal failure. Bone pain is often severe and accounts for most of the poor quality of life in affected patients. Moreover, pathologic fractures and hypercalcemia are also major causes of morbidity, and some mortality, associated with the disease. Bone destruction is the consequence of an uncoupling process associating increased osteoclastic resorption with normal or decreased bone formation. Excessive osteoclastic resorption is an early phenomenon, as opposed to the inhibition of osteoblastic activity which occurs later in the course of the malignancy. Early histomorphometric studies showing that increased numbers of osteoclasts are in close proximity of myeloma cells suggested that several factors produced either by tumor plasma cells or other cells (i.e. microenvironmental stromal cells) could be involved in the pathogenesis of skeletal disease. At this time, interleukin (IL-6), its soluble receptor, and IL-1β appear to be the most critical factors involved in the occurrence of lytic bone lesions. Other hematopoietic growth factors, mainly M-CSF and TGFβ, could also play a crucial role. Diagnosis and treatment of bone disease are essential in the management of MM patients. Second-generation bisphosphonates, which are potent inhibitors of osteoclastic activity, have been proven useful in slowering the progression of bone disease and treating MM-associated hypercalcemia. Third-generation bisphosphonates seem to be 2 to 3 logs more potent than the previous ones in preclinical models and hold promise for further improvement upon the reduction in skeletal morbidity in MM patients.

LECTURE

31
MDR. HOPES, DISAPPOINTMENTS AND PERSPECTIVES

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Knowledge, interpretation and exploitation of drug resistance suffers from a number of difficulties that are partly technical, partly biological, partly clinical, and eventually contribute to a false perception of the concept of drug resistance. We would all like a reliable method of assessing drug resistance, and that one resistance mechanism is operative in any single case that the mechanism is genetically-based and is a stable characteristics of the cells, and that there is a significant relationship with treatment outcome. Unfortunately methods are sometimes unreliable or not yet standardized and frequently indirect, more than one mechanism is usually activate, the expression of resistance can be phenotypic and not genetically based (as is usually the case with typical and atypical MDR), and clinical outcome depends on other variables besides treatment. Moreover we would all like to be able to classify a case as resistant or not, but the definition of resistance is difficult because the expression of resistance is variable (from high to low) and can concern only a small proportion of leukemic cells, and yet these cells can be crucial to leukemia maintenance and relapse. After all, when one makes an attempt to bypass drug resistance *in vivo*, the first obstacle is an increase of toxicity. Therefore although drug sensitivity is the basic requirement for any treatment and although one can hardly imagine curing cancer or leukemia with agents that are not toxic to cancer or leukemic cells, drug sensitivity and drug resistance are still looked upon with some suspicion and annoyance. At the last meeting of the American Society of Hematology, New Orleans 1999, drug resistance was not even mentioned in any of the educational sessions. Moreover, there is a tendency to analyze and to use drug resistance data not as a source of specific knowledge, but as a prognostic factor, like tumor size, proliferation indicators, cell phenotype, cytogenetics, age and so on, without paying attention to the fact that it is one thing is to predict a poor outcome and it is another to identify resistance, either specific or pleiotropic. In leukemia, a complex karyotype is clearly associated with a poor outcome but does not (yet) tell us why the cells are resistant, to which drugs they are resistant, and what the mechanisms are underlying the resistance. We think that there is little sense in affirming that a factor is prognostically more important than identification of drug resistance. The development of so-called risk-adapted strategies eventually leads only to an increase of treatment burden. The logic of this approach is limited, as is clearly evident in the elderly, in whom treatment intensification is impossible and should be replaced by treatment selection. Knowledge of drug resistance should help not only to select active drugs but also to avoid non-active drugs, and hence to limit toxicity. Leukemia of the elderly is still an example of refusing or ignoring the implications of drug resistance studies. By almost universal agreement, treatment of leukemia of the elderly is poorly effective and too toxic, but although drug

ORAL COMMUNICATIONS

session 1

001
THALIDOMIDE IN REFRACTORY/RELAPSING MULTIPLE MYELOMA

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During the past years there have been encouraging reports concerning the efficacy of thalidomide in advanced refractory/relapsed multiple myeloma (MM) patients. High dose thalidomide seems to cause some toxic effects, which can induce the patient to discontinue the drug. Therefore efficacy of thalidomide, used alone or in combination, and tolerance of the drug, must both be evaluated. We have enrolled 9 MM patients already treated with at least two lines of chemotherapy. Four of these (44 %) had received high dose chemotherapy with autologous hematopoietic stem cell support. Thalidomide was started at 200 mg daily and increased by 100 mg every two weeks according to tolerance and response. When the maximal tolerated dose was reached without response, high dose dexamethasone was added (one or two courses). One patient died before completing one month of therapy, with a initial reduction in M-protein and was not evaluated for response. The other 8 patients can be divided into two groups, according to disease phase. Group A includes patients with an increasing monoclonal component (MC) despite chemotherapy (refractory). Group B refers to patients in early relapse, as indicated by increasing levels of MC in two consecutive tests after a period of stable disease (relapsed). The Southwest Oncology Group (SWOG) criteria were utilized to evaluate response in group A patients: complete response (CR, >75% MC reduction); partial response (PR, 50-75% MC reduction); stable disease or minimal response (< 50% MC reduction); progressive disease (increase of MC). In group B, as MC levels were low, response was defined by any reduction in the paraprotein levels. The characteristics of the patients are listed in Table 1. Thalidomide was generally well tolerated at these doses, with minor side effects, so that all patients have continued therapy. Sedation and somnolence were transient, whereas proactive measures were successful in reducing constipation. During the treatment two episodes of deep venous thrombosis occurred in two patients, who had already experienced this problem. Two cases of impotence were reported, but this effect has never been reported, as far as we know, in any report concerning thalidomide. Regarding the 2 CR observed in group A , one was obtained and sustained without adding high dose dexamethasone. In the 6 responders initial response occurred early (within 1 month), except for in one patient, who showed a very good PR (>60% MC reduction) only after two months, at a dose of 600 mg and after combination with dexamethasone. For both groups a marked improvement of Hb values to normal levels was obtained and two patients became transfusion independent. In all anemic patients but one, EPO was administered at the dosage of 10,000 U twice a week for a brief period. It is noteworthy that none of these had previously responded to EPO alone. In conclusion in these cases thalidomide has been demonstrated to be an effective and relatively safe tool for controlling advanced multiple myeloma. Larger studies are needed to evaluate the drug's role, as a single agent or in association, in different phases of the disease, with specific attention being addressed to long-term side effects.

Table 1.

Group	*A (Refractory)*	*B (Relapsed)*
N. of patients	4	4
Sex (M/F)	4/0	1/3
Median age (Range)	62 (52-75)	52 (50-66)
Prior high dose chemotherapy	1	3
100/200/400/600 mg of thalidomide	0/1/2/1	1/1/2/0
High dose dexamethasone	3	0
Responder	3 (2CR+1PR)	3
Median follow up (average)	5 (4,5-5,5)	5 (2-7)
Hb levels <10 g/dL	3	1
Frequent side effects:		
constipation	4	4
somnolence	4	4
rush	1	1

002
REVERSE TRANSCRIPTION POLYMERASE REACTION MONITORING OF PML-RARα FUSION TRANSCRIPTS IN ACUTE PROMYELOCYTIC LEUKEMIA FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION

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The t(15;17) is the cytogenetic hallmark of acute promyelocytic leukemia (APL). The chromosome rearrangement creates two fusion transcripts: the PML-RARα on chromosome 15 and the RARα-PML on chromosome 17. The PML-RARα fusion gene is detected in all APL patients by RT PCR, which is used to monitor minimal residual leukemia (MRD). Therefore RT PCR was applied to 11 APL patients in long-term complete clinical remission (CCR) after allogeneic bone marrow transplantation (Allo-BMT). At diagnosis all patients showed a standard t(15;17) on conventional cytogenetics and a RARα/PML fusion signal on der(17) on FISH analysis. BY RT PCR 6 patients were BCR1 positive, 3 BCR2 positive and 2 BCR3 positive. All the patients were in CCR at the time of the transplant. Four patients had had a RT PCR positive test within the six months preceding allo-BMT, but they succeeded in entering a new CCR with negative RT PCR analyses following a standard induction chemotherapy performed before allo-BMT. Busulfan and cyclophosphamide formed the conditioning regimen given to the 9 patients who received marrow from a HLA identical sibling donor; TBI and cyclophosphamide was given to the 2 patients who received marrow from an unrelated donor. Two patients developed acute GvHD. One died of acute GvHD while the other one had a progressive mild chronic GvHD. Four patients had a *de*

novo mild chronic GvHD. Ten patients are alive in CCR after a follow-up ranging from 12 to 77 months. MRD was monitored every three months in the first year following allo-BMT and then every six months by a nested RT PCR having a sensitivity level of 10^{-4}. Seven patients always had negative RT PCR tests. Four of them developed mild chronic GvHD at various times after allo-BMT. A positive RT PCR was seen in four cases. One patient was positive just after the transplant up to day +136, but then RT PCR became negative; two other patients had alternative positive and negative tests (one of them developed mild chronic GvHD); the last one became positive five years post-transplant without any previous positive RT PCR analyses. None of these cases relapsed and they all are in CCR. In conclusion our results suggests that the demonstration of a PML-RARα transcript by RT PCR post-transplant is not always predictive of an impending relapse, according to a possible graft-versus-leukemia effect.

003
DIFFERENTIAL REGULATION OF P27 AND CYCLINS BETWEEN BONE MARROW AND MOBILIZED PERIPHERAL BLOOD CD34+ CELLS

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The aim of this study was to characterize the molecular cell cycle regulation of normal human CD34+ cells. We, therefore, analyzed the expression of the cyclin-dependent-kinase inhibitor (CKI) p27 on CD34+ cells from steady state bone marrow (BM) and G-CSF mobilized peripheral blood (PB) cells. Analysis of steady state BM CD34+ cells showed a consistent expression of p27 (Western blot) with a mean OD of 0.33 ± 0.1 (range 0.17–0.56), lower than that of normal resting PB lymphocytes (mean=1.63 ± 0.52, range 0.92-2.29). In contrast, G-CSF mobilized PB CD34+ cells lacked p27 at both Western blot and flow cytometry. We then analyzed p27 expression in CD34+ cells from G-CSF primed BM cells and found levels of p27 expression similar to those of BM steady state CD34+ cells. Among the other cell cycle regulatory proteins- cyclin A, B, D1, 2 and 3, cyclin-dependent-kinase (Cdk) 2 and 4 and Cdc2, and the other CKIs p15 and p16- only Cyc A and B showed a different expression between steady state BM (positive) and G-CSF mobilized PB (negative) CD34+ cells. *In vitro* culture of steady state BM and G-CSF mobilized PB CD34+ cells with G-CSF and stem cell factor (SCF) showed, in BM progenitors a transient p27 downregulation, associated with cell cycle activation, followed by a protein upregulation at 72 hours. Mobilized PB CD34+ cells, in contrast, progressed into the cell cycle with a constant absence of detectable p27 expression during the 72 hours of *in vitro* culture. In conclusion, our study demonstrates that G-CSF mobilized CD34+ cells consistently lack p27 expression, compared to their steady state and G-CSF primed BM counterparts. These results may help us to understand the kinetic characteristics of mobilized PB CD34+ cells, suggesting that differences in adhesion status of hemopoietic progenitor cells may influence their proliferative capacity.

004
VARIABLE REGION GERMLINE GENE USE AND LIGHT CHAIN ORGAN TROPISM IN PRIMARY AMYLOIDOSIS: DISCOVERY OF A NEW AMYLOID-ASSOCIATED GENE, 3R, AND THE PREFERENTIAL KIDNEY INVOLVEMENT OF LAMBDA VI LIGHT CHAINS

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Primary amyloidosis (AL) is characterized by extracellular deposition of fibrils (amyloid) constituted of monoclonal light chain (LC) variable region fragments. Amyloid deposition is progressive and leads to organ failure and death. Light chains, most frequently lambda, are secreted by a small clone of bone marrow plasma cells. Amyloid deposition is systemic but one organ, or a combination of a few organs, is preferentially involved, dominating the clinical picture. Prognosis and treatment are affected by the organ/s involved. The mechanisms underlying light chain V region (VL) amyloidogenicity and organ targeting are unknown, and characterization of VL gene usage may provide insights into these biologically and clinically relevant aspects. A recent report[1] described the association between the Vlambda 6 family (a strongly amyloidogenic germline family)[2] and kidney involvement; however, patients were selected (autotransplantation program) and sequencing bias (63% cloning success) could not be excluded. Analysis of gene usage in a general population of patients with AL amyloidosis has not been performed and the relative contribution of each VL germline gene to amyloidogenicity and organ targeting are still unknown. We, therefore, sequenced the monoclonal VL regions from 38 unselected lambda amyloid patients. Patients were enrolled at the co-ordinating center of a National Amyloid Program. The predominant organ involved was kidney (#14, 37%), heart (#10, 26%), peripheral nervous system (#4, 11%), liver (#3, 8%) muscle, gastrointestinal tract, skin, (#2 each, 5%), or lung (#1, 3%). Amyloid LC variable regions were isolated by an inverse PCR-based strategy[3] and compared to germline databases. A monoclonal light chain V region was isolated in all cases. LC variable regions manifested somatic mutations, but the degree of mutation varied among the different Vlambda families. We found the following gene family usage: lambda III (#20, 53%), lambda II and lambda VI (#7 each, 18%), lambda I (#3, 8%), and IV (#1, 3%). Twelve (40%) of the 30 lambda germline genes were used: 3r (#11, 29%), 6a (#7, 18%), 2a2, 3m, 3l (#3 each, 8%), 1b, 2b2, 2e, 3h (#2 each, 5%), 1e, 3j, 4b (#1 each, 3%). The most represented germline gene, 3r, rearranged with significantly higher frequency than expected (X^2, $p<0.01$). This same gene was associated to various amyloid organ targets. By contrast, the lambda VI family gene, 6a, the second most frequently observed, was associated to predominant kidney involvement (6 of 7 cases). In conclusion, our results demonstrate that the most frequently used family is the lambda III (53%), that two genes, 3r (29%) and 6a (18%), code for almost half of pathogenic light chains, and that 6a preferentially generates kidney involvement. Since 3r is rearranged at a frequency of 7-8% in the normal repertoire,[4] this same gene must have significant amyloidogenic properties.

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005
FIRST LINE THERAPY WITH FLUDARABINE COMBINATIONS IN 42 PATIENTS WITH EITHER POST-MYELODYSPLASTIC SYNDROME OR THERAPY-RELATED ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemias (AML) evolving from a myelodysplastic syndrome (MDS) or secondary to chemoradiotherapy frequently display unfavorable biological characteristics. This may explain the lower remission rate obtained with conventional chemotherapy. Recently, associating fludarabine and intermediate-dose Ara-C has produced interesting results, particularly in high risk AML patients. Here, we report on 42 secondary AML patients treated with a combination of fludarabine, intermediate-dose Ara-C, G-CSF with or without an anthracycline (FLANG, FLAG-IDA or FLAG). Overall, complete remission (CR) was documented in 14 patients (33%), and partial response (PR) in 12 (29%), while 10 patients were resistant (24%). Six patients (14%) suffered from early death. The presence of an unfavorable prognosis karyotype had a negative impact on the CR rate (20% compared to 50% for patients with an intermediate prognosis karyotype, p=0.05). Patients treated with FLAG, FLANG or FLAG-IDA had similar CR rates. At the time of this analysis, after a mean follow-up of 12 months, the mean duration of CR is 16 months (range 3-66) and the mean survival is 11 months (range 1-67). The median time to granulocyte recovery (neutrophils > $0.5x10^9$/L) was 20 days (range 12-39), and $50x10^9$/L platelets were reached at a median of 26 days (range 9-56). Taken together, these fludarabine-containing regimens proved to be effective and tolerable treatment for patients with secondary AML. Patients above 70 years of age may also benefit from this therapy. The problem of treating patients with adverse chromosomal abnormalities remains unresolved.

006
SINGLE PLATFORM VS DUAL PLATFORM ASSAYS FOR CD34+ CELL ENUMERATION

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Most techniques derive absolute CD34 cell count from the flow cytometric percentage of CD34+ cells combined with an assessment of the absolute nucleated cell count from a hematology cell analyzer, the so called dual platform assay.[1,2] Recently, absolute CD34+ cell counts have been derived from single platform instruments.[3] We performed CD34 determination on 71 samples from 27 leukapheresis (PBSC), 29 umbilical cord blood (UCB) and 15 peripheral blood samples drawn before apheresis (PB). Our dual platform (FCS) method was based on a direct immunofluorescence whole-blood staining using a phycoerythrin (PE)-labeled CD34 monoclonal antibody (HPCA-2 PE, Becton Dickinson, CA) plus lyse/wash technique. The absolute counts were derived from a leukocyte count on a hematology analyzer (Coulter JT3, Beckman). We used two methods of single platform assay, one was the IMAGN 2000 microvolume fluorimeter (Biometric Imaging, CA)[4] and the other was based on the ISHAGE protocol for flow cytometric CD34 determination or fluorescent microbeads at a known concentration (Stem-Kit, Coulter, FL, USA).[5] Within 30 minutes we were able to obtain absolute CD34 counts with the single platform as compared to one hour with the dual platform assay. By using Spearman's correlation, absolute CD34 counts from the Imagn 2000 and ISHAGE method (r= 0.93; p< 0.0001), from Imagn 2000/dual platform method (r=0.95; p<0.0001) and from the ISHAGE/dual platform (r=0.98; p<0.0001) were significantly correlated. By using matched pair-t-tests, the differences between Imagn 2000, ISHAGE and dual-platform were not significant. These same significant correlations were found within PBSC and PB subsets, as shown in the table below.

Samples = 71	CD34+ cells/mL mean ± SD			Spearman's Correlation		
	Imagn 2000	ISHAGE	FCS		R	P
PBSC n° 27	1951.7± 2519	2072±2744	2438±2908	ISHAGE vs Imagn 2000	0.96	<0.0001
				ISHAGE vs FCS	0.95	<0.0001
				Imagn2000 vs FCS	0.97	<0.0001
UCB n° 29	22.14±19.10	26.35±16.65	27.3±17.40	ISHAGE vs Imagn 2000	0.32	=0.065
				ISHAGE vs FCS	0.84	<0.0001
				Imagn2000 vs FCS	0.49	=0.125
PB n° 15	54.4± 91	78.4±113	87.2±118	ISHAGE vs Imagn 2000	0.90	<0.0001
				ISHAGE vs FCS	0.96	<0.0001
				Imagn2000 vs FCS	0.89	<0.0001

Conversely, in UCB samples only a slight correlation was found between Imagn 2000 and the two other methods, probably due both to the very low number of CD34+ cells and to the inclusion of debris and aggregates in the Imagn 2000 method. Our preliminary results indicate that the abovementioned single platform assays are feasibile, easy, rapid and comparable to classical dual platform methods and therefore they may be recommended for clinical transplantation practice.

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ORAL COMMUNICATIONS

session 2

007
CLINICAL RELEVANCE OF CYTOGENETIC STUDIES IN 420 PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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We report the incidence, morphologic characteristics and prognostic significance of chromosome abnormalities detected in 420 patients with myelodysplastic syndromes (MDS) studied at our Institution. A diagnosis of aquired sideroblastic anemia (ASIA) was formulated in 6 cases, refractory anemia (RA) in 163 cases, refractory anemia with excess of blasts (RAEB) in 124 cases, RAEB in transformation (RAEB-t) in 102 cases and of chronic myelomonocytic leukemia (CMML) in 44 cases. The overall incidence of chromosome abnormalities was 60%. Considering FAB classification cytogenetic defects were identified in one out of six cases with ASIA, in 73% of RA, in 76% of RAEB, in 92% of RAEB-t and in 11% of CMML. Therefore, the MDS subtypes with the lowest blast cell percentages also showed the lowest incidences of karyotype defects. A correlation with morphology was possible in a total of 63 cases. Twenty-three showed the 5q- syndrome, with the proximal breakpoint localized at band 5q13 and the distal one at 5q33. Within this group of patients, median age was 65 years and the male/female ratio was 8/15. In one case a trisomy 6 associated with a hypoplastic bone marrow was noted. Nineteen cases demonstrated a deletion of number 17 at band 17p11. In 17 the Pelger-Huet anomaly and vacuolated neutrophils were seen. Five cases had a rearrangement of band 3q26 and all had elevated platelet numbers. A t(5,12) was typically observed only in CMML cases, while a deletion or a translocation of 12p was seen in another 13 patients. A –7 or 7q- was identified in 62 cases, belonging to all MDS FAB subtypes. A 20q- was seen in 17 cases. Karyotype evolution occurred in a total of 42 cases. It occurred especially in RA patients; in RAEB and RAEB-t it was rarely seen. This datum suggests that RA is really a preleukemic condition. As far as prognosis is concerned, patients with the 5q- syndrome had the best outcome. Their median survival was 76 months and none transformed into AML. Patients with a normal karyotype had a similar median survival but 27/167 evolved into AML. Loss of the Y chromosome and 20q- were both associated with a good prognosis. Monosomy 7, 7q-, 17p- and complex karyotypes had the worst outcome with median survival ranging from 7 to 11 months and frequent leukemic evolution.

008
T-CELL DEPLETED STEM CELL TRANSPLANTATION FROM FULL HAPLOTYPE MISMATCHED DONORS IN PATIENTS WITH HIGH-RISK HEMATOLOGICAL MALIGNANCIES

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Allogeneic stem cell transplantation is a potentially curative treatment for hematologic malignancies. Patients with no suitable related or unrelated HLA matched donor have at least one full haplotype mismatch donor within the family. In September '99 we started a T-cell depleted mismatched transplant program in patients with high risk hematological malignancies.[1,2] Six patients have so far been enrolled in this study (2 ALL, 2 AML, 1 NHL, 1 CML-CB). The median age was 45 (range 28-62). One ALL patient was in II CR; 5 (1 ALL, 1 AML, 1 secondary AML, 1 NHL, 1 CML-CB) were in relapse or progression with chemoresistant disease. The conditioning regimen included 12Gy F-TBI (4 patients) or melphalan 160 mg/m^2 (2 patients) with thiotepa 10 mg/kg, fludarabine 200 mg/m^2 over 5 days, thymoglobulin (Imtix-Sangstat) 15 mg/kg over 5 days and cyclosporin A 1 mg/kg from day-13 to -3. No post-transplant immunosuppressive therapy was given for GVHD prophylaxis. Peripheral blood stem cells were mobilized from donors with 12 μg/Kg/die G-CSF (lenograstim) over 7 days and collected by leukaphereses. CD34+ cells were T-cell depleted by using the CliniMACS (Miltenyi System). The median number of CD34+ selected cells infused was 15.6x10^6/kg (range 7.8-17.7), whereas the number of CD3+ cells was 1.72x10^4/kg (range 0.39-9.4). Three patients (1 ALL, 2 AML) engrafted with full donor chimerism, reaching PMN>500/μL on day +10, +18, +31 and PLT> 20000/μL on day +12, +25 +31, respectively. On day +49 a graft failure was observed in the patient with secondary AML and a further immunosuppressive regimen (fludarabine+CTX+ATG) was administered, followed by a second infusion of CD34+ selected cells from the same donor (CD34+: 11.53x10^6/kg, CD3+:0.8x10^4/kg). He achieved a stable and durable engraftment, reaching PMN>500/μL and PLT>20000 on day +18 and + 19 respectively. None of the 3 evaluable patients developed acute GVHD. Pulmonary aspergillosis was documented in all of them and successfully treated with 5 mg/kg/day of Ambisome. The three patients are alive and disease free on day +180, +75 and +47. Three patients died: two (1 NHL, 1 CML-CB) with a poor performance status and high disease burden died of multiorgan failure, on day +1; 1 patient

(ALL) died of disseminated Aspergillosis on day +10. Patients enrolled in this transplant program had no therapeutic chance of cure with conventional therapy and their need for transplant was urgent. We conclude that this mismatched transplant program is feasible and offers a valid therapeutic option to patients with high-risk hematologic malignancies who do not have a suitable matched donor.

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009
CORRELATION BETWEEN TUMOR BURDEN AND SUCCESSFUL *EX VIVO* PURGING IN FOLLICULAR LYMPHOMA PATIENTS ASSESSED BY REAL-TIME QUANTITATIVE PC

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Purging procedures are increasingly being used to provide stem cell harvests devoid of contaminating tumor cells. In follicle center lymphoma (FCL) most approaches are successful in eradicating PCR-detectable disease only in a fraction of harvests undergoing *ex vivo* manipulation. It is presently unknown whether the achievement of a PCR-negative status following *ex vivo* purging could be associated with a smaller tumor burden compared to harvests that remain PCR- positive following the same procedure. To address this issue, we developed a real time PCR approach for quantitative measurement of tumor contamination using the Bcl-2 rearrangement. Real time PCR of the t(14;18) was always performed on triplicate samples. Standard curves were generated using ten-fold dilutions of cloned t(14;18) rearrangements starting from 10^6 copies. In order to normalize samples for DNA quantity and quality, real time PCR of the GAPDH gene was also performed. Our method proved effective for quantitative analysis and always gave rise to standard curves with correlation coefficiencies greater than 0.98. Sensitivity was 10^{-5}. Extensive evaluation of assay accuracy and reproducibility showed that real time PCR,can be considered as a robust and reliable method for measuring the degree of tumor contamination in patients' samples. Real time PCR was used to evaluate the relationship between tumor burden in peripheral blood progenitor cell harvests and purging effectiveness: a panel of 10 patients whose PCR-positive collections underwent *ex vivo* manipulation using the MaxSep System was analyzed before and after *ex vivo* manipulation and following autologous transplantation. Four collections were successfully cleared of minimal residual disease as assessed both by nested and real time PCR. Before *ex vivo* manipulation, these collections had a tumor contamination of 12 Bcl-2 rearrangements/1,000,000 diploid genomes (dg)(range: 4-25). In contrast collections from patients who remained PCR positive following the same procedure had a median tumor burden of 1,330 Bcl-2 rearrangements/1,000,000 dg (range: 282-8,372). Despite the small patient sample, we could observe a significant correlation between tumor burden and successful clearance of PCR-detectable disease (p=0.04). Following *ex vivo* manipulation, median tumor burden in collections in which the procedure failed to eradicate PCR-detectable disease was 60 Bcl-2 rearrangements/1,000,000 dg (range: 52-241). All patients autografted with PCR-negative stem cell grafts are presently in continuos clinical and molecular remission with a median follow-up of 1,610 days while 5 of 6 patients autografted with PCR positive harvests have relapsed. This study provides the first evidence that real time PCR can predict the effectiveness of therapeutic intervention in non-Hodgkin's lymphoma. Based on these findings, we foresee a more widespread use of this technique to evaluate the impact of different therapeutic approaches in patients with FCL.

010
LIPOSOMAL-DAUNORUBICIN DISPOSITION AND RENAL EXCRETION IN PATIENTS WITH ACUTE LEUKEMIA

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Liposomal formulations of anthracyclines have been developed to increase the drugs' delivery to solid tumors while reducing toxicity in normal tissues.[1] DaunoXome (DNX, NeXstar) is a liposomal-encapsulated preparation of daunorubicin registered for Kaposi's sarcoma treatment that was developed to allow a selective distribution of daunorubicin to tumor tissues in an attempt to improve its therapeutic index.[2,3] During prior *in vitro* studies DNX showed at least a comparable toxicity with free daunorubicin against leukemic cells.[4,5] The aim of our study was to determine DNX pharmacokinetics in 11 poor risk acute leukemia patients treated with DNX 60 mg/m^2 iv on days 1, 3, and 5. Blood and urine samples were collected at appropriate intervals after each of the three DNX administrations. The total amount of daunorubicin (free and entrapped) (t-DNR) and of its metabolite daunorubicinol (DNRol) were assayed by HPLC according to the method of Camaggi and co-workers with a few modifications.[6] The main pharmacokinetic parameters ($t^1/_2\alpha$ 4.54±0.87 h; Vdss 2.88±0.93 L/m^2; Cl 0.47±0.26 L/h/m^2) showed that in patients with acute leukemia liposomal-entrapped daunorubicin pharmacokinetics differ greatly from that observed for the conventional formulation. In fact, DNX produced mean plasma AUC levels (t-DNR AUC 456.27±182.64 µg/mLh) about 100 to 200-fold greater than those reported for the free drug at comparable doses due to a very much lower total body clearance. Volume of distribution at steady-state was 200 to 500-fold lower than for the free drug. Plasma AUC of DNRol (17.62 ±7.13 µg/mLh) was similar or even greater than that observed with free daunobubicin for comparable doses. The AUC ratio between metabolite and parent drug was 0.041. Cumulative urinary excretion showed that about 6% and 12% of the total DNX administered dose was excreted in urine as daunorubicin and daunorubicinol, respectively. No major toxicity was encountered. Therefore, pharmacokinetic char-

acteristics suggest that DNX may be more convenient than free daunorubicin in the treatment of acute leukemia. In fact, liposomal formulations may allow reduction of both daunorubicin uptake in normal tissues thus minimizing toxicity at least for the parent drug and guarantee unimpeded access to leukemic cells in the bloodstream and bone marrow, thus theoretically improving efficacy.

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011
AN IMMUNOTOXIN ANTI-CTLA-4, CONTAINING THE RIBOSOME-INACTIVATING PROTEIN SAPORIN AND A SCFV, IS SELECTIVELY TOXIC FOR ACTIVATED T LYMPHOCYTES

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Cytotoxic T-lymphocyte Ag-4 (CTLA-4 or CD152), a homolog of CD28, is a glycoprotein expressed on the surface of activated T-cells and shares its ligands, CD80 and CD86, with CD28. CTLA-4 is not expressed on resting T-cells and is transiently induced only after activation. Despite a quite low surface expression, CTLA-4 shows a much higher avidity for CD80/86 as compared with CD28.[1] Cross-linking of CTLA-4 has a negative regulatory effect on T-cells, by downregulating interleukin-2 (IL-2) production, interleukin-2 receptor (IL-2R) expression and cell cycle progression *in vitro*. Immunotherapy with monoclonal anti-CTLA-4 antibodies could be utilized for the suppression of the immune system (autoimmune diseases, transplant rejections and graft versus host disease-GVHD). The recombinant human monoclonal antibody scFv #83 recognizes an epitope present only on the CD152 dimer. This scFv is able to stain human T-lymphocytes stimulated either with anti-CD3 or anti-CD28 antibodies. In this report we describe an immunotoxin made with the scFv #83 and the ribosome-inactivating protein (RIP) saporin. Immunotoxins are chimeric proteins consisting of a toxin coupled to an antibody, by either chemical conjugation or genetic engineering. To date several clinical trials have been conducted, and some are still ongoing, to evaluate the anti-tumor efficacy of immunotoxins. The response rates observed in phase I/II trials have often been higher than those reported for some of the conventional drugs. In our studies as toxic moiety we chose saporin, a RNA N-glycosidase from plants which cleaves one or more adenine molecules from ribosomal RNA, thus damaging ribosomes in an irreversible manner.[2] This RIP and the scFv were linked via a disulphide bond between chemically inserted sulphydryl groups. The conjugate was purified by affinity chromatography on Sepharose rProteinA. After conjugation saporin maintained its enzymatic activity, evaluated on a cell-free protein synthesis system (rabbit reticulocyte lysate), and scFv #83 retained the same antigen binding properties of the native antibody. The ability of this immunotoxin to eliminate CTLA-4 positive cells was studied on several human cell lines, and on human T-lymphocytes, activated with anti-CD3 and anti-CD28 antibodies. RIP incremented its toxicity on target cells by 1-2 log upon conjugation with scFv #83. Better results were obtained on activated T-lymphocytes. After 72 h of incubation with immunotoxin, a high percentage of lymphocytes (85-90%) appeared apoptotic, even at the lowest tested dose (0.01 nM). In resting lymphocytes, the immunotoxin was unable to induce apoptosis even at the highest concentration used (10 nM). In a mixed leukocyte reaction, with human dendritic cells used as stimulator, the immunotoxin scFv #83/saporin completely inhibited lymphocyte proliferation, at a concentration of 10 nM. These results show that the immunotoxin scFv #83/saporin could be a good tool for experimental therapy of autoimmune diseases, transplant rejections and GVHD, provided that systemic toxicity is low grade and well tolerated.

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012
ALTERNATIVE SPLICING OF THE BCR GENE OUTSIDE THE MAJOR BREAKPOINT CLUSTER REGION IN PATIENTS WITH P210-POSITIVE CHRONIC MYELOID LEUKEMIA

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Patients with Philadelphia-positive (Ph+) chronic myeloid leukemia (CML) usually have BCR-ABL rearrangements involving BCR exons 13 or 14 (e13 and e14, or b2 and b3), and ABL exon 2 (a2) resulting in the hybrid protein P210: on chromosome 22 the breakpoint is located in the so called *major breakpoint cluster region* (M-bcr). In rare cases the breakpoint on chromosome 22 is located downstream of M-bcr, in a region called m-BCR, and involves BCR exon 19 (e19) and ABL exon 2, which maintains its reading frame and gives origin to protein P230. We found that a P230-positive CML patient was also expressing some e14a2 (P210) transcript, so we decided to investigate whether other CML

cases positive for the e13/e14a2 (i.e. P210) transcripts were indeed P230 (e19a2 cases) expressing also the classical P210 hybrid protein. We therefore used a set of primers located downstream to M-bcr, in BCR exon 15 (e15) and tested 100 classical CML patients at diagnosis. Using a nested RT-PCR we found three cases with bands shorter than the P230-positive control. The sequence of one of these bands showed a fusion between BCR exon 18 (e18) and ABL exon 2, a rearrangement that does not maintain the ABL correct reading frame. Our data suggest that approximately 2-3% of CML cases with the e13/e14a2 (P210) transcripts have a breakpoint 3' to the classical M-bcr region and also express small amounts of alternative BCR-ABL rearrangements involving various BCR genes.

ORAL COMMUNICATIONS

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013
MULTILINEAGE INVOLVEMENT IN THE 5Q- SYNDROME: A FISH STUDY ON BONE MARROW SMEARS

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The involvement of a pluripotent progenitor cell was demonstrated to occur in MDS with normal karyotype or with numerical chromosome aberrations, but the pattern of lineage involvement by the 5q31 deletion in the 5q- syndrome is unknown. Bone marrow (BM) smears from 8 patients with the 5q- syndrome were studied by a modification of the FISH technique that allowed for direct visualization of the cell morphology. A commercial 5q31 probe (Vysis Inc.) was used simultaneously in dual-color experiments with a chromosome-5-centromeric probe in BM smears from 8 patients with the 5q-syndrome. As additional internal controls a chromosome-7-centromeric probe and a 7q31 probe were used. To establish the sensitivity limit of this approach 5 normal BM smears were studied. All 8 patients had the 5q-chromosome as the sole anomaly in 45% to 75% of the interphase cells. *a) Normal controls.* The presence of the expected 2-red and 2-green signals in more than 70% cells was consistently observed in well spread areas with preserved cell morphology, a minority of cells having clumped chromatin. In the 5 controls the percentage of erythroid and granulocytic cells having a false 5q31 deletion was in the range of 5-10% (median value 7%). The ploidy of megakaryocytes ranged between 4N and 16N, with a 5-12% rate of false 5q31 deletion (median 7%). *b) Patients.* 20-40 erythroblasts were analyzed in all cases: they consisted mostly of proerythroblasts and basophilic erythroblasts. In all patients a clone carrying the 5q31 deletion was detected (35-50% of the cells, median 45%); 20-50 granulocyte precursors were scored, with 5q31 deletion in all cases ranging between 40%-50% (median 45%). The proportion of neutrophils carrying the 5q deletion was consistently lower than the corresponding value that was observed in promyelocytes. In all patients 20-25 megakaryocytes were analyzable, with an overall incidence of 52-68% of the 5q31 deletion. Equal proportions of large multilobular megakaryocytes and of hypolobular megakaryocytes characteristic of the 5q- syndrome were scored: the latter cells showed the 5q31 deletion more frequently than the former cells. Few cells with uncondensed nuclear chromatin pattern, two or three prominent nucleoli with cytoplasmatic hypogranulation were seen in each sample carrying the 5q31 deletion in 66% to 100% of the cases (median 83%). We arrived at the following conclusions: i) the transformation in the 5q- syndrome involves an undifferentiated progenitor cell, consistently retaining the ability to proceed along multiple differentiation pathways; b) monolobated megakaryocytes are part of the abnormal clone.

014
LECAM1/ICAM1 RATIO IDENTIFIES A DISTINCT PATTERN ON NORMAL AND MYELODYSPLASTIC BONE MARROW CD34+ CELLS

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The abnormal growth and proliferation observed in myelodysplastic syndromes (MDS) could be secondary to defective adhesive properties within the stem cell compartment.[1] With this in mind, a three color flow cytometric assay was used to compare the expression of integrins (CD49b, CD49d, CD49f, CD11a, CD11b, CD11c, CD18), selectins (Lecam1, Leu8), ICAM1 and Thy1 on CD34+ progenitor cells from bone marrow (BM) of 23 patients affected by MDS and 13 healthy donors. The expression of adhesion molecules (AM) was measured as percentage of positivity, mean intensity of fluorescence (MIF) and AM Index (AMI = product of MIF and percent positive cells). Considering age (19/23 >60 years, median 70), FAB subtypes (16/23 RAEB or RAEB-t), number of cytopenias (13/23 ≥ 2 cytopenias), BM blast infiltration (12/23>10% of BM blasts, median 10%), IPSS score (4/7 Int-2+High Risk), the vast majority of the patients (19/23, 83%) had at least one poor-risk feature. With the group of healthy donors serving as control, we observed that, among MDS patients, the selectin Lecam1 was defective on CD34+ cells in terms of either percentage, or MIF and AMI (p<0.02 for all comparisons), whereas ICAM1 was significantly more expressed in terms of percentage (p=0.012). Given the mutually exclusive relationship between Lecam1 and ICAM1 both in MDS and normal BM, we tried to verify whether specific values of the Lecam1/ICAM1 AMI ratio could serve to distinguish normal from MDS CD34+ progenitor cells. In fact, a greater Lecam1/ICAM1 AMI ratio for normal BM compared to MDS samples was observed [median 41.13 (range 3.9-80) vs. median 0.24 (range 0.008-3.7), p<0.001]; this finding suggests that a low Lecam1/ICAM1 AMI ratio (<3.7) may identify myelodysplastic patterns. Significantly, the AMI ratio converterd from 0.33 to 12.12 in 1 patient after a chemotherapy-induced remission. In addition, as leukemic transformation progresses, the AMI ratio decreases (median 0.046, range 0.005-0.19, p=0.23) mainly due to an overexpression of ICAM1 as confirmed in 3 patients (AMI ICAM1 3794 vs. 352. p=0.008). In conclusion, Lecam1 is defective in the stem cell compartment of MDS patients and this may explain in part the abnormal growth pattern observed in this disease;[2,3] furthermore, the evaluation of the Lecam1/ICAM1 ratio could be helpful at diagnosis, during the clinical course of disease and the follow up after therapy as a marker of minimal residual disease.

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015
SECOND CHRONIC PHASE BEFORE TRANSPLANTATION IS CRUCIAL FOR IMPROVING SURVIVAL OF BLASTIC PHASE CHRONIC MYELOID LEUKEMIA

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The prognosis of patients with blastic phase chronic myeloid leukemia (BP-CML) is extremely poor, even when allogeneic stem cell transplantation (allo-SCT) is performed in this phase. Fludarabine plus high-dose cytarabine has shown valuable activity in acute myeloid leukemia (AML). Since successful outcome after transplantation seems to depend, in AML and in chronic phase CML, on disease status at the time of transplantation, we tested whether use of FLAN induction before allo-SCT may be useful in BP-CML. From January 1988 to June 1998, 20 patients with BP-CML were studied: 10 patients received FLAN induction chemotherapy before proceeding, if a suitable HLA donor was found and clinical conditions were adequate, to early allo-SCT, whereas 10 patients were submitted to BMT without remission induction. Overall 8/10 (80%) patients achieved second chronic phase after 1 course of therapy with FLAN; 1/10 (10%) showed a partial response and 1 (10%) was refractory. The refractory patient did not proceed to allo-SCT. Nor did one patient who obtained second chronic phase but lacked a suitable HLA donor. A further patient who achieved second chronic phase but experienced early relapse with rapid regrowth of the disease was not submitted to allo-SCT. Thus 7 patients (6 in second chronic phase and 1 with partial response) were submitted to allo-SCT within 3 months after FLAN. Of these, the patient who had shown only partial morphologic response to FLAN obtained karyotypic, morphologic and molecular second chronic phase after allo-SCT, but relapsed within 3 months of the transplant. Of the other 6 patients transplanted in second chronic phase, all obtained molecular remission, four are still in second chronic phase, with intervals ranging from 10 to 54 months, while one died of infection having relapsed 14 months after SCT, and one died of transplant-related complications in second chronic phase. The mean duration of second chronic phase and survival (analyzed on an intention-to-treat basis) after allo-SCT are both significantly longer than in the group of 10 BP-CML patients submitted to allo-SCT without FLAN remission induction treatment [22.4 (range 1-61) vs 3.5 months (range 1-10) and 22.7 (range 2-61) vs 6.4 (range 1-16) months respectively], even though 9/10 of the patients not submitted to FLAN obtained morphologic second chronic phase after BMT, and 4 and 3 obtained cytogenetic and molecular second chronic phase, respectively. We conclude that FLAN induction therapy followed by early allo-SCT appears effective in the treatment of BP-CML and could provide a possible cure for BP-CML patients, deserving wider study in the context of a multicenter trial.

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016
PROGRAMMED CELL DEATH IN THE ERYTHROID COMPARTMENT. QUANTITATIVE EVALUATION AFTER BONE MARROW TRANSPLANTATION FOR HOMOZYGOUS BETA THALASSEMIA

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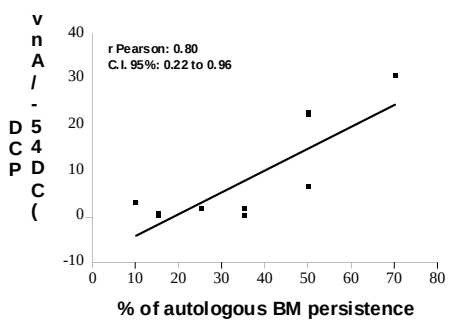
Accelerated intramedullary programmed cell death (PCD) of erythroid precursors contributes to the severe anemia characterizing homozygous β thalassemia. Because the genetic defect is expressed in the hematopoietic marrow, bone marrow transplantation (BMT) represents an important and successful option in the cure of such a disease. Persistence of residual host hematopoietic cells (RHC), referred to as mixed chimerism (MC), has been described after marrow transplantation in beta-thalassemia. In this report we tested the hypothesis that after BMT for β thalassemia, the extent of erythroid precursor PCD correlates with the extent of RHC persistence. Bone marrow samples were taken from 8 long-term survivors (3-10 years) of BMT for homozygous β thalassemia. Patients were evaluated for MC by DNA-based techniques (RFLP, PCR-VNTR) or fluorescence *in situ* hybridization analysis for the Y chromosome. PCD was evaluated by FACS analysis using annexin V reactivity on the erythroid precursors (CD45-). The figure shows the significant direct correlation observed after allogeneic BMT between the amount of PCD, evaluated as absolute number ($x10^5$/mL) of early apoptotic erythroid precursors (CD45-/AnV+), and the extent of autologous bone marrow persistence. Quantitative evaluation of PCD in the erythroid compartment could be a useful tool for evaluating the extent of MC in patients after BMT for homozygous β thalassemia. *This work was supported by the Berloni Foundation against the Thalassemia.*



017
PERIPHERAL BLOOD STEM CELL TRASPLANTATION IN THE ELDERLY WITH MULTIPLE MYELOMA

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Multiple myeloma hits patients older than 60 years in more than 70% of cases and a great proportion of them are between 60 and 70 years. A proposed therapeutic program devoted to ameliorate the outcome of myeloma patients should include those over 60 years. In the last years more and more aggressive treatments, including myeloablative therapy followed by autologous peripheral blood stem cell (PBSC) transplantation have been proposed with curative intent; however most programs exclude patients older than 60 years. In an effort to demonstrate the feasibility of aggressive approaches also in older patients, we present our experience in patients with multiple myeloma older than 60 years observed and uniformly treated in a single institution. There were 18 patients, treated from April 97 to May 2000, aged between 60 and 73 years (mean 67.5). The whole program included 4 courses of VAD therapy followed by mobilization of CD34 cells with cyclophosphamide at 5 g/m^2, collection of a minimum number of CD34 cells of 2.5x10^6/Kg, myeloablative therapy with busulfan 12 mg/kg over 4 days and melphalan 140 mg/m^2 the fifth day, reinfusion of CD34 cells two days later and, finally, interferon maintenance at the maximum tolerated dose starting 2 months after transplantation. No patients failed to mobilize and yielded a mean number of CD-34 positive cells of 6.5x10^6/kg (range 2.5-13.1); the engraftment was recognized in all patients with PMN >500 reached at 9.9 days mean (range 6-13). One death 5.5% related to the procedure after hematologic recovery was recorded in a patient 60 years old who developed interstitial pneumonia; 4 episodes of Gram + sepsis without consequences were documented during cytopenia, 5 fever of unknown origin were also recorded, grade III-IV mucositis was recorded in one third of patients. All patients showed a response to the procedure with a complete remission obtained in 5 patients; 5 patients progressed and three patients died of their disease. At a mean follow-up of 16 months 14 patients are alive, 3 in complete remission, 9 with stable disease and 2 in progression; 4 have died. We conclude that myeloablative therapy in over 60-year old patients with multiple myeloma is feasible with acceptable toxicity; whether such an approach will provide a prolongation of survival needs to be demonstrated by randomized studies, however this study may provide basic information in order to design prospective studies.

018
DETECTION OF CIRCULATING ANEUPLOID CD38+ PLASMACELLS AND CD19+ LYMPHOCYTES IN MULTIPLE MYELOMA PATIENTS

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Cells belonging to the myeloma clone are frequently detectable in the peripheral blood of the majority of patients with multiple myeloma (MM) either by molecular biology and by flow cytometry techniques. DNA aneuploidy has been used as a genetic marker of malignancy in MM, and can be detected by flow cytometry in combination with monoclonal antibodies. Plasmacells are defined by high CD38 expression and variable expression of CD19. The combined use of surface staining CD38 and DNA staining with propidium iodide (PI) is an accurate method for identifying monoclonal aneuploid plasmacells in MM patients. The presence of circulating myeloma cells has been correlated with the stage of the disease, and a role in relapse after autologous hemopoietic stem cell transplantation has been hypothesized. CD19 is a pan B-lymphocyte antigen that appears early in B-cell ontogeny and disappears with differentiation to plasmacells. If CD19 positive B-lymphocytes are part of the myeloma clone, there should be evidence of myeloma specific genetic and phenotypic markers in this population. Therefore, if myeloma plasmacells lack CD19 expression, then CD19 positive cells represent a phenotypically distinct population of B-lineage cells that can be characterized by the presence of the genetic changes associated with myeloma. Even though the presence of plasmacell precursors has been suspected, circulating aneuploid CD19+ lymphocytes have rarely been detected in MM patients with CD38+/CD19- plasmacells. By using multi-dimensional flow cytometry with DNA content measurement in combination with CD19 or CD38 monoclonal antibodies, we analyzed 25 MM patients. Aneuploid plasmacells were detected in bone marrow aspirates from 9 out of 25 patients (36%). The plasmacell phenotype was CD38+/CD19- in 7 out of the 9 patients. In 5 out of 7 patients (71%) we were able to detect CD38+ circulating plasmacells (0.43% median ; 0.02% SD). A small number of CD19+ aneuploid cells were detected in 2 out 5 patients (0.003% median ; 0.001% SD). In order to detect a sufficient number of CD19+ aneuploid cells we had to analyze more than 1x10^6 circulating lymphocytes. By using this technique we were able to detect around 3 aneuploid cells over 100,000 events. In this study we detected circulating plasmacells in 71% of the MM patients and we were able to detect circulating aneuploid CD19+ lymphocytes. The significance of this finding is controversial and open to different interpretations.

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019
MLL LEUKEMIA WITH A NEW THREE-WAY TRANSLOCATION T(6;11;7)(Q27;Q23;Q22)

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Translocations involving the chromosomal band 11q23 are demonstrated in infant acute lymphoblastic leukemias, in acute myeloid leukemias (AML), as well as in myelodysplastic syndromes and lymphomas. The majority of these translocations cause the rearrangement of the MLL gene, mapped at band 11q23, which breaks within a region of 8.3 kb spanning exons 6-8. We report a case of AML - M1 in a 64-years old female. She had never been exposed to topoisomerase II inhibitors or to other genotoxic agents. The patient did not respond to treatment with Mini-MICE (mitoxantrone, etoposide, cytosine arabinoside) and she died in first aplastic phase because of widespread cerebral thrombosis. The cytogenetic analysis performed on bone marrow cells showed a 11q23 deletion, an apparent t(6;7) translocation and trisomy 8 in all metaphases. The Southern blot analysis with the 0.83-kb BamHI MLL cDNA fragment showed two bands or rearrangement in addition to the germline fragment, indicating that the MLL gene was broken inside the classical cluster region. FISH analysis was first done with a combination of two PAC clones, labeled with two different fluorochromes, which detect both the 3' and 5' MLL region, with a minimal overlap in the breakpoint (A. von Bergh *et al.*, Genes Chromosome Cancer 2000; 28, 14-22). In interphase cells, a fused spot corresponding to the normal MLL gene and two single-color spots corresponding to a rearranged MLL were demonstrated in 78.7% of cells. Metaphase-FISH showed that the 5' region of MLL was retained in the der(11) chromosome, whereas the 3' MLL was translocated to the der(7) chromosome. A dual-color FISH analysis on metaphases with a 7q36qter probe and a centromeric chromosome 6 probe demonstrated that the 7q was translocated on the der(6); using a 6qsubtel probe together with a 11 satellite DNA, we found that the chromosome 6q was translocated on the der(11). Due to the translocation of 6q to 11q23 band, involvement of the AF6 gene was suspected; indeed, RT-PCR for MLL and AF6 (located on 6q27) was positive for the presence of a MLL/AF6 fusion. FISH and molecular results demonstrated in our patient a MLL/AF6 fusion resulting from a new three-way translocation, t(6;11;7)(q27;q23;q22).

020
DIFFERENTIATIVE THERAPY + LOW-DOSE CYTOTOXIC DRUGS AS A MAINTENANCE, POST-REMISSION TREATMENT IN POOR PROGNOSIS AML AND MYELODYSPLASTIC SYNDROMES: PRELIMINARY RESULTS

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Acute myelogenous leukemia (AML) patients more than 60 years old and myelodysplastic syndrome (MDS) patients uneligible for allogeneic bone marrow transplantation (BMT) and with more than 10% bone marrow blasts have a poor prognosis, in spite of the possibility, for 50-60% of them, of achieving a complete remission (CR) after intensive chemotherapy. Indeed, CR is of short duration in these patients (usually less than one year)(*Beran M et al.: J Clin Oncol 17: 2819, 1999; Estey EH et al.: Blood 93: 2478, 1999*). Our previous studies (*Ferrero D et al.: Leuk Res 20: 867, 1996; Blood 94 suppl. 1, 1999*) demonstrated that a combination therapy with 13-cis retinoic acid (20-40 mg daily) + dihydroxylated vitamin D3 (1-1.5 µg daily), as a continuous treatment, + cycles of 6-thioguanine or 6-mercaptopurine (40-60 mg daily for 15-20 days every 5 weeks) can improve hematologic parameters and prolong survival in about 50% of MDS patients with blast excess uneligible for intensive chemotherapy. We, therefore, applied the above described therapy (with some modifications), as a maintenance treatment, to poor prognosis AML and MDS patients who had achieved a CR but were not eligible for allogeneic BMT. We have so far treated 15 patients with a median age of 66 years (range 27 -73). Eight patients had AML (in one case after previous MDS), 4 RAEB-t and 3 RAEB with more than 10% bone marrow blasts. In 4/15 cases the disease was related to previous cytotoxic chemotherapy. Thirteen patients started the treatment in first CR and 2 in second CR, achieved after different protocols of standard chemotherapy. Eight patients had received post-remission consolidation chemotherapy and started maintenance 1-4 months after CR achievement, the other 7, because of age and/or poor performance status, proceeded directly to maintenance therapy. Two MDS patients received 13-cis retinoic acid + dihydroxylated vitamin D3 only, while in 7 patients these drugs were associated with intermittent 6-thioguanine, as described above. In a further 6 patients, the 6-thioguanine cycle was replaced, once every 3-4 months, by a combination of low dose s.c. ARA-C (8 mg/m^2 x 2/day) + 6-mercaptopurine (40 mg/daily) for 14 days.

Therapy was well tolerated, the most frequent complaint being moderate lip and mouth dryness. No treatment-related death occurred. Symptomatic but reversible hypercalcemia occurred in one patient, while transient grade 2-4 thrombocytopenia (and grade 1-2 neutropenia) was detected in patients receiving ARA-C. Median CR duration and survival were 19 (2-35+) and 26 (4-41) months respectively. Ten patients relapsed and died of their hematologic disease, one patient died of a second tumor after 22 months of CR. Four patients (2 AML, 1 RAEB-t, 2 RAEB, including one therapy-related), all out of the 6 whose treatment included ARA-C, are alive and in continuous CR at 6, 16, 23 and 35

months from CR achievement. Two of these five had not received consolidation chemotherapy after CR. In conclusion, about 60% of our patients, treated with a cheap and well tolerated therapy, have obtained a longer than expected survival and CR duration. The present results need, of course, to be confirmed by a larger case population and a longer follow-up. However, the association of differentiating agents + low dose chemotherapy is probably worthy investigation as a maintenance treatment in poor prognosis myeloid malignancies.

023
GENETIC MODIFICATION OF HUMAN T-CELLS WITH CD20: A STRATEGY TO PURIFY AND LYSE TRANSDUCED CELLS WITH ANTI-CD20 ANTIBODIES

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T-lymphocytes are of paramount importance for the optimal treatment of bone marrow-transplanted patients. The possibility of genetically engineering T-lymphocytes could contribute to improve their handling in a clinical setting. In particular, one would like to find optimal strategies for rapid selection of transduced cells and, in addition, to make T-lymphocytes susceptible to death *in vivo* in case of graft-versus-host disease (GVHD). We explored the possibility of introducing a single human gene that can at the same time drive immunoselection of the infected cells and make them susceptible to drug-mediated lysis. A retroviral vector was constructed which contains the human CD20 cDNA under the control of Moloney murine leukemia virus (MoMLV LTR). Freshly isolated mononuclear cells were infected for three consecutive days in the presence of PHA and hrIL-2 and acquired a CD3/CD20 double phenotype with a mean percentage of 15.9, ranging from 6.5 to 31.7. Transduced T-lymphocytes grew and expanded *in vitro* like mock infected cells, and CD20 expression was maintained for several months with no change in the growth curve of the cells. CD20-expressing T-lymphocytes can be positively immunoselected using microbead-conjugated anti-CD20 monoclonal antibody and a magnetic separator (Miltenyi Biotech, Germany) which allows a high and reproducible level of purity. *In vitro* exposure to chimeric humanized monoclonal anti-CD20 antibody (rituximab, Roche) in the presence of complement, results in effective and rapid killing of the transduced CD3/CD20 double positive human T-cells. In addition several different mutants of the CD20 molecule have been generated to explore the possibility of transducing a molecule completely devoid of any possible signaling activity in the context of T-lymphocytes. The feasibility of selecting and killing cells transduced with these CD20 mutants is presently being investigated. This approach represents a new and alternative method of gene manipulation with a *suicide gene* for the production of drug-responsive T-cell populations, a crucial step for the future management of GVHD developing after bone marrow transplantation.

024
ALPHA-INTERFERON AS AN ALTERNATIVE TO ALLOGENEIC BONE MARROW TRANSPLANTATION IN THE TREATMENT OF P-190BCR-ABL POSITIVE ADULT ACUTE LYMPHOBLASTIC LEUKAEMIA

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Treatment of P190 BCR-ABL+ acute lymphoblastic leukemia (ALL) patients remains problematic: one possibility is to use biological response modifiers such as α-interferon (α-IFN), which is known to be active in chronic myeloid leukemia (CML). We used α-IFN to treat 10 adult P190 BCR-ABL+ ALL patients (8 newly diagnosed; 2 in first relapse). All received a remission induction chemotherapy (modified L-20 protocol). Patients achieving morphologic, immunologic and cytogenetic complete remission (CR) were then submitted to a rotational consolidation regimen lasting six months. When no HLA identical donor was available, patients aged <55 years underwent stem cell harvest followed by autologous transplantation; patients aged > 55 years received standard maintenance treatment for six months. In the second year, maintenance treatment (all ages) was based on cycles of α-IFN (3 MU three times a week for 6 weeks) alternated with methotrexate/6-mercaptopurine continuously for up to two years from first demonstration of CR. Thereafter, patients maintaining CR had the same schedule of α-IFN (6 weeks on, 6 off). Eight patients (6/8 first diagnosis, 2/2 relapsed) obtained morphologic, immunologic and cytogenetic CR with persistent molecular positivity. Two with an HLA identical donor had allogeneic bone marrow transplantation. Six proceeded with chemotherapy: 1 experienced early relapse, 3 were autotransplanted, and 2 received maintenance treatment. Five patients then received alfa-IFN as scheduled. All 5 are in continuous morphological and cytogenetic CR, with a longer mean duration of maintained morphologic CR (mean 46 months; range: 20-88) than in previous reports of Ph+ ALL patients treated with chemotherapy regimens (excluding allogeneic BMT). α-IFN thus appears effective in this poor-risk subset of patients. This well-tolerated IFN-containing maintenance treatment could be considered to reinforce intensified programs based on autologous stem cell transplantation as an alternative to allogeneic transplantation in P190BCR-ABL+ ALL patients, (and by extension for Ph+ ALL patients) lacking an HLA-matched donor.

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ORAL COMMUNICATIONS

session 5

025

HIGH-DOSE ARA-C GIVEN WITH AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELL SUPPORT FOSTERS THE HARVEST OF OPTIMAL AMOUNTS OF CIRCULATING HEMATOPOIETIC PROGENITORS

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Between 1996 and 1999, 111 patients with hematologic malignancies underwent aggressive chemotherapy programs including the sequential administration of high-dose (hd) cyclophosphamide (CY) (7 g/m^2) and hd-Ara-C (2 g/m^2 twice a day for 6 consecutive days) and final intensive consolidation with peripheral blood progenitor cell (PBPC) autograft. Patients were aged between 18 and 60 years; 106 patients had non-Hodgkin's lymphoma (52 diffuse large cell, 2 Burkitt, 5 anaplastic CD30 positive, 18 mantle cell, 18 follicular, 1 marginal cell, 4 transformed, 6 high-grade T-cell), 4 had Hodgkin's disease and 1 had amyloidosis. Seventy-eight patients were at disease onset, whereas the remaining were at first or subsequent disease relapse. A PBPC harvest was carried out following both hd-CY and hd-Ara-C. To minimize hematologic toxicity, a PBPC reinfusion (1-2x10^6 CD34+ cells/kg) was carried out following hd-Ara-C. PBPC mobilization was assessed in any case in terms of total CD34+ cells/kg collected with 1 or more harvest procedures following both CY and Ara-C. In 61 out of 111 patients, CFU-GM analysis (total CFU/Kg collected) was also carried out. According to the amount of PCPC collected, 96 out of 111 patients were considered *good mobilizers* since more than 2x10^6/kg total CD34+ elements were collected following high-dose CY; in this group, mobilization following CY and Ara-C was almost identical: median total CD34+ elements x10^6/kg collected were 17.6 (2.3-93.9) and 21.65 (0-107.4) respectively; similarly, median total CFU-GM x10^4/kg collected were 83 (19-860) and 121 (1.5-883) respectively. Beside the good-mobilizers, there were 15 patients unable to mobilize adequate amounts of progenitor cells following CY: these patients were considered *poor mobilizers*. Harvest was carried out even in the presence of low mobilization. All PBPC collected were employed for reinfusion following the subsequent hd-Ara-C course. Interestingly, all 14 evaluable poor mobilizer patients yielded very good harvests following Ara-C. The median quantity of collected CD34+ x 10^6/kg was 10 (2-36.9), versus 2 (1-2) following hd-CY; analogously, the median quantity of collected CFU-GM x 10^4/kg was 61.9 (23.6-167), versus 1.21 (0-2.5) versus following hd-CY. PBPC collected in this subset of patients allowed satisfactory engraftment following autograft. The data from this experience imply the following conclusions: (i). the use of hd-Ara-C with PBPC reinfusion allows satisfactory mobilization, even if delivered at short-interval from a previous hd-chemotherapy course; (ii). this approach is particularly effective in those patients with non-optimal PBPC mobilization at the first hd-treatment. This approach may increase the number of patients able to undergo PBPC autograft consolidation. In addition, PBPC collection following repeated intensive chemotherapy courses contributes to a more extensive *in vivo* purging effect, making this program particularly suitable in those patients at high-risk of tumor cell harvest contamination. The concomitant use of humanized monoclonal antibodies could perhaps amplify the *in vivo* tumor cell clearing prior to PBPC collection.

026

OXALIPLATIN INDUCES ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS IN HUMAN MYELOMA CELLS

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We investigated the activity of oxaliplatin (L-OHP), a third-generation platinum coordination complex of the 1,2-diaminocyclohexane (DACH) family, against human multiple myeloma (MM) cells with different sensitivity to interleukin-6 (IL-6). The exogenous IL-6-dependent XG-1, the IL-6-partially dependent XG-1a, and the IL-6-independent U266 and IM-9 myeloma cell lines were used in these experiments. *In vitro* cytotoxicity assays were performed by the trypan blue exclusion test and hemocytometric cell counts. Induction of apoptosis was analyzed with propidium iodide, annexin-V staining and the Mebstain method. We demonstrated that 24 hours of treatment with L-OHP, at clinically achievable concentrations (IC_{50}: 5-10 μM), inhibits the growth of myeloma cells. This effect is related to the induction of apoptosis and is not antagonized by IL-6, which is the major growth and survival factor for MM. The combined treatment of L-OHP with dexamethasone resulted in the increase of the anti-myeloma effects of both compounds. After 24 hours of culture, the mean growth inhibition of the four cell lines was 49+/-10% in the L-OHP-treated cultures, and 32+/-3% in the cultures incubated with dexamethasone (10 μM). The combination of L-OHP + dexamethasone produced an additive inhibitory effect in all the cell lines (69+/-9% of inhibition). Our results provide a rationale for the experimental use of L-OHP in the treatment of patients with MM.

027

THERAPEUTIC STRATEGY FOR *DE NOVO* ACUTE MYELOID LEUKEMIA BASED ON FLUDARABINE-CONTAINING REGIMENS: RESULTS OF A PROSPECTIVE SINGLE CENTER STUDY

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After a preliminary trial on patients with poor risk acute myeloid leukemia (AML), we assessed the value of a therapeutic strategy for patients of any age *de novo* AML based on fludarabine-containing regimens as induction therapy followed by autologous or allogeneic transplantation whenever

appropriate. We tested a therapeutic program especially designed for elderly people, with the aim of reducing non-haematologic toxicity. In the younger subgroup of patients we were particularly interested in evaluating feasibility of high-dose therapy. Sixty-eight consecutive patients with *de novo* and untreated AML entered the study between June 1995 and April 1999 (median age 60 years). Patients < 60 years (N. 25) received fludarabine, ara-C, idarubicin and G-CSF (FLAG-Ida) while patients aged 60 or older (N. 43) were administered a similar regimen including mitoxantrone instead of idarubicin (FLANG). Patients achieving complete remission (CR) received post-induction therapy. Non-hematologic toxicity of FLAG-Ida was very low. Twenty-one patients (84%) out of 25 aged 59 or less achieved CR, whereas the final CR rate of elderly patients was 58%. Six patients (all over 60) died during induction. Thirteen patients under 60 years of age underwent autologous (N = 9) or allogeneic (N = 4) bone marrow or peripheral blood progenitor cell (PBPC) transplantation. After the mini-ICE regimen a mean of $7.8x10^6$ CD34+ cells/kg were collected with an average of 2 aphereses. In one patient the low CD34+ cell yield did not allow us to give high-dose therapy. In patients < 60 years the 3-year projected disease-free survival (DFS) and survival were 41% and 46% respectively. Elderly patients had a poorer outcome (18% DFS at 2 years). Good-intermediate and unfavorable karyotype patients differed significantly as far as DFS (24 % vs 0%; $p = 0.002$) and overall survival (OS) (46% vs 0%; p=0.0006) are concerned. Both FLAG-Ida and FLANG proved to be feasible and well tolerated. Our data confirm the prognostic relevance of karyotype. Though limited by the small number of patients our study shows that allogeneic or autologous transplantation after fludarabine containing regimens is feasible and well tolerated. Both acute and chronic GVHD were mild. In the group of younger patients the overall strategy provided good antileukemic activity. In patients over 60 years of age the FLANG regimen displayed low toxicity but the final outcome was not satisfactory, thus confirming the need for more effective post-induction therapy.

028
NOVEL TYPES OF BCR-ABL TRANSCRIPT IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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We have identified two novel BCR-ABL fusion transcripts in patients with chronic myeloid leukemia (CML). The first patient was diagnosed as having chronic phase Ph+ CML, underwent allogeneic bone marrow transplantation from an HLA-identical sibling donor, relapsed at 12 months post-BMT and showed no response to two escalating doses of donor lymphocyte infusion. RT-PCR analysis was performed at hematologic relapse, as no samples from diagnosis were available. No amplification products were seen when primers for the P210 proteins were used, whereas using primers for the e1a2 (P190) transcript, a band of higher molecular weight (195 Kd) with respect to the classical e1a2 control was observed. Sequencing revealed the presence of an in-frame fusion consisting of part of BCR exon 3, 44 nucleotides derived from ABL intron 1b and ABL exon 2. This rearrangement has therefore generated an abnormal BCR exon e3 in which the 3' sequence has been substituted by 44 nucleotides derived from the ABL intron 1b, creating a new BCR exon 3 that can be spliced to ABL exon 2 maintaining the correct frame of translation of the ABL sequences. The second patient had chronic phase CML at diagnosis. Molecular analysis performed using the primer set for P210 protein revealed, at the first step of RT-PCR, a band of 540 bp, 123 bp higher than the classical b3a2 rearrangement. Sequencing confirmed this finding, as a 123 nucleotide stretch was interposed between BCR exon 14 and ABL exon 2. This fragment was not derived from the BCR gene, or from the ABL gene; a search of the Gene-Bank database did not show any homology with known human genes. In any case, since the fragment encodes for a 41 amino acid polypeptide without stop codons, the ABL reading frame is maintained.

029
BONE MARROW TRANSPLANTATION IN CHILDREN WITH HEMATOLOGIC MALIGNANCIES. A SINGLE-CENTER EXPERIENCE

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Between July 1980 and December 1999, 111 children with acute leukemia were transplanted in the BMT Centre of Pesaro. Their mean age was 8.8 years (range 1-16). Fifty-nine patients were transplanted from HLA matched (56 related and 3 unrelated) donors [group A]; 52 children from HLA 1-3 antigens mismatched parents [group B]. One patient from each group received a 2nd BMT from the same donor. Diagnoses were: AML (n=17), ALL (n=40), MDS (n=2), with 51% (n=30) in advanced phase of disease in group A , and: AML (n=19), ALL (n=25), MDS (n=8), with 92% (n=48) in advanced or refractory disease in group B. Twenty-five patients were prepared with cyclophosphamide+total body irradiation, all others with busulphan+cyclophosphamide, in 10 of whom VP-16 was also added. ATG or ALG before transplant was given to 47 children in group B. For GVHD prophylaxis long MTX (n=31), CsA alone (n=7), sMTX+CsA (n=21 in group A, and n=52 in group B) were given. *Results.* The incidence of severe acute GVHD (grade III-IV) was 19% in group A and 40% in group B. Chronic GVHD was mild in 10, and moderate in 2 patients in group A, while in group B 11 patients had mild, 4 moderate and 1 severe chronic GVHD. Overall relapse, transplant-related mortality (TRM) and disease-free survival were respectively: 36%, 27% and 37% in group A, 25%, 58% and 17% in group B. In group A, post-transplant relapse of original disease was more frequent in patients transplanted in relapse than those in complete remission (50% vs 21%, p=0.01), and event-free survival was significantly higher in patients in CR than those in relapse (52% vs 23%, p=0.02). In group B age less than 7 years was associated with higher EFS (40% vs 8%, p=0.01) and lower TRM. The major cause of death was relapse of underlying disease in group A, and TRM (GVHD, infections, toxicity) in group B. In conclu-

sion, bone marrow transplantation from an HLA identical donor can cure approximately 50% of children transplanted in CR, while only 20% of children with advanced or refractory leukemia benefit from transplantation, either from HLA matched or mismatched donor, without any difference in EFS (23% vs 19%).

030
CLINICAL SIGNIFICANCE OF APO1/FAS AND CD23 SERUM LEVELS IN B-CHRONIC LYMPHOCYTIC LEUKEMIA

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B-cell chronic lymphocytic leukemia (CLL) shows variable clinical outcome and therefore biological parameters need to be added to current staging systems in order to predict the indolent or the aggressive course of the disease.[1] Reports from the literature have demonstrated that soluble CD23 is implicated in the progression of the disease;[2] moreover, B-CLL overexpresses Bcl-2 gene product or soluble APO1/Fas and therefore is more resistant to chemotherapy-induced apoptosis.[3,4] In order to define whether these mechanisms play a role in determining the heterogeneous B-CLL clinical activity, we performed APO1/Fas, Bcl-2 and CD23 immunoenzymatic assays on 168 patients, (median age 64.2 years, 94 males and 74 females), all fulfilling the recommended diagnostic criteria with dim SIg and CD5+/CD23+ immunological pattern. Forty-five patients had low modified Rai stage, 116 intermediate stage and 7 high stage. Forty-eight were treated with chlorambucil at conventional doses and 48 received 6 courses of fludarabine monophosphate. The thresholds of positivity were set at 7.1 ng/mL for APO1/Fas, >40 U/mL for CD23 and >240 U/ml for Bcl-2. CD23 values were closely related to both Bcl-2 (p=0.004) and APO1/Fas (p<0.001), while a trend for a correlation was noted between APO1/Fas and Bcl-2 (p=0.157). A level of β2 microglobulin higher than 2.2 mg/mL was significantly associated with both CD23 >40 U/mL and APO1/Fas >7.1 ng/mL (p<0.001 and =0.001, respectively). Furthermore, the presence of three or more inthrathoracic/abdominal enlarged lymph nodes (>3 cm in diameter) was correlated with both CD23 >40U/mL and APO1/Fas >7.1 ng/mL (p<0.001 and =0.001). A lymphocyte doubling time (LDT) <12 months was observed in 14 patients and 12/14 had Bcl-2 >240 U/mL (p=0.010). CD23 >40, APO1/Fas >7.1 and Bcl-2 >240 were strongly correlated with intermediate/high modified Rai stages (p<0.001, p=0.010 and p=0.011, respectively). Forty-eight of the 72 (66.9%) patients with CD23 <40 U/mL and 49/69 (71%7 of those with APO1/Fas <7.1 ng/mL did not require any therapeutic approach; conversely, 63.3% (60/95) of the CD23 >40 U/mL cases and 54.3% (50/92) of APO1/Fas >7.1 ng/mL patients required continuous chemotherapy (p<0.001 and =0.001). Moreover, a significant lower complete remission (CR) rate was detected in patients with beta2-microglobulin >2.2 ng/mL (38.9% vs 90%; p=0.010), in CD23 >40 U/mL (30% vs 83.3%; p<0.001), in Bcl-2 >240 U/mL (29.4% vs 68%; p=0.027) and in APO1/Fas > 7.1 ng/mL (39.1% vs 60.9%; p=0.038) patients treated with fludarabine. A significantly shorter survival at 8 years was found in beta2-microglobulin >2.2 ng/mL (p= 0.00031), in CD23 >40 U/mL (p=0.0027), in bcl-2 >240 U/mL (p=0.005) and in APO1/Fas >7.1 ng/mL patients (p=0.010). In conclusion, high CD23, APO1/Fas and Bcl-2 levels identify patients in advanced clinical stage, often with bulky disease and poor response to fludarabine. These biological parameters might be used to further stratify B-CLL patients into novel risk classes in order to identify those candidate for aggressive therapeutic approaches.

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ORAL COMMUNICATIONS

session 6

031
PRELIMINARY RESULTS OF TOPOTECAN-CYTARABINE REGIMEN IN PATIENTS WITH HIGH RISK SECONDARY ACUTE MYELOID LEUKEMIA

Beltrami G, Pierri I, Gatto S, Ballerini F, De Totero D, Pietrasanta D, Quintino S,Varaldo R, Venturino C, Grasso R, Miglino M, Canepa L, Clavio M, Gobbi M

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The outcome of patients with secondary and refractory acute myeloid leukemia (AML) is generally poor. Recently, regimens including topotecan, a topoisomerase I interacting agent, have been reported to achieve promising preliminary results. We tested the feasibility of the combination topotecan + cytarabine (TA) in 12 high risk AML patients. Topotecan (1.25 mg /m^2) was administered as a continuous i.v. infusion for 5 consecutive days; cytarabine (1g/m^2) was given i.v in a 2- hour infusion on the same days, within 12 hours after the beginning of topotecan. Patients achieving complete response (CR) were administered two further courses of TA with a 50% reduction in the dosage of both drugs. Partial responders were treated with a second course of the same TA regimen. In 10 patients AML was secondary to a myelodysplastic syndrome (5 RA, 2 RAEB, 3 RAEB t), in 2 to a chronic myeloproliferative syndromes (idiopathic myelofibrosis and essential thrombocythemia). Eight patients received TA as first line therapy, 4 as salvage treatment (1 prior regimens). The median age of patients was 61 (range 33-73). Chromosomal abnormalities were documented in 7 out of 12 patients (5 complex karyotypes, 1 monosomy 5 and 1 monosomy 7). Thrombocytopenia (Plt < 50x10^9/L)

occurred in 8 patients. Three patients (all untreated) achieved CR, after two courses of TA; 3 patients obtained partial response (in one of these TA was administered as salvage therapy); 6 patients did not respond. No severe extrahematologic side effects were recorded. The main toxicity involved the gastrointestinal tract. Mortality in the first 4 weeks was 27% (2 deaths from disease progression, 1 from haemorrhagic stroke). At the time of analysis only 1 patient is still alive after a median follow-up of 15 months. In conclusion, TA proved effective in high risk AML when employed as first line therapy. Patients with refractory AML were less sensitive to the regimen. In 2 of the 3 patients achieving CR we documented the disappearance or marked reduction of the percentage of metaphases carrying the poor prognosis karyotypic alteration. These preliminary results encourage us to extend our experience and to test alternative schedules of treatment.

032
SAFETY OF AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION IN PATIENTS WITH MULTIPLE MYELOMA AND CHRONIC RENAL FAILURE

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Patients with multiple myeloma (MM) and chronic renal failure have generally been excluded from myeloablative therapy programs followed by hematopoietic stem cell support because of the potential increase in transplant-related morbidity and mortality. We report here on our experience treating 6 MM patients with moderate to severe renal insufficiency, with autologous stem cell transplantation. One of these patients had required chronic hemodyalysis since the diagnosis of MM had been made. Peripheral blood stem cell collection was performed with either cyclophosphamide 5.5 to 7g/m^2 + G-CSF, 5μg/kg/day (patients 1-3, 5 and 6) or G-CSF, 15μg/kg/day alone (patient #4). Four patients (#1-4) received autotransplant as front-line therapy, while the last two patients were treated in relapse, which occurred following prior autologous stem cell transplantation in support to melphalan, 200μg/m^2 (#5) or maintenance therapy with alpha-interferon (#6). High-dose chemotherapy administered as preparation to transplant included busulfan 12μg/kg + melphalan 80μg/m^2 (pts #1-3 and 6) or melphalan 80m^2/mq alone (#4 and #5) in order to reduce mucosal damage. Following transplant, prompt and sustained recovery of hematopoiesis was documented in all the patients; 500 PMN/mm^3 and 20,000 platelets/mm^3 were reached after a median of 13 and 14 days, respectively. None of the patients suffered from WHO grade 3-4 infectious complications. Transplant-related toxicity included grade 3-4 oral mucositis (pts #1, 4 and 5) and veno-occlusive disease (pt #3). Renal function either improved or remained stable throughout the transplant period. All the patients but one responded to therapy, 3 of them are progression free after 2, 15 and 26 months; two relapsed after 16 and 4 months and one died of cholangiocarcinoma 7 months after transplant, while still in remission. Although our experience is limited so far, these results appear promising and support the investigational use of myeloablative therapy in MM patients with chronic renal failure.

033
ISOLATION AND EXPANSION OF ENDOTHELIAL CELLS FROM HUMAN BONE MARROW PRECURSORS

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The existence of the *hemangioblast*, the common precursor of hematopoietic and endothelial cells, has not been definitively proven in adult life; however, numerous findings support the hypothesis that endothelial cells (ECs) can originate from hematopoietic stem cells. In this study we report that bone marrow AC133+ cells, a subset of CD34+ hematopoietic progenitors, can differentiate into ECs. In particular, immunomagnetically separated AC133+ cells (MACS, Miltenyi) were grown on fibronectin-coated flasks in M199 medium supplemented with FBS, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) for 3-4 weeks (up to confluence), with an average±SD expansion of 8±4. Phenotypic analysis showed that most cells were CD45-, CD14- and expressed several endothelial markers (Ulex europaeus agglutinin-1, von Willebrand factor (VWEF), P1H12, CD105). These cells were further purified by immunomagnetic separation using Ulex-FITC and anti-FITC microbeads and the sorted cell population was expanded with VEGF: highly purified endothelial cells, expressing Ulex, CD105, vWf and P1H12 and showing Weibel-Palade bodies and a high proliferative capacity (up to 2400 fold increase) were obtained. These purified ECs were also co-cultured with CD34+ hematopoietic progenitor cells in parallel with a purified fibroblastic monolayer. After 3 weeks of culture, cells both in the supernatant and in the adherent layer were seeded in methyl-cellulose to assess the CFU-GM output. CD34+ cells grown on endothelial cells gave rise to 355±75 CFU-GM colonies, while cells grown on fibroblasts 1191±123 CFU-GM colonies. These results, taken together, indicate that: a) ECs can be obtained from bone marrow hematopoietic progenitor cells; b) with multiple purification steps, highly purified EC cultures can be obtained; c) ECs co-operate with fibroblasts in supporting hematopoiesis. In addition, these data support the hypothesis that endothelial progenitors are present in adult bone marrow and may contribute to neo-angiogenesis.

034
SUSCEPTIBILITY TO APOPTOSIS AND P27KIP1 EXPRESSION IN STABLE AND PROGRESSIVE CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) cells are characterized *in vivo* by prolonged survival and accumulation, while it has been reported that *in vitro* they die rapidly with an

increased susceptibility to apoptosis. The role of apoptosis in CLL is, therefore, still contradictory and it is also unclear whether apoptosis plays a role in the heterogeneous clinical pattern of the disease. In the present study, we investigated the leukemic cell susceptibility to enter apoptosis in primary fresh samples from 75 CLL patients in stable (STD) or progressive disease (PRD), upon culture of neoplastic cells in standard medium conditions (10% fetal calf serum (FCS) and in the presence of autologous serum (AS). We also analyzed the kinetic differences by measuring the cell cycle distribution and expression of the negative cell cycle regulator p27KIP1 (p27). Cell susceptibility to enter apoptosis, evaluated after 24 hours of culture, showed a significant difference between STD (mean=21.23±15.5%) and PRD (m=14.79% ±7.7%) cases both in standard medium (p=0.04) and, to a greater extent, in AS (mean=23.03±17.9% and 11.27±7.6% respectively) (p=0.01). Normal B lymphocytes showed a high susceptibility to apoptosis with a mean value of 59.57±16.5% and 53.47±32.8% in FCS and in AS, respectively. These values were significantly greater than those observed in both groups of CLL, although significance (p=0.01) was reached only in PRD cases. In addition, cell kinetic analyses performed on fresh CLL samples showed that, in contrast to STD samples, PRD cases were characterized by higher quiescence, based on a lower RNA content (p=0.04) and, especially, on significantly higher p27 expression (p=0.03). In fact, when CLL cells were cultured in the presence of AS, PRD samples did not progress into cell cycle, while STD samples exhibited a significant increase of G1 cells (p=0.02). The results of this study document that CLL cells, particularly those from PRD cases, are characterized by a markedly reduced susceptibility to apoptosis and by a higher quiescence and p27 overexpression. These data help us to understand the mechanisms underlying the accumulation process typical of CLL, as well as the events regulating disease progression. The parallel cell cycle arrest of PRD CLL cells may represent an event concurring with the decreased susceptibility of CLL cells to apoptosis. These observations also point to a link in CLL between p27 and susceptibility to apoptosis, which bears potential therapeutic implications.

035
EXPRESSION AND REGULATION PATTERN OF FAS, FAS LIGAND, CD30 AND CD30 LIGAND IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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The cellular pathways downstream of Fas/Fas ligand (FasL) and CD30/CD30 ligand (CD30L), molecules belonging to the TNF/TNFR superfamily, have been implicated in relevant immune activities including functional polarization, terminal differentiation and regulation of cell turnover. Inflammation as well as immunity involve close interactions between endothelial cells (EC) and immunocompetent cells based on either cytokine-induced or cell-to-cell signals, so that we can expect that some cytokines and cytokine receptors are shared by immune cells and EC. This study was aimed at analyzing expression and role, if any, of Fas/FasL and CD30/CD30L on the part of EC. Human umbilical vein endothelial cells (HUVEC) were isolated as described by Jaffe and grown in medium 199 supplemented with 20% FBS. For comparative purposes, the human T-cell leukemia line Jurkat and the human T-cell lymphoma line Karpas-299 as well as peripheral blood lymphocytes were analyzed and cultured in standard conditions, when needed. Cells were stimulated with recombinant interleukin-1 (IL-1), TNF-α, IL-2, IL-4, interferon (IFN)-gamma, CD40L, progesterone, dexamethasone, GM-CSF or erythropoetin and evaluated by flow cytometry (Fas/FasL, CD30/CD30L), RT-PCR analysis (CD30/CD30L mRNA) and propidium iodide/annexin staining (apoptosis evaluation). We found that HUVEC expressed 1) Fas in resting conditions and this expression was markedly upregulated by IFN-γ; 2) FasL at lower levels than on Jurkat cells; 3) CD30L mRNA and CD30 molecules at low levels and its expression was enhanced by CD40L. By contrast, CD30 was never expressed on HUVEC. Finally, cell-to-cell interactions between CD30/Fas-positive cell lines and HUVEC led to the delivery of pro-apoptotic signals at least towards the cell line cells. The demonstration that HUVEC can express CD30L and that CD30L can be upregulated through CD40L suggests a novel, interesting regulatory mechanism implying a possible functional re-direction, including induction of pro-apoptotic signals, of CD30-positive cells interacting with HUVEC *in vivo*.

036
FISH ANALYSIS ON FRACTIONATED CELL POPULATIONS IN SEX-MISMATCHED ALLOTRANSPLANTATION AFTER NON-MYELOABLATIVE TREATMENT

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High-dose chemotherapy and radiation are typically used as a preparative regimen for bone marrow transplantation, but produce considerable morbidity and mortality. A new strategy utilizes a lower dose, non-myeloablative regimen to provide sufficient immunosuppression to prevent graft rejection, allowing engraftment of allotransplanted cells that produce a graft-versus-malignancy effect. Allogeneic T-cells, infact, act to eliminate residual hematopoietic and malignant cells of the host, but this process takes months to complete. In three patients who received a bone marrow transplantation after a non-ablative regimen from a sex-mismatched sibling, we studied, by FISH, chimerism in bone marrow and purified cell populations (T-lymphocyte, granulocytes) with X/Y probes. Granulocytes (GN) were purified by Ficoll separation, whereas T-lymphocytes were purified by fluorescent sorting after coupling with a anti-CD3 MoAb. Patient #1 is a 62-year old man with a diagnosis of AML-M4Eo in first relapse; patient #2 is a 54-year old female with AML-M1 with infectious complications, in first remission; patient #3 is a 52-year old patient with a long history of multiple myeloma. Different conditioning regimens were used, and engraftment was evaluated after 1 month in all patients; in patient #1 whole bone marrow was studied at months 2,

3 and 6; in patients #2 and #3 subpopulations were also analyzed at month 2. For FISH analysis we used a fluoresceinated centromeric probe for the X chromosome and a probe for Yq directly labeled with Cy3 (Cytocell Ltd, UK). At month 1, recipient cells acconted for from 3% to 11.3% of cells in BM, from 3.5% to 9.5% in GN and from 10.2% to 31.2% in T-lymphocytes. Two months after transplant the percentage of host cells decreased in all samples from patient #3, whereas it remained stable in patient #2. Complete engraftement was observed at month 6 in patient #1. In all three patients FISH analysis performed on BM and granulocytes showed a similar incidence of chimerism, whereas on T-lymphocytes we always observed the presence of a higher percentage of recipient cells . X/Y FISH on fractionated cell populations may represent an easy tool for evaluating progressive engraftement of donor cells after non-myeloablative allotransplantation.

ORAL COMMUNICATIONS

session 7

037
CHARACTERISTICS OF REAL-TIME AND COMPETITIVE RT-PCR QUANTIFICATION OF BCR-ABL TRANSCRIPTS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Some chronic myeloid leukemia (CML) patients can achieve a complete cytogenetic response (CCR), defined as the disappearance of Philadelphia chromosome-positive metaphases, which is the clinical goal of treatment with interferon-α(IFN). We used both competitive RT-PCR[1] and real-time TaqMan to quantify BCR-ABL[2-6] transcripts in 323 bone marrow and peripheral blood specimens collected from 84 CML patients (40 at diagnosis and 44 after achieving CCR with IFN)[2] (median age 47.5 year, range 18-65; median Sokal score 0.9, range 0.53-2.78). Total ABL, GAPDH, and β2microglobulin transcripts were quantified, as internal controls and expressed as BCR-ABL transcript/μRNA and as BCR-ABL/ABL, BCR-ABL/GAPDH and BCR-ABL/β2 microglobulin ratios. All 44 CCR patients had evidence of residual disease. Wide variations in the amount of BCR-ABL transcript were found at diagnosis, ranging from 17,300 to 750,000 with competitive RT-PCR and 30,900 to 398,000 with real-time TaqMan (median values 78,000 and 102,000, respectively). Median value of BCR-ABL/ABL was 8.86 while BCR-ABL/β2microglobulin ratio was 0.10576 (β2microglobulin being the most stable internal standard RNA control gene). Amount of BCR-ABL transcript at diagnosis was associated with the number of blast cells and Sokal's score. The median BCR-ABL/μgRNA at the time of maximal α-IFN response with real-time TaqMan was 4 (range, 3-4,600) and was significantly lower in patients who remained in CCR than in those who had a major karyotypic response (4,490 versus 4, $p<0.0001$). Our findings show that the level of residual disease falls with time in complete responders to α-IFN. Our competitive RT-PCR and real-time TaqMan assay both provide highly sensitive and reliable methods for monitoring CML patients and predicting rsponse to α-IFN therapy response. Our results confirm the greater resolution and enhanced sensitivity of Real-Time TaqMan analysis for the easy detection and quantification of BCR-ABL.[7]

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038
HEMATOPOIETIC PROGENITOR GROWTH EVALUATION AFTER COMBINED KIDNEY AND BONE MARROW TRANSPLANTATION IN AN ANIMAL MODEL

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It has been postulated that the result of a solid organ transplant is conditioned by the bidirectional traffic of immunocompetent cells between donor and recipient with the persistence of immunocompetent donor cells in recipient's lymphoid organs (microchimerism). This phenomenon seems to be increased, associating donor bone marrow (BM) cell infusion with solid organ transplantation. The aim of our study was to evaluate, in an animal model, hematopoietic progenitor cell growth after kidney transplantation (KT) alone (group A: 3 evaluable cases) or kidney plus bone marrow transplantation (4 evaluable cases: group B). The animals, seven outbred unrelated piglets, were transplanted with equivalent identical donors. HLA incompatibility was assessed by mixed lymphocyte cultures. Two subjects in the group A died, the first one at day +17 because of peritonitis and the second one at day + 64 of heart failure following pneumonia, while in group B only one animal died, after 65 days, of bowel obstruction. In group B the mononuclear BM cells ($1.3\pm0.83x10^8$/Kg), harvested from donor iliac crest and separated on Ficoll, were frozen at –80°C, and infused with a central venous catheter at day +7 from KT. Immunosuppression was achieved with intravenous FK 506 (0.6 mg/Kg/die) and oral mycophenolate mofetil (10 mg/Kg bid) from day 0 (kidney transplant) till day +30 when it was stopped. In the follow-up the animals were monitored till day +90 and were then sacrificed. Hematopoietic progenitor cell cultures were performed in methylcellulose with erythropoietin (4 U/mL) and GM-CSF (100ng/mL) on days: 0, +15, +30, +45, +60, +90. The results are reported in the following Table:

Table 1.

days from KT	*0*	*15*	*30*	*45*	*60*	*90*
A CFU-GM°	84±43	106±76*	11	20	22	
BFU-E°	67±32	65±81	702	62	166	
CFU-GEMM°	8	1±1	0	0	2	
B						
CFU-GM°	28.5±16	223±45*	113±24	45±22	207±107	151±245
BFU-E°	116±68	184±44	103±153	48±25	36±21	108±192
CFU-GEMM°	2.7±2.8	3±2	1±1	2±2	2±2	2±2

*°x10^5 plated cells; *group A vs group B: p<0.05.*

In group B 3 out 4 subjects were alive and well at 90 days. This fact underlines a better survival in group B then in A, in which only one animal was alive 36 days after KT and the other two cases died of infectious diseases.

Moreover, the *in vitro* proliferative activity was higher in group B than in A, although statistical significance was observed only for CFU-GM growth at day +15. These results suggest good tolerance of the BM infusion with small toxicity in the recipient hematopoietic compartment. In conclusion BM infusion seems to ameliorate the survival rate after solid organ transplantation but more tests and molecular microchimerism evaluation should be performed to confirm this hypothesis.

039
THE PROPORTION OF PERIPHERAL BLOOD LEUKEMIC CELLS DETECTED BY IMMUNOCYTOCHEMISTRY CORRELATES WITH THE CLINICAL STATUS IN HAIRY CELL LEUKEMIA

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Immunohistochemistry (IHC) on bone marrow (BM) trephine samples is considered the most reliable diagnostic tool and a sensitive method for the detection of residual disease after treatment of hairy cell leukemia (HCL). In a previous study we showed that peripheral blood (PB) immunocytochemistry (ICC) highlights hairy cells (HC) and allows the diagnosis of HCL in all the cases at presentation. In this study, using a panel of monoclonal antibodies (McAb) directed to HCL-associated antigens, we compared the PB ICC with the BM trephine IHC before and after treatment with DCF and IFN in combination. CD22, CD25, CD103 and DBA.44 McAb were utilized on PB cytospins, while CD20 and DBA.44 were used on BM trephine biopsies. Immunomorphology staining was used for 31 patients at diagnosis, 20 patients in complete remission (CR) and 28 patients with *active disease* (partial responders and progression) for a total of 159 PB and 159 BM samples. PB circulating HC were easily identified in all cases at diagnosis. Among the HCL-associated antigens, CD22 accounted for the highest percentage of positive HC (30.3±4.0%). Less than 1% PB HC was observed in all the 57 cases in CR. In the 71 cases with *active disease*, the percentage of PB CD22 positive HC was 14.5±1.8%. Compared to PB ICC, a higher percentage of CD20 positive HC was observed in the BM trephine at diagnosis (69.5±3.1%) and in patients with *active disease* (39.1±3.7%). All samples in CR had ≤ 10% BM leukemic infiltration. When the expression of the HCL-associated antigens was evaluated on the polyclonal lymphoid population, an increased percentage of DBA.44 positive B lymphocytes was observed after therapy in the majority of cases. PB ICC can help in monitoring disease status and reduce the need for BM trephines in HCL. *(S. Masi was supported by a fellowship from FIRC).*

040
ROLE OF 1, 25α DI-HYDROXY VITAMIN D3 AND ITS NUCLEAR RECEPTOR IN NORMAL HEMATOPOIESIS

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Several studies performed in our and other laboratories have demonstrated the capacity of 1,25-α–dihydroxy-vitamin D3 (VD) to induce monocytic differentiation of leukemic myelo- (M2 type) and mono-blasts (M5 type). This differentiation is achieved through a specific interaction with the corresponding nuclear receptor (VDR), that heterodimerizes with RXR, and activates the transcription of VD primary response genes.

Although the biological effects induced by VD in leukemic hematopoiesis have been intensively studied in the past years, it is not known whether VDR and its ligand are involved in the regulation of normal hematopoiesis. To assess the effects exerted by VD on normal hematopoietic cells, we cultured human CD34+ hematopoietic stem cells, purified from cord blood samples, in the presence of cytokines such as stem cell factor (SCF), Flt3 ligand (Flt3-l), interleukin 11 (IL-11), interleukin 6 (IL-6) and Interleukin 3 (IL-3). This combination of cytokines has, in fact, demonstrated to achieve optimal expansion of CD34+ cells, coupled with myeloid differentiation, characterized by the appearance of both granulocytic and mono-macrophagic morphologic and immunophenotypic markers. The effects induced on these primary hematopoietic cells by treatment with pharmacological (10^{-8} M) to physiological (10^{-10} M) levels of VD, were then analyzed. Our results clearly indicate that physiologic concentrations of this vitamin are able to: 1) induce a remarkable mono-macrophagic differentiation of CD34+ stem cells, as demonstrated by the appearance of a macrophagic morphology and by the induction of CD14 and CD11b surface antigens; 2) down regulate the expression of CD34 antigen and the CD34+/CD38- highly undifferentiated fraction of CD34+ cells; 3) enhance the proportion of mono-macrophagic progenitors (CFU-M), as assessed by clonogenic assay in methylcellulose perfomed on CD34+ cells following VD stimulation. Western blot analysis, performed on nuclear extracts of primary hematopoietic cells, has shown that VDR is strongly induced upon VD treatment, suggesting that the effects exerted by VD on normal hematopoiesis are genomic rather than non-genomic. We are planning experiments to assess whether VD is able to synergize with growth factors such as GM-CSF and M-CSF, which are physiologically involved in monocytopoiesis.

041
CYTOGENETIC ABNORMALITIES IN NEWLY DIAGNOSED MULTIPLE MYELOMA

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Few cytogenetic studies have been reported in multiple myeloma (MM), however the prognostic impact of specific abnormalities has been assessed specifically concerning short survival of patients with aberrations of chromosome 13 (monosomy or partial deletions) and chromosome 11 (reciprocal translocations or partial deletions). To date, we have studied the karyotypic pattern of 62 newly diagnosed MM patients, enrolled in the "Bologna 96" protocol (which compares 1 vs 2 autologous stem cells transplants). Fifty-five cases (88.7%) had a successful cytogenetic analyses, an abnormal karyotype was detected in 25 patients (45.5% of evaluable patients). Almost all patients showed a complex pattern: the number of affected chromosomes ranged from 1 to 17 (median number: 5). The most common numerical aberrations were chromosomes 3, 5, 9 and 15 trisomies and chromosome 13 monosomy; while the structural aberrations involved mostly 14q, 11q, 12p and 1 and 16 chromosomes. The involvement of 14q32 was the most common abnormality in MM. We found cytogenetic involvement of the 14q32 in 32% of abnormal cases; these included 3 patients with the classical t(11;14)(q13;q32), 1 patient with t(1;11;14)(q21;q13q32), another one with t(11;14;17) (q13;q32;q21) and 2 patients with unknown additional material on 14q32. Aberrations of chromosome 13 have been described in 6 patients in conventional cytogenetic studies. To establish the frequency and the clinical impact of deletions of chromosome 13 in MM, we performed interphase FISH with a probe specific for D13S319, a gene locus distal to rb-1 on 13q14. The studies were performed as dual-color hybridizations combining the D13S319 probe with a centromere-specific reference probe. The number of observed interphases ranged from 176 to 716 (median number: 511). We found a deletion in 17 of 44 patients (38.6%); the frequency of aberrant cells ranged from 9.9% to 59.1%. FISH analysis found a deletion of 13q14 in 10 out of 25 patients with apparently normal or non-informative karyotypes. Twelve patients with abnormal karyotypes but 2 normal chromosomes 13 were also disomic for 13q14 on FISH analysis. A longer follow-up of these patients is necessary to define the possible unfavorable role of chromosome 13 aberrations and other abnormalities on response to therapy and survival in MM.

042
ANALYSIS OF P53 GENE MUTATIONS IN MULTIPLE MYELOMA AT DIAGNOSIS

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Multiple myeloma (MM) is a clonal hematologic disorder whose pathogenesis is ascribed to a multistep carcinogenic process. One of the critical targets of genetic lesions seems to be p53, a nuclear phosphoprotein involved in the control of cell proliferation.[1] Several groups have reported an increased frequency of p53 mutations in patients with advanced MM.[2-4] In this study we investigated the frequency and the location of p53 gene mutations in a large series of newly diagnosed MM patients who were subsequently treated with conventional chemotherapy (VAD and high dose cyclophosphamide) followed by one or two autologous peripheral blood stem cells transplantations. Genomic DNA obtained from bone marrow specimens of 90 patients at diagnosis[5] was analyzed by primer-specific amplification of exons 5 to 8, followed by direct automatic sequencing. A total of 20 point mutations were detected in 15 out of 72 evaluable patients (20.8%). Ten mutations fell in exon 5, six in exon 6, one in exon 7 and three in exon 8. Eleven mutations caused an amino acid substitution in the DNA-binding domain of the protein; the remaining nine mutations were silent. In addition, our analysis revealed 2 GT to GC transversions in the splice donor site at the exon 8/intron 8 border. These shifts presumably cause intron 8 to be retained in the processed mRNA, with subsequent premature termination due to the presence of a stop codon after 93 nucleotides in the intron. The result should be a truncated protein lacking the last 87 amino acids at the C-terminus (comprising the oligomerization domain, nuclear localisation signals and acetylation sites) which should be substituted by the 31

amino acids encoded by intron 8.

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ORAL COMMUNICATIONS

session 8

043
IMPAIRMENT OF THE COMPARTMENT OF STROMAL CELLS IN MYELODYSPLASTIC SYNDROMES

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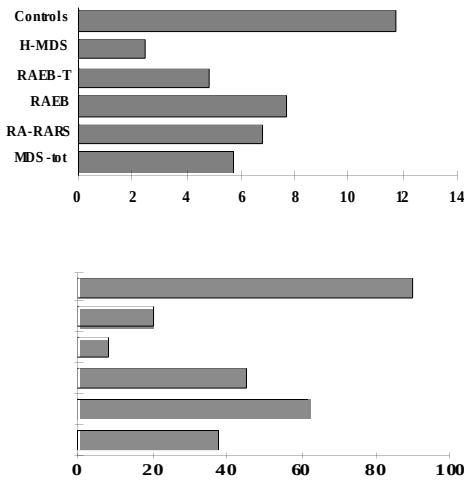
We assessed the *in vitro* behavior of bone marrow stromal cells (BM-SC) in myelodysplastic syndromes (MDS) with a particular focus on hypoplastic MDS (hMDS). H-MDS is an entity characterized by cytopenia, BM hypoplasia sharing similarities with severe aplastic anemia (SAA), except for the presence of a normal number of CD34+ cells, occurrence of abnormal localized immune precursors, dysmegakaryocytopoiesis and involvement of -7/7q- chromosome. Thirty-two cases were considered: 10 refractory anemia (RA), 3 RA with ring sideroblasts (RARS), 8 RA with excess of blasts (RAEB), 5 RAEB-t and 6 cases of hMDS. BM cells were cultured with long-term culture medium to evaluate the mesenchymal clonogenic precursors (CFU-F) and the stromal layer confluence capacity in a T25 flask. MDS stromal cells gave evidence of defective cell growth capacity vs controls (see Figures 1 & 2). In hMDS, CFU-F significantly (Wilcoxon test) differed from that in controls, RA/RARS (p=0.02) and RAEB (p=0.01) groups; the same difference was maintained considering the stromal confluence (p=0.01). An increased apoptosis process involving both the hemopoietic compartment and BM-SC has been reported in MDS, with the maximum rate occurring in the RAEB-t samples (*Raza et al., Blood, 1995*). Our *in vitro* findings confirm an impairment of BM-SC in MDS, suggesting how hMDS differs from both MDS or SAA, in which the stromal cell compartment seems to be spared, adding a further clue that differentiates these two similar forms of BM failure.



044
EXPRESSION OF CYCLIN D1, D3 AND B IN NON-HODGKIN'S LYMPHOMA AND ITS CORRELATION WITH PROLIFERATIVE INDEX (KI67)

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Cyclins are proteins which have been implicated in the control of mitosis in all eukaryotes. Ki-67 is a nuclear-associated proliferation antigen and it is expressed in all cycle phases except for G_0. We want to define the expression of those cyclins in non-Hodgkin's lymphomas (NHL) tissues, and evaluate their correlation with proliferative index and their expression in these diseases. Using the APAAP method we tested the following antibodies in 53 cases of NHL: anti-cyclin B (a mitotic phase cyclin) anti-cyclin D1, anti-cyclin D3 (a G_1 phase cyclin) and anti-Ki67. Cyclin D1 was expressed in 13 cases (24%), it was present in all 7 mantle cell lymphomas with high positivity. Cyclin D3 was expressed in 10 patients, being present in four mantle cell lymphomas and at low expression in some large cell lymphomas; it was not present in follicular lymphomas. These two cyclins were characterized by a prevalent expression in disease in advanced stage (> 80%), symptomatic conditions (> 80%) and with high values of LDH (> 70%). Cyclin B, with nuclear positivity, was present (more than 60% of positive cells) in 32 patients, Ki67 was positive in 22 cases. Ki67 was strongly expressed in all large cell lymphomas but not in follicular ones, it was significantly positive in patients with high LDH values. Cyclin B was also present in follicular lymphomas (53%) and in mantle cell lymphomas (6 out 7). There was no

relationship between B1 positivity and Ki67 expression in nodular lymphoma. In particular those cases positive for B1 did not show an increased Ki67 value. According to proliferative index cyclins D1 and D3 were not correlated with proliferative index; in contrast cyclin B was strictly associated with Ki67 expression: its presence in a portion of follicular lymphomas will allow identification of a more aggressive nodular pattern.

045
BONE MARROW STROMAL CELLS IN ACUTE PROMYELOCYTIC LEUKEMIA: COMPARISON WITH OTHER ACUTE MYELOGENOUS LEUKEMIA SUBTYPES

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Acute promyelocytic leukemia (APL) with t(15;17), significantly differs from other acute myelogenous leukemias (AML) by the prognosis which has been significantly improved after introduction of retinoic acid, as a differentiating agent. In an attempt to clarify whether APL differs from AML with regards to the stromal cell compartment too, bone marrow mononucleated cells (BM/MNC) from 21 untreated AML patients (M0: 1; M1: 4; M2: 4; APL: 6; M4: 2; M5:4) were plated in long-term culture. The fibroblastoid clonogenic precursors (CFU-F/ 10^6 BM/MNC; Collagen I/II/III+, CD68+) and the endothelial colonies (CFU-En CD31+, factor VIII+) content were assessed after 15 days by an immunohistochemical technique. Fibroblast confluence , in the T25 flask, was evaluated after 40 days by inverted microscope. CFU-F % conference p (Wilcoxon) AML (all cases) (mean±s.d) 4.4±4.7 50±30 0.01 non-APL AML 3.4±2.9 38.1±31.2 0.01 APL 7.6±2.1 71±11 n.s Controls 12.3±4.4 90±10 Considering CFU-En, the non-APL group displayed a 3.8-fold higher value than the control group (1.1±1 vs 0.28±0.4; p=0.05), while this difference was not present in APL samples (0.5±1.1). In conclusion, the biological characteristc of bone marrow stromal cells was found to be different in APL, in comparison with other AML subvarieties. This may be due to the different nature of the diseases. Moreover, we give evidence of an high angiogenesis rate in AML which appeared more prominent in non-APL samples.

046
THE VALUE OF PCR IN THE DIAGNOSIS AND MONITORING OF EARLY STAGE OF MYCOSIS FUNGOIDES TREATED WITH INTERFERON PLUS PUVA

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Polymerase Chain Reaction (PCR) analysis of T-cell receptor (TCR) gene rearrangements has contributed significantly to more accurate diagnosis and staging of mycosis fungoides (MF). Recent data suggest that this assay may be able to monitor tumor response to therapy, and detect early relapse of malignant lymphomas during clinical remission. In this work, we have planned a prospective study designed to demonstrate clonal T-cells in skin and peripheral blood samples of patients with early stages of MF who were treated with a combination of IFNα2b plus PUVA. PCR amplification of T-TCR-γ chain gene was performed on DNA extracted from formalin fixed and paraffin-embedded skin biopsies in a total of 35 specimens obtained from 13 patients with MF (6 in stage IA, 4 in stage IB, 3 in stage IIA), both at diagnosis and at the end of therapy. At diagnosis, clonal TCR-γ rearrangements were found in 8 out of 13 patients (61.5%); during the follow-up period the same monoclonal band was detected in biopsy samples from four patients, while in the other four cases monoclonality was lost. Three out of the four patients with evidence of monoclonality also showed clinical and/or histologic persistence of the disease during the follow-up period. All the patients in whom TCR-PCR analysis failed to detect the malignant clone at the end of the therapy, achieved clinical and histologic stable remission. On the other hand, of the five cases that were negative by PCR at diagnosis, two showed clinical and histologic relapse (but still without evidence of monoclonality when relapse occurred), while the other three remained in durable clinical remission. In addition to skin biopsy samples, 21 blood specimens from 12 patients were analyzed by the PCR technique to investigate possible extracutaneous spread of MF. The peripheral blood was free of disease by PCR in 11 out of 12 patients. In conclusion, our data suggest the value of PCR for early diagnosis and evaluation of treatment of those lymphomas carrying TCR-γ gene rearrangements that are detected by the primers utilized. It is remarkable that IFNα2b plus PUVA combination therapy may eradicate all malignant cells at molecular level in a group of complete responders. In the early stages of MF, skin clonality after therapy may imply that residual malignant cells maintain a potential for clinical relapse. Therefore the availability of molecular analysis could identify patients who might benefit from additional therapy.

047
SUSTAINED REMISSION OF REFRACTORY T-CELL PRO-LYMPHOCYTIC LEUKEMIA WITH CAMPATH-1H

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Campath-1H, a genetically engineered human IgG1κ monoclonal antibody directed against the CD52 antigen, is currently being used in clinical trials for lymphoid malignancies and autoimmune disorders. We report the successful use of campath-1H in a patient with refractory T-cell pro-lymphocytic leukemia (T-PLL). In October 1996, a 45-year old man presented without constitutional symptoms; physical examination showed spleen enlargement (21 cm below the costal margin), while CT scan of chest and abdomen revealed multiple small-volume lymphoadenopathies. Blood count showed thrombocytopenia ($59x10^9$/L) and atypical lymphocytosis ($3.3x10^9$/L), CD3+, CD2+, CD5+, CD7+, CD8+, CD45RA+; the neoplastic cells were Vβ3-positive by flow cytometric and molecular analysis. Furthermore, cytogenetic investigation showed an abnormal karyotype with multiple alterations (45, XY, 7p+, 7p+, 8p+,

-9, -13, -14, +Mar, +Ring); bone marrow was heavily infiltrated by neoplastic cells, moreover diffuse fibrosis was detected. The patient was substantially refractory to conventional therapy (an oral combination of prednisone and methotrexate) and relapsed early after an initial response to cladribine. Since the spleen appeared to be the main site of disease, splenectomy was carried out in September 1997. In June 1998, due to the consistent rise in lymphocyte count and the development of severe thrombocytopenia, the patient received two courses of campath-1H (kindly supplied by PFK Europe) as salvage therapy. Campath-1H was administered as a 30 mg 2-hour iv infusion three times a week for a maximum period of three weeks per course. T-PLL cells were rapidly eliminated from blood and bone marrow, and the patient achieved complete remission in November 1998. Adverse events were minimal except for a profound, but transient pancytopenia with the following nadir blood counts: WBC $1{,}880 \times 10^9$/L, PMN $1{,}150 \times 10^9$/L, lymphocytes $0{,}240 \times 10^9$/L and PLT < 10×10^9/L. During the follow-up period the light microscopy showed a striking increase (up to $4 \times 10e^9$/L) of large granular lymphocytes with the immunophenotype characters of NK cell (regulatory cells controlling the growth of the tumor clone?). T-cell receptor analysis by immunophenotyping excluded Vβ3 lymphocytes in the peripheral blood and bone marrow; in contrast, the presence of minimal residual disease was shown by cytogenetic and molecular analysis (45, XY, 7p+, -9, +Mar and CDR3 Vβ3+). Up to April 2000 the patient continues to be in clinical CR and the cytogenetic analysis carried out on the leukapheresis product, collected after mobilization with cyclophosphamide/G-CSF, did not show any alterations. We conclude that campath-1H is an active agent for young T-PLL patients candidate for autografting procedures.

048
AC133 EXPRESSION AS A PHENOTYPIC MARKER OF EARLY HEMATOPOIETIC PROGENITOR CELLS IN UMBILICAL CORD BLOOD

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Umbilical cord blood (UCB) is a rich source of very immature hematopoietic progenitor cells (HPC). Because of the limited volume of cord blood in each sample, it is critical not only to ascertain the number of stem cells available but also to provide qualitative information concerning the proliferative potential of CD34+ UCB. AC133 is a novel antigen which has been found to be selectively expressed on $CD34^{bright}$ HPC and rapidly downregulated as stem cells differentiate. In our study we analyzed AC133 expression on CD34+ cells from 22 UCB and 14 leukapheris products obtained from patients with hematologic malignancies after mobilization with chemotherapy + G-CSF. AC133 expression was also correlated with the plating efficiency of CD34+ cells, determined dividing colony-forming cells by the number of plated CD34+ cells. The analysis was performed on mononuclear cells by flow cytometry (FACSCalibur, BD) in a three fluorescence setting using CD34 APC, AC133 PE, HLA-DR PercP; in each sample at least 500 CD34+ events were acquired in a logical gate combining a fluorescence/SSC plot and a forward-scatter characteristic (FSC)/SSC plot. The results were obtained as mean fluorescence index (MFI), expressed as the ratio of sample mean channel: control mean channel. AC133 MFI was slightly higher in umbilical cord than peripheral blood (21.5 ± 10.6 vs. 17.1+/-5.6, respectively). In UCB samples CD34 + mean value percentage was 0.39 ± 0.22%; we found a good correlation between AC133 and CD34 MFI (r=0.8, p=0.000) and between AC133 and HLA-DR MFI (r=0.73, p=0.001). Moreover AC133 MFI was also directly related to CFU-Mix number (r=0.57, p=0.026). These results suggest that AC133 identifies a subset of CD34+ cells enriched of highly immature HPC and its quantitative determination could be useful to provide further information concerning the degree of immaturity of HPC in UCB samples.

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SIMULTANEOUS ANALYSIS OF GENETIC RISK FACTORS FOR THROMBOPHILIA

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Thrombophilia is a genetic predisposition to develop recurrent deep venous thrombosis (DVT) or arterial thrombosis in young age. Common genetic risk factors are factor V Leiden (FV L) and a variant of prothrombin gene (PT G20210A), which are also frequent in the general population (about 5 and 3% respectively). Several other genetic and acquired factors are involved in the thrombotic process. A polymorphism in exon 13 of the FV gene, FV A4070G, was recently shown to influence circulating FV levels and to contribute to the activated protein C (APC) resistance phenotype, but its role in the thrombotic process is still controversial. We studied 50 patients with venous thromboembolic disease (31 with DVT, 10 with pulmonary embolism, 4 with superficial vein thrombosis, and 5 with retinal vein occlusion). All patients were studied for acquired risk factors (surgery, immobilization, hormone therapy…) and had laboratory tests for plasma levels of AT III, protein C, protein S and APC resistance. DNA was isolated from peripheral blood lymphocytes by standard methods. Mutations FVL, FV A4070G and PT G20210A were searched for by specific DNA amplification and digestion (by Mnl I, Rsa and Hind III, respectively) in all cases; we determined homocysteine levels and specific mutations in methylenetetrahydrofolate-reductase (MTHFR) and cystathionine-beta-synthase (CBS) genes in selected cases. SSCP and direct sequencing were performed by standard methods. FV Leiden was positive in 9/50 (18%) of patients with venous thromboembolic disease, PT G20210A was present in 7/50 (14%) in agreement with literature data; we also examined the impact of the FV A4070G in our limited population. This mutation was present in 6/50 (12%) of patients in the absence of FV Lei-

den. One patient was compound heterozygote for FV L and FV A4070G. All patients had APC resistance. The FV A4070G was present in 5/34 (14%) of patients without other mutations. A pedigree exemplifies thrombosis as a multigenic-multifactorial event. The proband is a 17-year old boy who developed arterial thrombosis and recurrent DVT with pulmonary embolism. The patient had no mental retardation, ectopia lentis or osteoporosis. Homocysteine was 352 µMol/L and homocystinuria was present. The patient was a heterozygous carrier of FV Leiden, CBS I278T and MTHFR A667V. Family analysis showed segregation of the same defects in the father, who was also an A677V MTHFR homozygote. All family members were asymptomatic and none had a history of thrombosis. CBS mutations A114V, R125Q, E131D, P145L, G307S were excluded in the proband. SSCP and sequencing of the CBS gene exons is in progress to ascertain the presence of the second mutation.The family highlights that complex genetic interactions may occur in patients with thrombophilia.

050
NUCLEAR FACTOR-ERYTHROID 2 (NF-E2) EXPRESSION IN CD34-DERIVED MEGAKARYOCYTIC CELLS OF ESSENTIAL THROMBOCYTHEMIA: COMPARISON WITH THE NORMAL COUNTERPART AND CELL LINES WITH MEGAKARYOBLASTIC OR ERYTHRO-MEGAKARYOBLASTIC FEATURES

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Essential thrombocythemia (ET) is a chronic myeloproliferative disorder characterized by lineage-specific expansion of the megakaryocytic and platelet compartment. The cause of ET is unknown. Since few consistent cytogenetic abnormalities have been reported, little is known about the existence of any underlying molecular lesions. The nuclera factor-erythroid 2 (NF-E2) transcription factor has recently been found to play a pivotal role in terminal megakaryocyte maturation and platelet production. Despite its importance in regulating globin gene transcription, targeted disruption of the gene encoding the p45 NF-E2 subunit leads to severe thrombocytopenia but little or no alteration in erythropoiesis. Therefore, NF-E2 could have a pathogenetic role in ET megakaryocytopoiesis, which notoriously suffers alterations. In the present study, we investigated *in vitro* expression of NF-E2 transcription factor in megakaryocytic cells from ET patients. For this purpose, primary CD34+ hematopoietic progenitor cells taken from the bone marrow of ET patients and healthy donors were induced to differentiate along the megakaryocytic lineage in liquid cultures for 14-16 days by addition of 100 ng/mL thrombopoietin. NF-E2 expression pattern was monitored at both the mRNA and protein levels by quantitative reverse-transcription polymerase chain reaction (real-time RT-PCR) and immunofluorescence, respectively. Parallel experiments were also performed on megakaryoblastic and erythro-megakaryoblastic cell lines (JURL-MK1, JURL-MK2, HEL, B1647, MO7e, K-562). We found that CD34-derived megakaryocytic cells from normal donors express NF-E2 transcription factor and the expression did not significantly vary during the course of megakaryocyte maturation. As far as regards the two isoforms of the NF-E2 gene (a and f), our mRNA analysis suggests that the a isoform is much more highly expressed than the f isoform in normal and malignant megakaryocytic cells. Both isoforms of NF-E2 mRNA significantly decreased in ET CD34-derived megakaryocytic cells with respect to their normal counterparts, although the f isoform was less reduced than the a isoform. The reduction has also been observed in cell lines. In particular, real time RT-PCR documented that the mean NF-E2a/GAPDH normalized ratio of normal CD34-derived megakaryocytic cells (0.31) was 3.47-fold that observed in ET samples (0.094) and 19.3-fold that of cell lines studied by us (0.016). By comparison, the variations in the mean NF-E2f/GAPDH ratio were smaller. In particular, while the mean value of normal megakaryocytic cells (0.031) was 2.38-fold higher than in ET CD34-derived megakaryocytic cells (0.013), it was only 6.2-fold higher than in cell lines (0.005). Despite the reduction in the NF-E2 mRNA isoforms, NF-E2 protein expression (MIF), as evaluated by a double labeling system (CD41 PE; NF-E2 FITC MoAbs), did not significantly vary in ET CD34-derived megakaryocytic cells or in the investigated cell lines with respect to normal CD34-derived megakaryocytic cells. Therefore, it is possible that different levels of protein turnover and/or mRNA stability could at least partially account for this finding. In conclusion our data suggest that CD34-derived megakaryocytic cells of ET show NF-E2 alterations, which seem to be more related to the malignancy state than to the process of platelet production or megakaryocyte maturation, since they were observed not only in ET megakaryocytes but also in cell lines with megakaryoblastic or erythro-megakaryoblastic pattern.

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MISMATCHES INVOLVING AMINO ACID SUBSTITUTIONS AT POSITION 116 OF HLA CLASS I MOLECULES CAN INFLUENCE THE OUTCOME OF BONE MARROW TRANSPLANTATION FROM UNRELATED DONORS

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Class I molecules interact with peptides through six specific pockets that harbor the peptide residues facing downward. A slight difference in the sequences in one of these pockets can change the binding requirements, and result in a different set of bound peptides. Residue 116 is fundamental, since it forms the bottom of pocket F, that harbors the C-terminal amino acids. of the peptide, and substitutions at position 116 may or may not alter the spatial conformation of the pocket. We defined variations at position 116 involving the replacement of a small residue with a large one or vice versa as relevant or as irrelevant. We then tested whether HLA Class I mismatches involving relevant or irrelevant allele substitutions at position 116 had a different influence on the outcome of bone marrow transplantation (BMT) from unrelated donros. Ninety-four donor/recipient pairs were

typed by PCR-SBT for the HLA-A, -B, -C, -DRB, -DQA, and -DQB loci and all were identical for class II . We then identified 3 subgroups of patients: 64 were identical also for class I whereas 32 had mismatches at the level of class I. The mismatched pairs were then examined for the amino acid substitution at position 116 of the HLA Class I a chain and classified as irrelevant substitutions (n=15), those not altering the spatial conformation of the F pocket, and ii) relevant substitutions (n=16), as those involving changes altering the spatial conformation of the F pocket. Pairs having a mismatch in two loci were put in the group with relevant substitutions when at least one of the amino acid changes could be attributed to this group. The 3 groups, matched, relevant and irrelevant mismatched, were comparable for diagnosis, phase of the disease, patient age and conditioning regimen. The risk of developing acute GvHD grade III-IV was 21% in matched pairs, 13% for irrelevant substitution and 41% for relevant class I substitutions (p=0.05). Transplant-related mortality (TRM) was 26%, 25% and 70% (p=0.0004) . Survival was superimposable in matched (70%) or irrelevant mismatched pairs (72%) , but significantly poorer in pairs with relevant mismatcheds at position 116 (23%) (p=0.002). This study suggests that irrelevant substitution at position 116 does not change the outcome of unrelated BMT when compared to class I matched pairs. On the contrary relevant substitutions had a significant impact on GvHD and overall outcome.

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T-CELL ATTRACTING CHEMOKINES ARE PRODUCED BY CHRONIC LYMPHOCYTIC LEUKEMIA B-CELLS AFTER CD40 STIMULATION

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B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the progressive accumulation of resting B lymphocytes, which are, at least *in vivo*, resistant to apoptosis. Such resistance can not be explained only by intrinsic defects of the neoplastic cell, but seems to be due to a non-dispensable role of the microenvironment in which the disease arises. It has now become clear the fundamental (though not sufficient) role of by-stander, non-tumoral cells and of the numerous extrinsic factors produced by them, in the onset and progression of the disease. There is substantial evidence that CD40 stimulation rescues B-CLL cells from apoptosis and induces proliferation, showing that malignant B-cells retain the capacity to respond to microenvironmental signals, such as those delivered by helper CD40L+ T cells, always present in involved bone marrow (BM) and lymph nodes. Since chemokines are known to be fundamental in regulating the migration and the homing of normal lymphocytes within the different lymphoid organs, we hypothesized that also malignant B-cells could produce chemotactic molecules capable of attracting by-stander cells that can in turn favor the growth of the neoplasia. In order to explore this possibility, we examined whether B-CLL cells could produced chemokines, particularly T-cell chemoattractants. We performed an extensive study of chemokine expression by RT-PCR in CD19+/CD5+ malignant B-cells from peripheral blood (PB) or lymph nodes (LN) of 28 B-CLL patients. The percentage of leukemic B cells ranged between 92 and 97% of total cells. No chemokines could be detected in primary leukemia cells with the exception of IL-8 and, in some patients, of faint bands corresponding to cDNA for MIP-1α and RANTES. Interestingly, the CC-chemokines MDC and TARC were expressed in 4/4 and 2/4 cases, respectively, in which the CLL cells were FACS-purified from involved LNs. This evidence prompted us to examine whether a physiologic signal available in the LN microenvironment as CD40L, could alter the chemokine expression profile of circulating B-CLL cells. For this purpose, we cultured B-CLL cells in the presence or the absence of soluble CD40L, for up to 3 days. No change in the low level of expression of either MIP-1a or RANTES was observed, while in contrast, CD40 ligation induced strong expression of both MDC and TARC. This induction was observed at mRNA level and, in case of MDC, confirmed at protein level, by ELISA. In the supernatants of stimulated cells, secreted MDC was present at a concentration as high as 28.8 ng/ml. As this molecule has been described as a very efficient chemoattractant for activated T-cells, we tested the supernatants obtained from stimulated CLL cells for their capacity to attract CD4+ T-cell lines, known to express CCR4, the specific receptor for MDC and TARC, as shown by calcium mobilization and migration assay. In conclusion, we show that B-CLL cells do not constitutively express significant levels of chemokines but that CD40-crosslinking of these tumor cells induces the expression and production of the chemokines MDC and TARC. These molecules can mediate the chemoattraction of activated CD4+ helper T- cells. This evidence supports a scenario in which malignant B-cells attract by-stander, non-tumoral cells that provide relevant survival signals to the leukemic clone, and, in turn, stronger chemokine production. This would give rise to a vicious circle, leading to progressive accumulation of the neoplastic cells.

053
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS AND ROLE IN THE RETINOIC ACID SIGNALING PATHWAY OF A NOVEL RETINOID IN ACUTE PROMYELOCYTIC LEUKEMIA

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Retinoids are modulators of cellular proliferation and differentiation in many cell types and their diverse effects are mediated by three distinct isoforms of receptors RAR (α, β, γ). In this study we characterized a novel derivative of retinoic acid, hydroxy-hydhro-phenantrene (HHP-RA), m.w. 256 (pat. TO/ 98/A000808) and compared its activity to that of known retinoids. The new molecule and known retinoids have similar chemical properties, by studies with HPLC and spectrophotometer analysis, but the new compound does not possess the typical sensitivity to light. Cell lines, HL60, NB4, as well as cells obtained from 6 cases of acute promyelocytic leukemia (APL) were cultured in RPMI 1640 with 15% FCS for 3, 4, 6 days, supplemented with the new derivative of retinoic acid 10μM, 100 nM, 1 μM or ATRA at the same

doses. Our results showed that HHP-RA blocked proliferation to the same extent as ATRA at 1 μM and 100 nM doses, while it resulted toxic at the 10 μM dose. Cell counts indicated a significant decrease (50%) in proliferation in cells treated with the novel derivative. By cytofluorimetric analysis after 4 days of culture, 11.72% of cells treated with HHP-RA 1 μM were in S-phase, compared with 42.49% of control culture cells. Consistent data were obtained with ATRA 1 μM. Annexin V tests demonstrated induction of cell line apoptosis: 30% compared with 9.96% of controls in HL 60; the percentage was not significant in NB4 cells or in primary APL cultures. These data were supported by the observation of apoptotic bodies as well as of a consistent increase of pre-G1 peak in cell-cycle flow cytometric analysis. Moreover, we performed transactivation in COS-1 cells transiently transfected with the expression vectors pSG5/RARα and PSG5/RXR and the luciferase reporter gene pRAREβ-luc, showing that when RARα and RXR were coexpressed in transiently transfected COS-1 cells the activation of the RARβ promoter that contains a DR5 RARE was enhanced 3 fold after exposure to HHP-RA 10^{-6} M and 4 fold when RXR only was expressed. In fact studies with fractionation of HL 60 and NB4 nuclear extracts over FPLC showed that the new molecule binding affinity for the endogenous nuclear receptors was less strong for RARα than that ATRA. These observations helped to analyze whether the new compound could imply different therapeutic strategies, possibly as an alternative to the known retinoids in the treatment of resistant APL.

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MOLECULAR REMISSION AFTER ALLOGENEIC OR AUTOLOGOUS TRANSPLANTATION OF HEMATOPOIETIC STEM CELLS FOR MULTIPLE MYELOMA

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Ablative chemotherapy[1] followed by bone marrow transplantation[2-4] either performed with an autologous[5] or allogeneic graft has been shown to increase the duration of multiple myeloma patients' overall survival.[5-7] To assess the clinical relevance of minimal residual disease (MRD) in patients with multiple myeloma (MM), 50 patients were monitored in complete clinical remission (CCR) after autologous[8,9] or allogeneic stem cell transplantation.[7] Molecular remission (MCR) can be obtained in a relatively high proportion of MM patients who have achieved CCR after allografting and in a smaller fraction of patients after autografting. MCR was associated with prolonged relapse free survival: these patients could have a rather favorable clinical outcome. Stringent molecular monitoring using clonal markers based upon rearranged immunoglobulin heavy-chain genes was done in 44/50 MM patients in CCR. Molecular remission was defined as >1 consecutive negative PCR results. Twelve out of 44 (27%) molecularly monitored patients achieved MCR; 4/12 turned PCR+ and 1/4 relapsed. Patients achieving MCR had a significantly lower relapse rate (16% vs. 41%; $p < 0.05$) and higher relapse free survival (110 vs. 35 months; $p < 0.005$). Fourteen of 26 allografted patients with CCR were molecularly evaluated: 7/14 achieved MCR and did not relapse; 1/7 of the remaining patients relapsed. Thirty out of 47 autografted patients with CCR were molecularly evaluated: 5/30 (16%) achieved MCR; 2/5 turned PCR negative and 1/2 relapsed. Ten of the 25 remaining patients relapsed. On these non-randomized groups, the higher MCR rate after allografting was statistically significant ($p < 0.01$; Fisher's exact test). MCR can be obtained in a relatively high proportion of MM patients who have achieved CCR after allografting and in a smaller fraction of patients after autografting. In about a quarter of MM patients who achieve CCR after transplantation, it may be possible to keep the disease burden constantly below the PCR threshold.[10] Since MCR was associated with prolonged relapse free survival, these patients could have a rather favorable clinical outcome.

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CHROMOSOME 17 SHORT ARM DELETION IN ACUTE MYELOID LEUKEMIA FOLLOWING ESSENTIAL THROMBOCYTHEMIA

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Chromosome 17 short arm deletion is the most frequent karyotype defect in acute myeloid leukemia (AML) following essential thrombocythemia (ET), occurring in about 40%-50% of such cases. Therefore we decided to evaluate its incidence by applying conventional cytogenetics (CC) along with FISH in 12 cases of AML occurring after ET. The other aim of the study was to establish whether the chromosome abnormality might be induced by a previous treatment. At diagnosis all patients had had a normal chromosome pattern. A RT-PCR analysis had excluded the presence of BCR/ABL transcripts in all the cases. These cases had developed AML after a median time of 104 months (range 78-118). The diagnosis was AML-M2 in 2 cases, M4 in 6 cases, M5 in 3 cases and M7 in one case. Five cases did not yield analyzable metaphase. Six patients had a complex karyotype, while one had a single abnormality, i.e. an interstitial long arm deletion of chromosome 5. CC demonstrated a 17p- in three out of the 6 patients with fully analyzable mitoses. Chromosome 7 rearrangements were observed in 5/6 cases. FISH was performed with a p53 DNA probe and with a 17alfa centromeric probe. The interphase FISH cut-off value was determined by examining ten normal subjects without 17p- and by calculating the number of cells carrying two signal due to the alphoid probes, but with only one signals due to the p53 DNA probe. FISH allowed the detection of 17p- in 4/7 fully karyotyped cases and in 3/5 patients without anayzable cells on CC. Therefore the incidence of 17p- in our cases was 50%. From a morphologic point of view a Pelger-Huet anomaly was seen in 9 cases. As far as chemotherapy is concerned 4 cases had received only pipobroman, while 3 had received both pipobroman and hydroxyurea. In conclusion the incidence of 17p- in our patients is similar to that reported by other groups. No conclusion about the role of chemotherapy can be drawn. Our data do not support the suggestion that 17p- development is caused by previous treatment with hydroxyurea.

056
MOLECULAR AND CLINICAL STUDIES IN HEMOCHROMATOSIS TYPE 2 AND 3

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Hemochromatosis (HFE) is an autosomal recessive disorder characterized by progressive iron loading that, if untreated, causes organ damage (cirrhosis, diabetes, endocrine dysfunctions and cardiopathy) in midlife. The HFE gene has been cloned on chromosome 6p and two mutations have been described, C282Y and H63D. Most patients are homozygous for C282Y mutation in northern Europe. In Italy only 64% of the patients are C282Y homozygous, showing that the disease is more heterogeneous. Juvenile hemochromatosis (JH or hemochromatosis type 2) differs from typical HFE. JH leads to severe iron loading and organ failure at age < 30 years and maps on 1q (HFE2 locus). Recently a few patients with a clinical diagnosis of hemochromatosis without HFE mutations and without JH presentation (hemochromatosis type 3) have been identified and a new locus (HFE3) on 7q22 has been reported. A non-sense mutation (Y250X) in transferrin receptor 2 (TFR2) is present in these patients in the homozygous state (*Camaschella et al., Nat Genet 2000; 25:14-5*). The aim of this study was to investigate the molecular and clinical differences between HFE2 and HFE3. We studied 18 HFE2 patients belonging to 12 different families (9 from Italy and 3 from the UK) and 11 HFE3 patients belonging to 3 unrelated Italian families. DNA was isolated from peripheral blood lymphocytes by a standard method. C282Y and H63D mutations were assessed on specific DNA amplification and restriction enzyme digestions (RsaI and MboI, respectively). Linkage to chromosome 1q was established by microsatellite analysis of 1q markers (D1S442, D1S2344, D1S1556, D1S498). Linkage to chromosome 7q was established by using D7S651, D7S647, D7S2498, D7S2480, D7S734, D7S662 markers and by TFR2 intragenic polymorphic repeats. Scanning of TFR2 exons was performed by PCR and direct sequencing. Y250X mutation was analyzed by PCR and MaeI digestion. All HFE2 patients with a clinical phenotype of JH were linked to chromosome 1q. HFE3 patients had mutations in TFR2, homozygous Y250X (6 cases) or a new mutation (88insC) that causes a stop codon in TFR2 at aminoacid 60 (E60X) (5 cases) Mean age at presentation was 22 years in patients with HFE2 (range 13-31) and 40 years in HFE3 (range 27-45). Mean transferrin saturation was 90% in HFE2 and 85% in those HFE3 and mean serum ferritin was 2,869 mg/L and 1,810 mg/L respectively. Cardiopathy was present in 8/18 (44%) HFE2 patients and in no HFE3 patient. Hypogonadotrophic hypogonadism was present in all the HFE2 patients and in 2/11 (18%) of HFE3 patients. Diabetes was present in 11/18 (61%) and in 1/11 (9%), respectively. Cirrhosis was present in 8/15 (53%) of HFE2 and in 3/11 (27%) of HFE3 patients. HFE2 patients had the more severe phenotype. HFE3 patients had a more varied clinical phenotype, in some cases characterized by mild iron overload. Our data show that

hemochromatosis is heterogeneous in Italy. The variability of the clinical phenotypes may be accounted for by the presence of different genes, different mutations and modifier genes.

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SEVERE CYTOPENIA WITH FULL DONOR CHIMERISM AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION MIMICS ACQUIRED APLASTIC ANEMIA

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We describe 10 patients with hematological malignancies undergoing unmanipulated allogeneic hematopoietic stem cell transplantation (HSCT) who developed severe pancytopenia after transplantation. All patients had two main features: an empty marrow and full donor chimerism. Nine were recipients of an unmanipulated HSCT from a matched unrelated donor and 1 from an HLA identical sibling. The source of stem cells was bone marrow in all patients. The conditioning regimen consisted of cyclophosphamide plus total body irradiation (n=6), cyclophosphamide and thiothepa (n=1) or cyclophosphamide plus thiothepa and fludarabine (n=3). Graft-versus-host disease (GvHD) prophylaxis consisted of cyclosporin A with methotrexate. Median grafted cell dose was $3.3x10^8$/kg. Median CFU-GM of inoculum was $1.3x10^4$. Treatment with ATG: five patients were treated with antithymocyte globulin (ATG, IMTIX, 1.25 mg/kg/day x 5 days) at a median interval of 380 days after BMT (166-1155): one is too early after ATG to evaluate; 2/4 evaluable patients showed complete recovery of cytopenia. Four out of 5 patients are alive 1-36 months post -ATG Control group: five patients did not receive ATG and were used as controls, One patient recovered hematopoiesis after prolonged treatment for CMV infection, 4 remain cytopenic. *Conclusions.* These patients with severe cytopenia and aplasia have features closely resembling those of acquired severe aplastic anemia (SAA) . Response in 2/4 evaluable patients to ATG further supports this view. Recent progress in the pathogenesis and treatment of acquired SAA may, perhaps, be exploited to improve understanding and treatment of poor graft function after allogeneic HSCT.

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SURVIVIN EXPRESSION IS UPREGULATED UPON CD40 STIMULATION IN B-CHRONIC LYMPHOCYTIC LEUKEMIA

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B-chronic lymphocytic leukemia (B-CLL) is characterized by a relentless accumulation of monoclonal CD19+ CD5+ B cells. B-CLL represents the paradigmatic example of human malignancy primarily involving defects in the apoptotic death program. Genetic defects of the neoplastic cells and/or external stimuli may both influence defective apoptosis. The microenvironment likely plays a prominent role since the malignant cells progressively accumulate *in vivo*, whereas they rapidly undergo apoptosis when cultured *in vitro*. We investigated which molecular mechanisms might govern the defective apoptosis of malignant cells and what, if any, could be the role of the microenvironment. For this purpose, we investigated the expression and modulation of the family ofi inhibitor of apoptosis proteins (IAP), originally identified in baculovirus. IAP are known to suppress apoptosis induced by a variety of stimuli through caspase and pro-caspase inhibition, therefore representing the last chance for a cell to escape its apoptotic fate. We analyzed IAP expression in mononuclear cells from peripheral blood or bone marrow (BM) of 30 B-CLL patients. Among the tested genes, 4 of them (cIAP1, cIAP2, NAIP and XIAP) were consistently positive in all the samples analyzed. In contrast, survivin expression was undetectable in the majority of cases (25/30, 83%). We next considered the possibility that physiologic stimuli available to B-cells in the microenvironment might modulate IAP expression. Since CD4+ T-cells are present in involved BM and lymph nodes (LN), we focused our attention upon the interactions between the B-cell-associated molecule CD40 and its natural ligand (CD40L, CD154), expressed by activated T-lymphocytes. To assess the effect of CD40 stimulation, we set up time course experiments with B-CLL cells cultured in the presence or absence of human soluble CD40L. The expression of cIAP1, cIAP2, NAIP and XIAP remained unmodified after CD40 engagement, while survivin expression was upregulated after 48-96 hours of stimulation. As the *in vitro* data were pointing toward a role of CD40 ligation in modulating survivin expression, we evaluated the *in vivo* expression of survivin in LN and BM biopsies of B-CLL patients. In involved LN, survivin was positive in the malignant B-cells present in the so-called pseudo-follicles. These data were confirmed in BM biopsies where survivin positive B-cells were found in clusters. A role of *in vivo* CD40 stimulation seems to be supported by the presence of a high number of activated CD4+ T-cells in these neoplastic lesions. These observations indicate that a signal through CD40 might be responsible for the induction of the apoptosis-resistant phenotype in B-CLL cells invading the lymphoid tissues. These data provide a link between microenvironmental factors, here represented by CD4+ T-cells, and the apoptosis resistance of B-CLL cells.

059
IN VITRO CHEMOSENSITIVITY OF THE HUMAN B-LYMPHOBLASTOID CELL LINE WIL2-S TO ANTINEOPLASTIC AGENTS

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The *in vitro* drug sensitivity testing of human tumor cells to different cytotoxic agents has been used preclinically and clinically in order to select chemotherapy regimens for specific diseases. In this study, anticancer drugs were examined for their inhibitory effects on cell growth of WIL2-S, a suspension culture of human B-lymphoblastoid cells, phenotypically characterized by the expression of the following antigens: HLA-DR+, CD5-, CD20+ and CD95+ (*Beletskaya IV, et al.*,

FEBS Letters 1997; 412:91-3), in order to select those agents with enhanced cytotoxic activity that might be used in the rational development of treatment combinations of lymphoid neoplasms. METHODS. The antineoplastic agents examined in this study were chosen for their ability to be active in various phases of the cell cycle and were as follows: nucleoside (arabinosyl-cytosine) and folic acid analogs (methotrexate), alkylating agents (temozolomide, thio-tepa), inhibitors of topoisomerase I (topotecan, irinotecan) and topoisomerase II (etoposide, idarubicin). The WIL2-S cell line was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and $1x10^5$ cells were plated in 1 mL of medium into each of 24-well plates for cell culture. Graded concentrations of drugs were added to each well as follows: arabinosyl-cytosine 0.005-10 μg/mL, methotrexate 0.01-10 μg/mL, temozolomide and thio-tepa 0.01-10 μg/mL, SN-38, the active metabolite of irinotecan, and topotecan 0.001-1 μg/mL, etoposide 0.001-1 μg/mL, and idarubicin 0.01-10 μg/mL. Each concentration was examined in quadruplicate experiments. Cells were exposed to the cytotoxic drugs for 6 hours, then the medium was replaced with drug-free medium and the cells grown for 48 hours at 37°C in 5% CO_2. At the end of incubation, surviving cells were counted, and the concentration of each drug that produced a 50% inhibition of cell growth as compared to control, drug-free cultures (IC_{50}) was calculated by CalcuSyn software (Biosoft, UK). RESULTS. The IC_{50} values of the drugs examined in this study on WIL2-S cell line were as follows: arabinosyl-cytosine 1.5-22 ng/mL, methotrexate 3.8-64 ng/mL, temozolomide 0.28-0.89 μg/mL, thiotepa 2.74 μg/mL, topotecan 2.9-4 ng/mL, SN-38 0.19-0.46 ng/mL, etoposide 73-105 ng/mL, and idarubicin 43 ng/mL. CONCLUSIONS. These results provide evidence that the WIL2-S lymphoblastoid cells show a distinct chemosensitivity to cytotoxic agents with highest activity displayed by topoisomerase I inhibitors (topotecan and irinotecan [SN-38]) followed by nucleoside (arabinosyl-cytosine) and folic acid analogs (methotrexate), topoisomerase II inhibitors (etoposide and idarubicin), and finally by the alkylating agents (temozolomide and thio-tepa). These data are not unexpected because the lymphoblastoid WIL2-S cells are characterized by enhanced growth fraction with a significant amount of cells in the S phase of the cell cycle. These results might be useful in selecting rational drug combinations based on the mechanism of action and cytotoxic activity of anticancer drugs.

060
MOLECULAR PROFILE OF EPSTEIN-BARR VIRUS IN AIDS-RELATED NON-HODGKIN'S LYMPHOMA

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Epstein-Barr virus (EBV) is associated with a variety of neoplasms and is believed to be a major risk factor for AIDS-related non-Hodgkin's lymphoma (AIDS-NHL) development. However, the role of EBV in the disease pathogenesis is unclear. Polymorphic sequences in the EBV genome appear to have biological and pathogenetic importance, and several studies have attempted to associate particular EBV strains to specific EBV positive malignancies. In this study, we aimed at defining the EBV genotype in a well characterized panel of 89 AIDS-NHL samples (all EBV positive) including 52 AIDS-related primary nervous system lymphomas and 37 systemic AIDS-NHL. Viral genes with oncogenic potential, such as EBNA-1, LMP-1, EBNA-2 and –3C, were analyzed by PCR and direct sequencing. The EBV genotype detected in AIDS-NHL was correlated with the clinical and epidemiological features of tumor, i.e. disease site, tumor histology and risk factor for HIV infection. Sequence analysis of EBNA-1 was performed on 51 AIDS-NHL samples in order to distinguish the two major subtypes, namely prototype and variant, which have been suggested to bear different oncogenic potential. A prototype EBNA-1 sequence was detected in 39/51 (76.5%) AIDS-NHL whereas the variant EBNA-1 subtype was carried by 4/51 (7.8%) cases. In the remaining cases (8/51; 15.7%), multiple EBNA-1 prototype sequences were detected, consistent with multiple EBV infection. The representation of EBNA-1 subtypes in AIDS-NHL was consistent with that found in the general population. However, comparison between EBNA-1 subtype distribution and tumor histology revealed that variant EBNA-1 subtypes clustered with AIDS-Burkitt's lymphoma (4/12; 33%), whereas were consistently negative in all AIDS-diffuse large cell lymphoma (DLCL) analyzed (n=29) ($p<.05$). Analysis of LMP-1 C-terminal region revealed a full length gene in 39/78 (50.0%) AIDS-NHL, whereas the delta30LMP-1 was present in 27/78 (34.6%) cases. Eight out of 78 AIDS-NHL (10.3%) displayed the delta69LMP-1 gene, which has been reported to display peculiar growth activity and transforming properties for B-cells *in vitro* and *in vivo*. Analysis of EBNA-2 and –3C led to definition of the EBV strain type-1 in 48/65 (73%) AIDS-NHL, while type-2 EBV occurred in 13/65 (20%) cases. A double infection was found in 4 samples. No preferential occurrence of a specific LMP-1 variant or EBV type was observed in AIDS-NHL. In conclusion, although most EBV polymorphisms in AIDS-NHL reflect the overall distribution detected in the general population, the distribution of specific EBNA-1 subtypes may vary among the different categories of AIDS-NHL. The finding that prototype and variant EBNA-1 sequence are not randomly shared by AIDS-DLCL and AIDS-Burkitt lymphoma suggest that the viral gene status may be relevant to the pathogenesis of at least some B-cell malignancies.

ORAL COMMUNICATIONS

session 11

061
CELL DOSE IS A MAJOR PREDICTOR OF OUTCOME IN PATIENTS UNDERGOING UNRELATED BONE MARROW TRANSPLANTATION

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We analyzed the impact of cell dose on the outcome of 73 patients undergoing an unrelated donor (UD) bone marrow transplant (BMT). The median age of the patients was 29 years (18-49), the median interval diagnosis-BMT 1209 days (201-45,10). The diagnosis was chronic myeloid leukemia in 57 patients and acute leukemia in the remainder. All patients were prepared with cyclophosphamide (CY) and total body irradiation (TBI) (9-12 Gy) and received cyclosporin methotrexate for GvHD prophylaxis. All donors were matched by high resolution molecular typing for HLA A, B and DRB1. The dose of cells infused was ≤2, ≤3, ≤5, ≤6.7x10^8/kg respectively in 8, 19, 35 and 11 patients. The distribution of age was the same in these 4 subgroups with 50% of the patients under 25 in the ≤2x10^8 kg and 45% in the ≤6.7x10^8/kg group. The disease was in early phase in 50% of patients in all subgroups (p=0.9). The actuarial survival at 3 years is 50%, 59%, 66%, 90%. In multivariate COX analysis with donor gender/age, recipient gender/age, disease diagnosis and phase, interval diagnosis transplant, cell dose, this last was the only significant predictor at the p=0.02 level. When acute GvHD was introduced in the COX model, the cell dose became more predictive (p=.0001). The transplant related mortality (TRM) was equally predicted in COX analysis (p=0.02). The relapse rate in the 3 groups was not different (p=0.1). This study further confirms the importance of cell dose in reducing TRM after unrelated BMT: it appear that a high cell dose (>5x10^8/kg) is desirable. Bone marrow harvest can be optimized and donor/transplant Centers should be aware of the major impact of cell dose on survival.

062
ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR ADULT PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA: PREDICTIVE ROLE OF MINIMAL RESIDUAL DISEASE MONITORING AND ITS CLINICAL RELEVANCE

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Acute lymphoblastic leukemia (ALL) comprises 20% of acute leukemias in adults. Some data support the use of more intensive therapy, including bone marrow transplantation (BMT), in patients with bad prognostic factors such as high white blood cell counts, age > 30 years, unfavourable cytogenetic abnormalities and a long interval to achieve remission The graft-versus-leukemia (GVL) effect may reduce relapse risk after BMT, according to data which report a lower relapse rate in patients with GVHD compared to those without GVHD. The main purpose of our work was to study a group of ALL patients who underwent BMT and to evaluate the relapse rate according to minimal residual disease (MRD) positivity, grade of acute and chronic GvHD and chimerism rate analyzed by blood group, karyotype and simple tandem repeats (STR). We have developed a PCR-based method that enable us to monitor clonogenic IgH VDJ rearrangement as a possible predictor of relapse in B-ALL patients. It allows the detection of one malignant cell in 10^5-10^6 normal cells. After having identified the specific Vh family sequence found in the neoplastic population at diagnosis, we performed a three step amplification based on the sequential use of oligonucleotides complementary to Jh leader, Fr1 and J consensus region. In order to overcome the main problem of differentiating the clonal band from physiologic polyclonal rearrangements, we used single strand conformational polymorphism (SSCP) which proved to be a simple and convenient method. Twenty-three ALL patients undergoing haematopoietic stem cell transplantation with available biological samples to evaluate MRD were included in our study (Table 1).

Table 1. Patients characteristics.

N. of patients	25
Sex (M/F)	11/12
Status at BMT:	
1st CR	13
2ndCR	8
REL	2
Cytogenetics	
Normal	14
t (9;22)	4
t (4;11)	2
complex	1
monosomy 17	1
trisomy 8	1
Bone marrow donor	
HLA-identical sibling	18
HLA identical father	1
Syngenic	1
Matched unrelated donor	2
Mismatched sibling	1
Conditioning regimen	
CTX-TBI	19
CTX-Thiotepa	3
Flu-CTX	1

Four patients received donor lymphocyte infusions (DLI7: two as a prophylactic measure, and two others following the appearance of a minimal quota of CD10 CD19 double-positive cells in peripheral blood during clinical complete remission (CR). Four patients had negative MRD before BMT and remained negative at all times after BMT; one of them had undergone syngeneic BMT. All these patients are alive and well at a median follow-up of 41 months. Nineteen patients were MRD-positive before BMT. Five of them

relapsed, with a median disease-free period of three months after BMT; they were MRD positive after BMT and had acute GvHD < 1. Four of them died of disease-correlated causes. The fourteen remaining patients are still in CR. Eight of them have now become MRD-negative, between one and eight years after BMT. Six of them remain MRD-positive but still in CR at a median time of 41 months. These patients had chronic GvHD grade 1-2. Two patients became MRD-negative after DLI. Two had a transplant-related death. Ten out of 15 patients studied were MRD positive and 100% chimeric by STR. Cytogenetic assessed chimerism (in heterosex transplants) cannot be an early predictor of the disease recurrence. So, in patients with MRD positivity after chemotherapy, the presence of MRD after BMT, if associated with chronic GvHD, is not predictive of relapse. Another open question is the role of BMT in MRD negative patients post chemotherapy. Are these patients already cured and do they really need BMT?

063
RANDOMISED CLINICAL TRIAL OF SINGLE VS DOUBLE AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION (BOLOGNA '96) FOR PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA

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Autologous peripheral blood stem cell (PBSC) transplantation (Tx) has shown to be superior to conventional chemotherapy in patients with multiple myeloma (MM) in terms of increased complete remission (CR) rate and extended survival, both disease-free and overall. In order to evaluate whether a double PBSC-Tx could represent an advantage over a single one, a multicentric randomized trial (Bologna '96) was designed. Treatment plan included VAD induction therapy, followed by cyclophosphamide 7g/m^2 + G-CSF and PBSC collection, and single vs double PBSC-Tx. High-dose melphalan (200 mg/m^2) was used before single or first (out of 2) Tx, while conditioning to second Tx included the combination of melphalan 120 mg/m^2 and Busulfan 12 mg/kg. From March 1996 to April 2000, 252 untreated MM patients from 25 Italian centers were enrolled in the trial. The present analysis includes 155 patients who either completed or interrupted the treatment; 85 and 75 were randomly assigned to receive a single (Tx-1) or a double Tx (Tx-2) respectively. Clinical characteristics were similar in the two groups of patients; median age was 52 yrs, 67% of patients were in stage III, median beta2 microglobulin was 2.7 mg/L. Off-study rates were 5% with VAD and 9% with cyclophosphamide; the probability of completing the assigned treatment program was 77% for Tx-1 and 71% for Tx-2. WHO grade III-IV non-hematological toxicity was mainly represented by oral and gastrointestinal mucositis (18% after Tx-1 and 23% after Tx-2). Stringently defined complete remission (disappearance of M protein at immunofixation analysis) was demonstrated in 5% of patients after VAD, 9% after cyclophosphamide, 24% after Tx-1 and 25% after Tx-2 by analyzing the data on an intention-to-treat basis. The corresponding CR+PR rates were 59%, 63%, 75% and 81% respectively. The final CR rate increased to 37% in patients who actually received Tx-2. Overall, treatment- related mortality did never exceed 5%. Projected probability of event-free survival was longer for patients receiving Tx-2 in comparison to those treated with Tx-1. The difference between the two groups was statistically significant, by performing an intention-to-treat analysis (p=0.001) and a landmark analysis at 5 months after Tx-1 (p=0.01). These results, though promising, should be cautiously interpreted. Final analysis of the study must be awaited to assess the superiority, if any, of PBSC-Tx-2 over PBSC-Tx-1 for the treatment of newly diagnosed MM patients.

064
CLINICAL AND MORPHOLOGIC CHANGES IN PATIENTS WITH PROGRESSED B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA ARE NOT ASSOCIATED BY IMMUNOPHENOTYPIC MODIFICATIONS

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B-cell chronic lymphocytic leukemia (B-CLL) is a neoplastic disorder characterized by a proliferation and accumulation of long-lived and immuno-incompetent lymphocytes in the peripheral blood, bone marrow and lymphoid tissues. Diagnostic criteria are based on morphologic aspects of the leukemic B-cells and on their antigenic pattern. It is well known that differential diagnosis with other chronic lymphoproliferative disorders in leukemic phase may be controversial in some cases and for this reason a useful immunophenotypic scoring system (CD5+/CD19+, CD23+, CD79b-, FMC7- and SmIg±) for B-CLL patients has been proposed. The aim of this study was to investigate whether clinical and/or cytomorphologic progression of the disease, as documented by the increase in the number of larger lymphocytes and/or prolymphocytes, was correlated with any change in the antigenic expression. For this purpose, 14 B-CLL patients, progressed 4-14 years from the diagnosis, entered the study. Immunophenotypic analysis was carried out on peripheral blood lymphocytes in flow cytometry (FACScan, Becton Dickinson) using a direct immunofluorescence technique by dual color staining employing a panel of conjugated monoclonal antibodies including CD5, CD19, CD20, CD23, CD79b, CD38, SmIg and κ/λ light chain. In 7 out of 14 patients, the immunophenotypic analysis was performed at diagnosis and at progression; in all cases the antigenic profile was assessed separately on the smaller and larger lymphocyte cell populations identifyied by FSC and SSC flow cytometry parameters. Overall results evidenced no changes in the antigenic expression in the intensity of fluorescence in patients studied at diagnosis and at relapse. Secondly, we found that small lymphocytes and larger prolymphocytes showed the same immunophenotypic pattern. In conclusion, with the caution due to the reduced number of cases analyzed, we demonstrated that the clinical and morphological progression observed in B-CLL patients does not lead to a modification in the original immunophenotypic pattern.

065
HUMAN ERYTHROPOIETIN ELICITS AN ANGIOGENIC RESPONSE *IN VITRO* AND *IN VIVO*

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Hematopoietic and endothelial cell lineages share common progenitors. Recent studies have indicated that several cytokines and interleukins formerly thought to be specific for the hematopoietic system, are also capable of affecting certain functions of endothelial cells. In this study, we investigated the angiogenic potential of erythropoietin (Epo), the main hormone regulating proliferation, differentiation, and survival of erythroid cells. Epo receptors (EpoRs) have been identified in the human EA.hy926 endothelial cell line by Western blot analysis. Also recombinant human Epo (rHuEpo) stimulates Janus Kinase-2 (JAK-2) phosphorylation, cell proliferation, and matrix metalloproteinase-2 (MMP-2) production in EA.hy926 cells and significantly enhances their differentiation into vascular structures when seeded on Matrigel. *In vivo*, rHuEpo induces a potent angiogenic response in the chick embryo chorioallantoic membrane (CAM). Accordingly, endothelial cells of the CAM vasculature express EpoRs, as shown by immunostaining with an anti-EpoR antibody. The angiogenic response of CAM blood vessels to rHuEpo was comparable to that elicited by fibroblast growth factor-2. Taken together, these data demonstrate the ability of Epo to interact directly with endothelial cells and to elicit an angiogenic response *in vitro* and *in vivo* and thus act as a *bona fide* direct angiogenic factor. Finally, our data suggest that the full action of Epo and hence the production of erythrocytes and their release into the blood are rendered possible by the convergence of two phenomena, namely (1) proliferation and differentiation of progenitor erythroid cells and (2) bone marrow angiogenesis.

066
DETECTION OF BCR-ABL GENOMIC REARRANGEMENT IN CHRONIC MYELOID LEUKEMIA PATIENTS IN COMPLETE CYTOGENETIC REMISSION

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Conventional and molecular cytogenetic studies are performed in clinical practice to monitor the effectiveness of treatment in chronic myeloid leukemia (CML) patients. In the majority of preliminary studies of FISH, the use of BCR-ABL DNA probes that detected only a single BCR-ABL fusion signal hampered the precision of the percentage of positive nuclei. New FISH probes (D-FISH probes) detect BCR-ABL fusion in interphase nuclei with a false-positive signal rate close to zero. Such probes have been employed in the current study. To date we have performed FISH in 13 patients (9 men and 4 women) in complete cytogenetic but not molecular remission, either after interferon-alfa (IFN-α) therapy (7 cases) or allogeneic bone marrow transplantation (BMT) (6 cases). At diagnosis, 12 cases presented the classical t(9;22)(q34;q11) translocation, without additional abnormalities and 1 patient had a complex chromosomal rearrangement: t(6;9;22)(p21;q34;q11) and an additional add(20)(p11). The type of chimeric transcript was assessed by qualitative RT-PCR experiments; all patients had the the typical b2a2 or b3a2 transcript. After treatment with IFN-α or allogeneic BMT, the monitoring of the disease mainly involved cytogenetic analyses. When the patient showed at least 50 Ph-metaphases and a positive RT-PCR assay, FISH was performed. When the material was sufficient, a minimum of 500 cells in interphase were scored for each sample by FISH. The final results were expressed as percentages of nuclei with fusion signals. Negative FISH control studies were performed on bone marrow from 7 patients with hematologic disorders other than CML. The cut-off limit for BCR-ABL positivity was calculated at 0.5% of positive nuclei. Persistence of the BCR-ABL genomic rearrangement has been shown in all patients: FISH detected 0.8 to 4.4% nuclei with a BCR-ABL fusion gene. Recent studies have shown that FISH can be positive also in patients in complete cytogenetic remission (CCR), whereas RT-PCR was negative or weakly positive. These findings, together with the molecular data, suggest that non-proliferating neoplastic cells persist in patients in CCR. Therefore, these patients need to be monitored by FISH and RT-PCR methods in order to evaluate the minimal residual diseases and the risk of relapse.

ORAL COMMUNICATIONS

session 12

067
ALLOGENEIC TRANSPLANTS WITH BONE MARROW OR PERIPHERAL BLOOD STEM CELLS IN ADULTS WITH CHRONIC PHASE. EXPERIENCE FROM THE BOLOGNE CENTER

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Peripheral blood stem cells (PBSC) have became, in the past few years, a major source of stem cells for allogeneic transplantation. One expectation of the procedure is a reduced incidence of relapse, related to the higher number of transfused T-lymphocytes, compared to bone marrow transplantation (BMT), via a GVL effect. We have examined the incidence of relapse (cytogenetic and hematologic) and chronic GVHD in 13 consecutive patients with chronic phase chronic myeloid leukemia (CML-CP), transplanted from fully HLA compatible siblings who received big busulphan/cyclophosphamide as conditioning and cyclosporin A/methotrexate for GVHD prophylaxis. As reference group, we analyzed our historic cohort of 26 patients treated in the same way. PBSC transplants were performed between November 95 and June 99, BMT transplants between Octo-

ber 89 and July 98. Twenty-six patients (mean age 37 ± 9 years, mean diagnosis - Tx interval 24 ± 12 months) received BMT; 13 patients received PBSC (mean age 35 ± 9 years, mean diagnosis - Tx interval 14 ± 6 months). Chronic GVHD was defined as mild, moderate or severe, with a minimum survival of 80 days; cytogenetic relapse was defined as any Ph+ occurring >6 months after transplant. Chronic GVHD occurred in 10/13 PBSC and 10/24 BMT (p=0.04, chi square test). In the PBSC group GVHD was mild (4 cases), moderate (1), severe (5); respective numbers for the BMT group were 6, 2, 2 (p=0.04, Kruskal-Wallis test), indicating more severe chronic GVHD in the PBSC group. Two relapses (one cytogenetic and one hematologic) occurred in the 24 BMT and one hematologic in the 10 PBSC (p=0.6). Actuarial disease-free survival of the BMT group is 79% (95% CI: 63 - 92) at 9 years; that of the PBSC is 78% (95% CI: 50-100) at 3 years. Despite the vastly increased incidence of severe chronic GVHD, PBSC transplants do not seem to offer any advantage over BMT in terms of relapse of the disease when conventional GVHD prophylaxis is applied in CML-CP.

068
ABERRANT METHYLATION OF DEATH-ASSOCIATED PROTEIN KINASE AND O6-METHYLGUANINE-DNA-METHYLTRANSFERASE GENES IN AIDS-RELATED NON-HODGKIN'S LYMPHOMAS

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Non-Hodgkin's lymphomas (NHL) represent a frequent complication of HIV infection and a major source of morbidity and mortality among patient affected by AIDS. The molecular pathogenesis of AIDS-related NHL (AIDS-NHL) is heterogeneous and is characterized by activation of proto-oncogenes, inactivation of tumor suppressor genes and viral infection by EBV and HHV-8. Aberrant methylation of CpG islands within promoter regions, causing inappropriate gene silencing, is an acquired epigenetic alteration that serves as an alternative to gene defects in tumor suppressor inactivation in many human cancers. Hypermethylation of the death-associated protein kinase (DAP-kinase) and of the O6-methylguanine-DNA-methyltransferase (MGMT) promoters have been recently reported in many human malignancies. DAP-kinase is a newly discovered serine/threonine kinase whose expression is required for apoptosis. MGMT is responsible for removal of O6-methylguanine adducts produced by alkylating agents and its loss of expression favors lymphomagenesis in MGMT-/- knockout mice. In this study, we have analyzed a panel of 84 AIDS-NHL representative of the clinico-pathologic spectrum of the disease for the presence of aberrant methylation of DAP-kinase and MGMT promoters using methylation specific-PCR. Aberrant methylation of DAP-Kinase occurred at sustained frequencies throughout the spectrum of AIDS-NHL, including 79% AIDS-related diffuse large cell lymphoma, 88% AIDS-related Burkitt's lymphoma, 88% AIDS-related Burkitt's like-lymphoma, 84% AIDS-related primary effusion lymphoma and 25% AIDS-related primary central nervous system lymphoma. Hypermethylation of MGMT occurred in 65% of AIDS-related primary effusion lymphoma and in 38% of AIDS-related diffuse large cell lymphoma, whereas it was restricted to 25% of AIDS-related Burkitt's lymphoma and 12.5% of AIDS-related Burkitt-like lymphoma. Hypermethylation of MGMT was apparently absent in AIDS-related primary central nervous system lymphoma. The implications of these observations are multiple. First, our results provide the first evidence that aberrant methylation is a mechanism involved in the inactivation of tumor suppressor genes in AIDS-NHL. Second, the high frequency of abnormal methylation of DAP-kinase indicates an important role for this molecular lesion in the development and/or progression of AIDS-NHL and suggests that DAP-kinase inactivation may synergize with EBV and/or HHV-8 antigens in deregulating apoptosis in these malignancies. Third, the frequency of MGMT methylation is heterogeneous in different subsets of AIDS-NHL, corroborating the existence of multiple pathogenetic pathways in these tumors. Finally, because MGMT status may influence tumor clone resistance to the cytotoxic effects of alkylating chemotherapeutic agents, the methylation status of MGMT may potentially provide a novel prognostic marker for these lymphomas.

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INTERLEUKIN-11 PREVENTS TH1 POLARIZATION THROUGH A DIRECT EFFECT ON HUMAN T-LYMPHOCYTES AND BY INHIBITING IL-12 PRODUCTION BY MONOCYTES, BUT NOT BY MYELOID DENDRITIC CELLS

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Interleukin-11 (IL-11) has recently been shown to suppress IL-12 production by macrophages and this finding has been associated with the prevention of acute graft-versus-host disease (GVHD) in a murine model. In this study, we investigated the immunomodulatory role of IL-11 on antigen presenting cells (APC) by evaluating its capacity to inhibit the production of IL-12 by interferon (IFN-γ) primed monocytes and by mature myeloid dendritic cells (DC). IL-11 inhibited IL-12 production by monocytes, but not by DC derived from CD34+ and CD14+ cells. Moreover, IL-11 did not affect dendritic cell production of TNFα, IL1β and IL-10 and their expression of maturation-associated surface antigens. No significant effect of IL-11 was observed on DC generation from CD34+ and from CD14+ cells in the presence of specific growth factors. RT-PCR analysis demonstrated the presence of IL-11 mRNA in highly purified monocytes and cell sorted monocyte derived-DC (Mo-DC). More interestingly, IL-11 mRNA upregulated during the differentiation from CD14+ cells to DC, resulting in the production of small amounts of IL-11 by Mo-DC after exposure to TNFα. A wide pattern of cell subsets were shown to carry IL-11 receptor mRNA: Mo-DC, CD14+,

CD19+, CD8+, CD4+, CD4+CD45RA+ cells. We then investigated the capacity of IL-11 to prevent type I cytokine production by naive T-cells through a direct effect on T-lymphocytes. IL11 prevented Th1 polarization of highly purified CD4+CD45RA+ T-cells primed with anti-CD3/CD28 antibodies, resulting in a significant increase of IL4 and a decrease of IFN-γ production. This effect was confirmed by IL-4 and IFN-γ flow cytometry intracellular staining. In particular, IL-11 increased the percentage of IL-4-producing cells in a similar way to IL-4, the most powerful Th2 polarizing cytokine. IL-11 induced CD4+cell proliferation under stimulation with antiCD3 antibodies and IL-2, revealing a direct effect on T-cells, which was IL-2 independent. The mitogenic effect of IL-11 was confirmed in allogeneic mixed leukocyte reactions (MLR), performed by coincubating CD4+cells and Mo-DC. In conclusion, these data demonstrate for the first time that human IL-11 is capable of preventing a cytotoxic Th1 response through a direct effect on T cells and by inhibiting IL-12 production by monocytes, but not by myeloid DC. DC were capable of producing small amounts of IL-11, revealing their possible function as a source of IL-11 in the T-cell immune response cytokine network. Additionally, IL-11 induced T-cell proliferation, showing a wide and complex spectrum of actions. IL-11 appears to be one very attractive cytokine with therapeutic potential for GVHD and for other diseases in which Th1 responses play a dominant pathogenic role.

070
DETERMINATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND HEPATOCYTE GROWTH FACTOR IN CHRONIC MYELOID LEUKEMIA: LOW LEVELS IN INTERFERON-TREATED PATIENTS

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Recent studies have shown that angiogenesis is increased in myeloproliferative diseases. On this basis we measured serum concentrations of two of the most important soluble mediators of angiogenesis, vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), in 25 patients affected by chronic myeloid leukemia (CML). Their median age was 55 years (range 26-77), median duration of disease 41 months (range 0-165). Twelve patients were receiving treatment with hydroxyurea (HU), 9 with interferon (IFN) and 4 were not treated at the time of evaluation. Serum VEGF and HGF levels were above the upper normal value in 12 and 11 patients, respectively. Although in some patients only one of the two factors was elevated, a correlation was present between VEGF and HGF (r=0.55, p= 0.004). In addition, VEGF correlated with platelet count (r=0.77, p < 0.0001), WBC (r=0.55, p=0.005) and LDH (r=0.5, p=0.01), while HGF correlated with WBC (r=0.88, p < 0.0001), LHD (r=0.76, p < 0.0001), splenomegaly (r=0.64, p=0.0006), platelet count (r=0.57, p=0.003), and hepatomegaly (r=0.46, p=0.02) and was inversely correlated with Hb (r=0.59, p=0.002). Thirteen patients in early chronic phase tended to have lower values of both angiogenic factors than patients in late chronic phase or accelerated disease (median VEGF 409 vs 1,255; median HGF 1,015 vs 1,615). However, these differences were not statistically significant. On the contrary, a significant difference was found when we divided our patients according to treatment: IFN-treated patients had much lower values of both factors than HU-treated and untreated patients (median value for VEGF 316 vs 1,692, p = 0.01; median value for HGF 841 vs 1,938, p = 0.02). Since angiogenic factors, especially VEGF, may be released under normal conditions in response to hypoxia, we also measured erythropoietin (EPO) serum concentration as an index of the hypoxic stimulus. However, neither VEGF nor HGF correlated with EPO, indicating that their increased concentration was independent of hypoxia. In conclusion, these data indicate that in half of our CML patients, serum levels of angiogenic factors are increased, especially in patients with more advanced disease. Moreover, our data confirm the anti-angiogenic activity of IFN and indicate that this effect could be exerted by lowering the levels of some important soluble mediators of angiogenesis.

071
SOUTHERN BLOT ANALYSIS FOLLOWED BY A MULTIPLEX RT-PCR STRATEGY TO IDENTIFY MLL/ALL1 REARRANGEMENTS AND FUSION GENES IN ACUTE MYELOID LEUKEMIA

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Rearrangements of the MLL/ALL1 gene at 11q23 are detectable in approximately 10% of adult patients with acute leukemia of either lymphoid or myeloid type. These fusions involve a variety of alternative partner genes, including a rearrangement with a partially duplicated MLL/ALL1 (so-called self-fusion). Interestingly, cytogenetic analysis frequently results in these cases in apparently normal karyotype, or aberrations other than 11q23 lesions, thereby suggesting the occurrence of cryptic alterations at this locus. We have recently developed a molecular strategy to detect these aberrations and to identify MLL/ALL1 fusion partners. This latter involves, as a first step, Southern blot analysis of MLL/ALL1 gene configuration. Cases showing additional restriction fragments compared to the germline control, are subject to subsequent multiplex RT-PCR analysis (modified following Pallisgaard *et al.*, Blood 1998; 92:574). As a first step, three reaction tubes are setup which include MLL/ALL1 fusion with ELL, AF6, AFX (vial n.1), AF1p, self-fusion, AF10, and AF17 (vial n.2), and AF4, AF1q, AF9, ENL (vial n.3). Oligoprimers to amplify the E2A gene are also added to each tube as an internal control. If an amplification product is obtained in one of the three tubes, then a series of RT-PCR reactions are performed in order to confirm and identify precisely the specific fusion gene. In the present study, 17 acute myeloid leukemia patients with MLL/ALL1 rearrangements were identified by Southern analysis and subjected to multiplex RT-PCR. In 13 of these 17 (76%), a fusion partner was identified. These included AF9 in 6/17 cases (35%), ELL in 2/17 (12%), AF10 in 2/17 (12%) and self-fusion in 3/17 (17%). No amplification was obtained using the above strategy in the remaining 4/17 cases (24%). In these 4 cases, karyotypic analysis showed a previously unrecognized involvement of chromosome band

10q22 (1 patient), a del (6q) in another, and was uninformative in the remaining 2 cases. At present, we are developing a race PCR strategy aimed at identifying novel fusion genes in cases showing rearranged MLL/ALL1 at Southern blot and no known fusion partner after multiplex RT-PCR. We conclude that Southern blot followed by the above multiplex RT-PCR analysis is a rapid and suitable strategy for identifying the most common MLL/ALL1 fusion genes.

072
MYELOID CELLS BEARING THE CHIMERIC PROTEIN BCR-ABL EXPRESS HB-EGF / DIPHTHERIA TOXIN RECEPTOR, BUT ARE INSENSITIVE TO THE DIPHTHERIA TOXIN PRO-APOPTOTIC EFFECT

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Heparin-binding EGF-like growth factor (HB-EGF) is a growth and/or chemotactic factor for smooth muscle, endothelial and normal/neoplastic epithelial cells, fibroblasts and astrocytes active as either a soluble or membrane-bound molecule. The latter possesses the unique property of being the receptor for diphtheria toxin (DT). Among myeloid/lymphoid cells, HB-EGF is expressed by monocytes (Mo), lymphocytes (PBL) as well as by *ex vivo* myeloid leukemic cells and a number of myeloid-, lymphoid-derived human cell lines. We have previously shown that primary human neutrophils (PMN) are characteristically negative for HB-EGF in basal conditions and that recombinant GM-CSF specifically induces and greatly enhances the production of HB-EGF in PMN and Mo, respectively as well as in *ex vivo* myeloid leukemic cells. Cells bearing the HB-EGF molecule on their membrane are usually highly sensitive to DT, which induces apoptotic cell death. We studied the human cell line K562 as well as *ex vivo* peripheral myeloid cells from 5 patients with p210-BCR/ABL chronic myeloid leukemia (CML) to evaluate expression of HB-EGF and sensitivity to DT, using molecular (RT-PCR cloning, Northern blot, flow cytometry, ELISA) and functional (mitogenic activity on BALB/c 3T3 cells), sensitivity to the pro-apoptotic effect of DT) approaches. The cell line K562 and the *ex vivo* CML cells expressed HB-EGF. Quite surprisingly, the normal counterparts of the latter were usually HB-EGF-negative, suggesting that the presence of the chimeric protein p210 may be involved in activating the HB-EGF promoter. It is likely that the chimeric protein p210 had the HB-EGF gene as a downstream target through activation of the Ras and MAP kinase pathway. Even more surprisingly, none of the p210-BCR/ABL-positive cell types which expressed membrane-bound HB-EGF were sensitive to a10^{-11} -10^{-8} M DT pro-apoptotic effect, in spite of the fact that DT was internalized. By contrast, when the normal counterparts of the CML cells were stimulated with recombinant GM-CSF or human bladder cancer cell line 5637 conditioned medium, which contains relevant amounts of carcinoma cell-derived GM-CSF, these normal myeloid cells upregulated HB-EGF acquiring a previously lacking sensitivity to DT. Thus, our data provide circumstantial evidence that HB-EGF is one of the molecules induced in CML cells by the biological activity of the chimeric protein BCR/ABL, which is also a powerful inhibitor of the pro-apoptotic effect of DT.

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073
IN VITRO AND *IN VIVO* STUDIES ON ANGIOGENESIS AND ANTI-ANGIOGENIC THERAPIES IN NON-HODGKIN'S LYMPHOMA

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We and others have reported several lines of evidence indicating that angiogenesis plays an important role in non-Hodgkin's lymphoma (NHL). In B-cell NHL, the extent of disease vascularization was found to increase simultaneously with disease progression, and in two different studies circulating levels of endothelial cell growth factors VEGF and b-FGF were found to correlate with survival independently of other known risk factors. We evaluated a panel of 11 B- and T-NHL cell lines and primary tumors and found that most of them produce VEGF (but not b-FGF) at concentrations that are within its known range of biological activity. Furthermore, all of the NHL cells expressed at least one of VEGF-related receptors Flt-1 and KDR. Remarkably, of the non-endothelial tumors studied so far, only some melanomas, leukemia, NHL and myeloma cells aberrantly express VEGF receptors. Thus, we investigated the presence of an autocrine pathway between VEGF and related receptors by NHL cell culture in the presence of a Flt-1/Fc chimera known to inhibit VEGF-dependent HUVEC proliferation. Using this approach, VEGF deprivation from culture medium was associated with 7-44% inhibition of NHL cell proliferation. In addition, we generated preclinical models of human NHL by transplanting NOD/SCID mice with the same NHL cell panel. Relevant neovascularization was found in most tumors, and tumor engraftment efficiency, time of engraftment and frequency of apoptotic/dead cells in tumors strongly correlated with VEGF production ($p<0.008$) and CD31+ tumor vessel frequency ($p<0.03$). NOD/SCID models of human B-cell NHL were used to investigate anti-angiogenic therapies. In a first series of NHL prevention studies we found that that chronic administration of green tea (known to have strong anti-angiogenic activity through the flavonol epigallocatechin-3-gallate) prevented 50% of Burkitt's Namalwa NHL and significantly inhibited NHL growth in two additional models. Notably, treatment with the chemotherapy drug cyclophosphamide (CTX) at the maximum tolerable dose (MTD) was unable to prevent Namalwa tumor occurrence. In a second series of studies, MTD CTX, rituximab and the anti-angiogenic drug endostatin were tested alone in NOD/SCID mice transplanted with Namalwa cells. All drugs delayed tumor growth, and CTX was the most effective in controlling bulky disease. In a third series of studies, we found that endostatin, given after MTD CTX or rituximab, effectively induced tumor stabilization. In fact, when mice given CTX or rituximab on day 3, 5 and 7 after transplantation were randomized to receive endostatin or PBS on day 15 to 19, in endostatin-treated mice tumor growth was prevented as long as the drug was administered. Furthermore,

endostatin administered on day 25- to 29 after tumor regrowth was still able to induce significant tumor regression, whereas CTX and rituximab were not effective. In the NHL models evaluated, the frequency of apoptotic endothelial and tumor cells was significantly increased in mice given anti-angiogenic drugs compared to in the other series. Our data underline the relevance of angiogenesis in NHL and indicate that sequential administration of MTD chemotherapy and endostatin seems promising for treating bulky NHL, while the less toxic sequential administration of rituximab and endostatin is promising for limited disease.

074
P210-DERIVED PEPTIDE VACCINE PLUS QS-21 AND GM-CSF IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA: RATIONALE AND PRELIMINARY DATA

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Chronic myelogenous leukemia (CML) presents a unique opportunity to develop therapeutic approaches using vaccination against a truly tumor specific antigen that is also the oncogenic protein involved in neoplasia. CML is in fact always characterized by the t(9;22) that results in the bcr-abl fusion oncogene and expression of a chimeric protein product p210. Previously we found that peptides derived from amino acid sequences crossing the b3a2 breakpoint in p210 elicit class I restricted cytotoxic lymphocytes (CTLs) and class II restricted specific proliferation of CD4 T-lymphocytes *in vitro* (HLA A3, A11, B8 and DR11, respectively). Moreover, b3a2-derived peptide-specific T-cells have been shown to kill and/or proliferate in the presence of CML blasts, thus suggesting that bcr-abl fusion sequences are naturally processed and expressed on CML cells. These *in vitro* data provided the rationale for developing peptide-based vaccines for this disease. Scheinberg *et al.* recently published the results of the first phase I dose escalation study of a multivalent breakpoint peptide vaccine plus the immunologic adjuvant QS-21. In this trial HLA restriction was not required and most of the patients had large tumor burdens. Nevertheless 3/12 patients treated (3/6 at the two highest dose levels of vaccine) generated peptide-specific T-cell proliferative responses *ex vivo* (n=3) and/or delayed-type hypersensitivity (DTH) responses (n=2). Peptide-specific CTLs have not been identified. As it is more likely that effective vaccination strategies will target patients with minimal tumor burden, we recently started a phase II trial including patients with appropriate breakpoint and HLA types and major or complete cytogenetic response. Furthermore, in order to improve peptide immunogenicity, we added low doses of GM-CSF to a fixed medium-high dose of CML peptides plus QS-21. Two patients have so far entered the study. Both of them showed a prompt and consistent DTH response already evident after the first 3 vaccinations of the 6 planned in the study. Neither of them had evidence of peptide-specific CTLs *in vitro*. One patient (HLA DR11) showed a significant peptide-specific T-cell proliferation. Although very preliminary our data suggest that patient selection and the addition of GM-CSF as an immunologic adjuvant, might result in a stronger immune response after peptide vaccinations which could translate into a measurable anti-tumor effect.

075
HIGH EXPRESSION OF CHROMOSOMAL FRAGILE SITES IN HEMATOLOGIC PATIENTS OCCUPATIONALLY EXPOSED TO HAZARDOUS CHEMICALS

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Background and Objectives. The rising incidence of cancer is mainly attributed to environmental exposure to a growing number of physical and chemical agents. Because of intrinsic structural characteristics several genetic loci might be more prone to damage and mutation. Chromosomal fragile sites (FS) are points on chromosomes that tend to break non-randomly when cells are grown in particular conditions: FS are considered unstable regions in the genome and might represent targets for diverse mutagens. Several epidemiological studies showed a higher incidence of leukemia in workers exposed to chemicals. Very few reports have focused on the cytogenetic aberrations of leukemia patients environmentally exposed to mutagens. We wanted to study possible correlations between occupational exposure to hazardous chemicals in patients diagnosed as having AML, ALL, MPD, MDS and 1) clonal chromosomal aberrations of bone marrow cells and 2) the incidence of non-clonal chromosomal aberrations (NCCA) and FS expression after aphidicolin co-culture. *Design and Methods.* Between January 1996 and December 1999, based on interviews, 80 patients out of 280, were categorized as having been exposed to chemical hazards (mainly pesticides, solvents and petroleum derivatives): 36 AML, 9 ALL, 15 MDS, 18 MPD and 2 cytopenias. Fifty-five out of 80 were exposed at the time of diagnosis, while 25 had been exposed in the past. For each case two control patients matched for age and diagnosis were selected. In cases and controls karyotype analysis was carried out on bone marrow cultures while the analysis of NCCA and FS expression was done on peripheral lymphocytes after incubation with aphidicolin. *Results*. Acute leukemia: exposed patients showed a higher incidence of abnormal karyotype; the rates of chromosome loss and deletion was also higher than those in controls (but not statistically significantly so) . Chromosome 3 aberration was much more frequent in exposed subjects (OD=3). Leukemia were more often secondary to a preleukemic condition in exposed patients. *MPD*. All patients categorized as exposed had CML(=14) idiopathic eosinophilia (=4). CML patients who had a history of exposure more often than matched controls presented with adverse prognostic features. *MDS* (=15): all exposed patients were categorized as RAEB, RAEB-T or CMML, cytogenetic profile was similar in exposed and not exposed patients. NCCA and FS expression: the incidence of NCCA was higher in exposed subjects than in never exposed patients and to patients who had been exposed in the past (OR=3.2 CI=1.6-12). The analysis of fragile sites expression showed that several FS were significantly more expressed in exposed pt: 1p32, 3p14, 4q31, 7q31, 7q32, 9p21, 14q24, 17q23. No correlation was found with clonal chromosome aberrations. *Interpretation and Conclusions.* The correlation between occupational exposure and the incidence of NCCA and FS expression was very marked in our series. Recently the coincidence of the FHIT oncogene with the

3p14 FS has been shown. The analysis of NCCA and FS expression, after aphidicolin co-culture, in subjects intensively exposed to mutagens seems to be a very effective tool for identifying and monitoring a population at risk because of environmental exposure. Furthermore it might reveal the direct effect of mutagenic agents on chromosomes highlighting a direct link between exposure, chromosomal damage and oncogene mutations.

076
EARLY ENGRAFTMENT OF SHORT-TERM *EX-VIVO* AMPLIFIED HUMAN CORD BLOOD CELLS IN NOD/SCID MICE

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Cord blood (CB) has been widely shown to be a reliable source of hematopoietic stem cells (HSC) for allogeneic transplant. From the Eurocord analysis on CB transplantation in both pediatric and adult patients, engraftment has been shown to be significantly affected by the cell dose infused. Therefore, the *ex-vivo* expansion of CB HSC, by increasing the number of donor cells/progenitors, might represent a promising approach to improve clinical results. With this purpose we previously standardized a short-term amplification culture system of CB CD34+ cells. We have now developed a NOD/SCID mice model in order to evaluate both the early engraftment potential of *ex-vivo* amplified cells and the ability of these cells to allow long-term hematopoietic reconstitution. Twenty-six NOD/SCID mice were transplanted with *ex-vivo* amplified CD34+ CB cells (duration of amplification: 7 days; input cell number: 4×10^4 cryopreserved CD34+ cells) cultured with flt3L/SCF/IL-3. The short-term engraftment capability of *ex-vivo* generated cells was evaluated 7, 14, 21 and 28 days after transplant by means of (1) cytofluorimetric detection of CD45+ cells in bone marrow (BM) and spleen samples and (2) molecular analysis of human DNA polymorphism on mice BM colony-forming cells. NOD/SCID mice (n=9) reinfused with 4×10^4 unmanipulated CD34+ cells were used as controls. In 7/26 (27%) mice transplanted with amplified cells and in 4/9 (44%) mice transplanted with unmanipulated CD34+ cells the engraftment was detected as early as 7 days after transplant and confirmed at each time point until 28 days. These preliminary results show that CD34+ cells amplified *ex-vivo* according to our protocol are able to induce early engraftment. The persistence of the proliferative potential of the human cells until 28 days may suggest that even long-term engraftment could be supported by the amplified CB cells.

077
NO PREFERENTIAL SENSITIVITY OF T(8;21) ACUTE MYELOID LEUKEMIA CELLS TO CYTOSINE ARABINOSIDE *IN VITRO*

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It has been reported that acute myeloid leukemia (AML) patients with inv (16) or t(8;21) abnormality show a better prognosis: in particular for inv(16) patients treated with high-dose cytosine arabinoside (ara-C) containing regimens, whereas, for t(8;21) the evidence in favor of high dose ara-C is contrasting. Concerning inv(16), we previously demonstrated that inv(16) AML cells are characterized by an increased sensitivity to ara-C *in vitro*, with higher incorporation of 3H ara-C into DNA and increase of induced apoptosis. In this study, we thus aimed at testing the *in vitro* sensitivity to ara-C of leukemic cells from patients showing the t(8;21), comparing 3 groups of cells: AML blasts with inv(16), with *intermediate* or with *unfavorable* karyotype at diagnosis. We analyzed blast cells from 58 patients who were diagnosed and treated in our Institution. The incorporation of 3_H ara-C into DNA was significantly lower for AML cells characterized either by *unfavorable* karyotype, or by t(8;21), if compared to either inv(16) (p=0.02) or normal karyotype (p=0.04). Nevertheless, ara-C induced apoptosis resulted concordant, with scarce or no increase after ara-C induction in t(8;21) AML cells. As well as a different molecular pathway, these data suggest that the mechanisms of response to chemotherapy for t(8;21) are probably different from those active in inv(16) AML cells.

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078
SALVAGE THERAPY WITH THALIDOMIDE FOR PATIENTS WITH ADVANCED RELAPSED/REFRACTORY MULTIPLE MYELOMA

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Extensive introduction of high-dose therapy with stem cell support has significantly improved the outcome of patients with multiple myeloma (MM) in terms of increased complete remission (CR) rate and extended survival, both disease-free and overall. Few options, however, are presently available for patients with primary refractory disease or those who relapse after single or double autologous transplantation. Thalidomide, a glutamic acid derivative with anti-angiogenesis properties, has been recently proposed as salvage treatment for such patients. At our Institution, we have started a therapeutic trial using thalidomide in relapsed/refractory MM patients. From October 1999 to May 2000, 18 patients (10males/8females) were enrolled in the trial. Their median age was 60 years, all patients were in stage III, median β2 microglobulin was 4.36 mg/L, median bone marrow plasma cell infiltration was 70%, and 11 patients had been previously submitted to one (n=3) or two (n=8) autologous stem cell transplants. Thalidomide was initially administered at a dose of 100mg/day; if well tolerated, the dose was increased serially by 200mg every other week to a maximum of 800mg/day. The median administered dose was 400mg/day. WHO grade > II toxic effects were constipation (40%), lethargy (26%) and skin rash (20%) At present, 14 patients are evaluable for response, 4 (28%) showed a > 50% reduction in serum or urine M protein and 4 (28%) showed a > 25% response. After a median 4 months follow-

up, 4/8 patients are alive and progression-free, 3 patients have relapsed, and 1 patient died of pulmonary edema while still in partial remission. These data confirm that thalidomide is active in relapsed/refractory MM and could thus deserve testing in combination therapy, perhaps as part of front-line treatment programs.

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079
SELECTIVE INHIBITION OF MEK1 KINASE DOWNMODULATES ERK ACTIVITY AND PROLIFERATION OF ACUTE MYELOID LEUKEMIA BLASTS

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In this paper, we report the results of the analysis of extracellular regulated kinases (ERKs), that are protein-serine/threonine kinases belonging to the mitogen-activated protein kinase (MAPK) superfamily, in twenty-five cases of primary acute myeloid leukemia (AML). Four human normal bone-marrows were also investigated. All the leukemic cases were examined at diagnosis by studying samples with more than 90% leukemic cells. FAB classification and immunologic and cytogenetic evaluations of the samples were performed. To detect ERK immunoenzymatic activity we selectively immunoprecipitated ERK1/2 kinase from 500 µg of cell lysates. Our results showed that ERK activity was very low or undetectable in the normal hematopoietic precursors taken from unfractioned bone-marrow. Conversely, all the leukemic samples had high levels of ERK activity. The range of the levels of ERK1/2 activity was between 93 and 170 units. Taken these values, we then investigated the functional relevance of high levels of ERK activity in AML. We made experiments modulating *in vitro* leukemic blasts taken from eight cases of AML by the MEK1 inhibitor PD 98059 (New England Biolabs, Beverly, MA, USA), that specifically inhibits ERK activity as no substrates for MEK1 have been identified other than ERK 1 and ERK 2. Different times of incubation and different concentrations of the compound were utilized. At a concentration of 40 µM, and incubating for 24h, we observed lower levels than in untreated samples of ERK1 and 2, dual phosphorylation detected by phospho-specific antibodies (New England Biolabs), and about 50% decrease of ERK1/2 immunoenzymatic activity. Tritiated thymidine uptake assay showed 43.56±6.8 decrease in proliferation in comparison with untreated controls. The decrease in proliferation was associated with the caspase-3 mediated cleavage of poly(ADP-ribose) polymerase (PARP) and with the appearance of the 89 kDa fragment that is an early sign of apoptosis, and with internucleosomal DNA fragmentation. Our results demonstrated that ERK activity may have a significant functional role in blast cell proliferation of primary AML. Furthermore, it is of interest that this biological approach to controlling neoplastic proliferation may exploit some mechanisms such as PARP cleavage utilized by common chemotherapeutic agents.

080
UNRELATED DONOR SEARCH AND TRANSPLANT FOR PATIENTS AFFECTED BY CHRONIC MYELOGENOUS LEUKEMIA. EXPERIENCE OF A SINGLE CENTER CONCERNING 30 PATIENTS

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With the aim of assessing the impact of unrelated donor (UD) bone marrow trasplantation (BMT) on survival, we evaluated the outcome of patients transplanted with those for whom a donor was unsuccessfully searched for. To evaluate the efficacy of transplant better, all patients were followed for at least 5 years. From September 1989 to July 1995 an UD was searched for 30 consecutive patients affected by chronic myeloid leukemia (CML) referred to our BMT Unit. Other patients, referred subsequently, are not included in this study because of the short follow-up. Patients characteristics: 19 were male (63.4%) and 11 female (36.6%); 22 (73.4%) were classified as standard risk (first chronic phase) and 8 (26.6%) as high risk (accelerated phase, blast crisis or second chronic phase); mean age was 32 years(range 10-47). Fourteen UD were identified (46.6%) and 13 patients were transplanted. One patient was not transplanted because of disease progression; 2 searches are still ongoing. In the group of transplanted patients, 6 had received, as first-line therapy, rINF-α combined with hydroxyurea (HU) in one case; the remaining 7 patients had received HU (six) and autologous bone marrow transplantation in one case. The mean interval from diagnosis to transplant was 29 months (range 13-61) and from formal search to transplant was 17 months (range 6-37). The conditioning regimen included 1320 cGy total body irradiation (ifTBI) and cyclophosphamide (CY) 120 mg/kg/dx2.

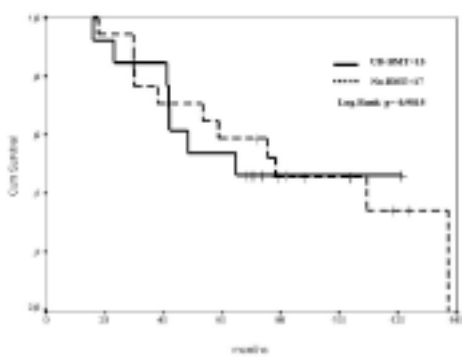

Figure 1.

Graft-versus-host disease (GvHD) prophylaxis protocol was cyclosporin A (CsA) and short/methotrexate (MTX) in

all cases plus antilymphocyte globulin (ATG) in 12/13. Patients not submitted to BMT were treated with chemotherapy and autologous (2 cases) or haploidentical (1 case) BMT. With a minimal follow-up of five years the overall survival of the 13 BMT patients is 46% while for those not transplanted it is 35% (p= ns). The difference between the two curves (Figure 1) is not statistically significant but only the transplant group curve shows a plateau after 5-10 years suggesting that UD-BMT offers a real chance of cure. In the BMT-group the only failure cause was transplant-related mortality. The main cause of death was severe GvHD (38.4%) while for patients not transplanted it was disease progression (58.8%) In long-term survivors of both groups performance status was 100% in 97% of cases.

081
CHEMOKINE RECEPTOR EXPRESSION IN ACUTE MYELOID LEUKEMIA CELLS

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The mechanisms regulating the trafficking of leukemic myeloid blasts from the bone marrow microenvironment to the peripheral blood and vice versa are very poorly understood. There is substantial evidence that the migration of normal hematopoietic progenitor and stem cells is a multistep process that requires the sequential engagement of specific chemotactic cytokines (chemokines) and their receptors. In accordance with this, it has been shown that the chemokine receptor CXCR4 is required for the retention of granulocytic precursors within the bone marrow microenvironment. This evidence prompted us to hypothesize that a differential expression of chemokine receptors (CRs) could account for the peculiar pattern of invasion and diffusion shown by leukemic cells. In line with this argument, it has been already reported that some acute myeloid leukemia (AML) cells express CXCR4, which appears to mediate the migration across bone marrow endothelium of the leukemic blasts in response to the specific ligand, SDF-1. Beside this, very little is known about the expression of other CRs by normal hematopoietic precursors and their malignant counterparts. We have analyzed, by FACS, the CR expression profile in AML patients at presentation. The CR expression was evaluated by staining leukemic cells with monoclonal antibodies specific for CCR1, CCR2, CCR5, CCR6, CCR7, CCR9, CXCR1 and CXCR4. Fourteen AML patients were studied and, according to the FAB classification, there were 7 patients with M4, 3 with M2, 3 with M1 and 1 patient with M0. Leukemic cells were obtained from the peripheral blood of AML patients and represented 79 ± 21% of the peripheral blood mononuclear cells. CCR1, CCR4, CCR5 and CCR7 were expressed in 0/14, 0/8, 1/14 and 0/11 of cases analyzed, respectively. In two cases of M4, CCR1 was expressed weakly by monocytic blasts. CCR5 was expressed in one case in the only M0 tested. CCR2, CCR6 and CXCR4 were expressed, with levels of mean fluorescence intensity (MFI) varying from patient to patient, in 11/14, 12/14, 13/14 of cases analyzed, respectively. CCR9 and CXCR1 were expressed in 6/14 and 7/14 of cases analyzed and in 5/7 and 7/7 of the M4 studied, respectively. In 2/7 of the M4 that were found positive for CXCR1, only the monocytic blasts contributed to CXCR1 expression. Finally, in 4/5 of the M4 that were found positive for CCR9, the MFI was significantly higher in the monocytic blasts. We found that also normal monocytes, such as AML-M4 cells, express CCR2, CCR6, CXCR1 and CXCR4, but they do not express CCR9. To summarize, in the 14 AML patients studied, leukemic cells expressed CCR2 and CCR6 but not CCR1, CCR4, CCR5 of CCR7. CCR9 and CXCR1 were positive only in M4, and particularly in monocytic blasts, in contrast to normal monocytes, positive only for CXCR1. To understand the relevance of these data better in relation to the migration and homing of AML cells, we are currently investigating chemokine-receptor expression by CD34+ cells, the functional activity of chemokine-receptor expressed by AML cells and the chemokine production of AML cells.

082
HERPES VIRUS THYMIDINE KINASE GENE AS A POTENTIAL TOOL FOR GENERATING A CONTROLLED GRAFT-VERSUS-HOST / GRAFT-VERSUS-LEUKEMIA EFFECT

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Generation of an efficient graft-versus-leukemia effect (GvL) in patients with hematologic malignancies who relapse after allogeneic bone marrow transplantation depends in part on the number of infused T-lymphocytes. At present GvL cannot be achieved without inducing concomitant graft- versus-host disease (GvHD); thus one strategy is to try to modulate this GvL/GvHD ratio. We engineered human T-lymphocytes with herpes virus thymidine kinase (HSV-TK) and neomycin resistance genes (NeoR), using an LXSN-derived vector which confers a ganciclovir-specific sensitivity to the transduced T-cells. Ten transduction procedures were performed using lymphocytapheresis products from healthy donors. Transduction efficiency was evaluated by semiquantitative PCR. We analyzed immunophenotyping, proliferation, interleukin 2 (IL-2) production, alloreactivity in a mixed lymphocyte culture and clonogenicity during different stages of retroviral infection and selection. Seven days selection with 0.6 mg/mL G418 resulted in a cell population which was inhibited by ganciclovir treatment (10 days ganciclovir 1mg/mL). Infected cells increased from 1-5% before selection to over 90% after G418 selection when viral supernatant infection was used, and from 30-40% to over 90% in the co-cultivation experiments. HSV-TK transduction and G418 selection did not change the lymphocyte phenotype. Trivial differences between controls and infected populations emerged at all timepoints demonstrating that retroviral infection has minimal effects on lymphocyte subset composition. The transduced selected lymphocytes expressed CD3 in 86.9 to 94%, CD4 in 54 to 83.6%, CD8 in 45 to 62%, HLA-DR in 55.5 to 86.7% of cells. CD16+ expression ranged from 4.3 to 4.7% and CD19+ from 0.6 to 3.2%. Transduced/selected populations, although less

responsive to IL-2 than control cells, retained their proliferative activity, alloresponsiveness, and ability to produce and respond to IL-2. Compared with control populations, their clonogenicity, assessed by limiting dilution assays was reduced after retroviral infection and G418 selection by 1.6 and 2.9 logs respectively with both viral supernatant incubation and co-culture procedures. These results show T-lymphocytes can be transduced on a large scale using tk-Neo vector. However retroviral vector infection and G418 selection significantly reduce T-lymphocyte clonogenicity as defined by limiting dilution analysis. Since this assay is a very sensitive method for detecting and quantifying functional T-lymphocytes, our results could have important implications for defining the number of engineered T-lymphocytes required to generate an efficient GvL.

083
TRANSFORMING GROWTH FACTOR BETA 3 INHIBITS CHRONIC MYELOGENOUS LEUKEMIA HEMATOPOIESIS BY INDUCING FAS - INDEPENDENT APOPTOSIS

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Transforming growth factor beta 3 (TGF- β3) is a potent suppressor of human hematopoietic progenitor cells. We compared the activity of TGF-β3 on highly purified CD34+ cells and more immature CD34+DR- cells from 15 patients with chronic myelogenous leukemia (CML) in chronic phase and 10 normal donors. Primitive hematopoietic progenitors were stimulated in liquid cultures and clonogenic assays by early-acting growth factors such stem cell factor (SCF) and interleukin 11(IL 11) and the intermediate-late acting stimulating factors interleukin 3(IL 3), granulocyte- macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO). Molecular analysis of bcr/abl mRNA was performed on single CML colonies by nested reverse transcriptase polymerase chain reaction (RT-PCR). Moreover, cell cycle analysis and assessment of apoptosis of normal and leukemic CD34+ cells were performed by propidium iodide (PI) alone and simultaneous staining with annexin V and PI, respectively. The colony-forming efficiency of CML CD34+ cells was generally inhibited by more than 90% regardless of whether the colony-stimulating factors were used alone or in combination. When compared to normal CD34+ cells, leukemic cells were significantly more suppressed in 6/8 culture conditions. The inhibitory effect on TGF-β3 on CD34+ cells was exerted within the first 24 hours of incubation as demonstrated by short- term pre-incubation followed by IL 3 and SCF stimulated colony assays. Evaluation of bcr/abl transcript on residual CML colonies incubated with TGF-β3, demonstrated a small subset of neoplastic CD34+ cell unresponsive to the inhibitory effect of the study cytokine. TGF-β3 demonstrated a greater inhibitory activity on primitive CD34+DR- cells than on more mature CD34+ cells. Again, CML CD34+ DR- cells were significantly more inhibited by TGF-β3 than their normal counterparts in 3/8 culture conditions. Kinetic analysis performed on CD34+ cells showed that TGF-β3 induces cell cycle arrest in G1- phase. However, this mechanism of action is shared by normal and leukemic cells. Conversely, TGF-β3 preferentially triggered the programmed cell death of CML CD34+ cells without increasing the proportion of leukemic cells co-expressing CD 95 (Fas-R) and this effect was not reversed by functional blockade of Fas-R. In conclusion, we demonstrate that TGF-β3 exerts a potent suppressive effect on CML cells which is partly mediated by Fas-independent apoptosis.

084
MOLECULAR IMMUNOGLOGULIN HEAVY CHAIN GENE ANALYSIS IN NODAL MARGINAL ZONE B-CELL LYMPHOMA

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Marginal zone B-cell lymphomas (MZL) are a distinct subtype of non-Hodgkin's lymphoma (NHL) which have been recognized and defined by the REAL classification.[1] MZL can be subdivided into three varieties: extranodal, nodal and splenic MZL. Very recently, the WHO classification clearly recognised nodal MZL (NMZL) as a distinct disease that must be distinguished from extranodal and splenic MZL with lymph node involvement and from the other small B-cell NHL.[2] Interestingly, a recent study recognized NMZL as the most common type of lymphoma in HCV-positive subjects.[3] In the present study we determined the nucleotide sequence of the tumor-related rearranged immunoglobulin heavy chain (IgH) in 10 cases of NMZL. The results were evaluated also on the basis of the presence of chronic HCV infection. Serum anti-HCV antibodies were detected in 5 out the 10 NMZL examined. Immunoglobulin VH genes were successfully amplified and directly sequenced in all the cases examined. VH sequences were found carrying somatic mutations in all cases with a sequence-identity rate compared with the closest germline gene ranging from 83.33 to 98.28% showing that the tumor cells were likely derived from mature B-cells that had participated in a germinal center (GC) reaction. The involved VH segments belonged to the VH4 family in 4 cases, to the VH1 family in 3 cases, to the VH3 family in 2 cases and to the VH2 family in 1 case. Statistical analysis of the pattern of somatic mutations suggests a role for antigen selection in 5 of the 10 cases examined. Interestingly, different VH segments were preferentially used in HCV-positive and HCV-negative patients: 3 out of 5 HCV-negative NMZL used a VH4-34 gene segment joined with different D and JH segments and 3 out 5 HCV-positive NMZL used a VH1-69 gene joined with a D3-22 and a JH4 segment with very strong similarities in the CDR3 regions of the 3 different cases. In conclusion, these data indicate: a) NMZL are derived from B-cells that have experienced the GC reaction with evidence of antigen selection in half of the cases; b) the presence of a VH1-69 segment in the majority of the HCV-positive NMZL cases with similar CDR3 sequences suggests the presence of a common antigen, probably a HCV antigen epitope, involved in the B-cell selection; c) the presence of a VH4-34 segment suggests a role of a yet unknown B-cell superantigen(s) in the selection of tumor precursor B-cell in HCV-negative NMZL.

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ORAL COMMUNICATIONS

session 15

085
HUMAN HERPESVIRUS-8 INFECTS BONE MARROW PROGENITORS AND EXERTS A MYELOSUPPRESSIVE EFFECT *IN VITRO* AND *IN VIVO*

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Infection with human herpesvirus-8 (HHV-8) has been so far implicated in the development of Kaposis's sarcoma, primary effusion lymphoma and the multicentric Castleman's disease of plasma cell type. Whether HHV-8 primary infection and/or reactivation may be linked to a non-neoplastic illness has yet to be determined. We describe the early occurrence of active HHV-8 infection (cell-free serum viremia), two months after autologous peripheral blood stem cell (PBSC) transplantation in one HHV-8 seropositive patient with non-Hodgkin's lymphoma. HHV-8 reactivation (viremia) was associated with the development of fever and bone marrow (BM) aplasia with plasmacytosis, in the absence of concomitant bacterial, fungal or other viral infections and without the occurrence of Kaposi's sarcoma. HHV-8 variant was classified as variant "A", based on the orf-K1 gene sequence. The expression of HHV-8 T0.7 transcript by *in situ* hybridization and latent nuclear antigen (LANA) by immunohistochemistry was documented in immature bone marrow progenitors in the aplastic BM but not in two other normal BM samples, previously collected from the same patient, before transplantation. We also describe the simultaneous occurrence of a serologically confirmed HHV-8 primary infection in two renal recipients, who had received twin grafts from the same cadaver donor. Sequencing analysis showed 100% identity of the hypervariable orf-K1 gene (variant "C") amplified in the donor (peripheral blood cells) and in the two recipients (cell-free serum). After 4 months, disseminated KS occurred in one, while fever, splenomegaly, and BM aplasia with plasmacytosis occurred in the other patient. The expression of HHV-8 LANA was documented in immature BM cells in the aplastic BM. Then, to evaluate the impact of HHV-8 infection on marrow cell differentiation and proliferation, normal bone marrow mononuclear cells were infected *in vitro* with the HHV-8 isolate we obtained after stimulation with tetradecanoyl phorbol acetate (TPA) of the BCBL-1 PEL cell line. In a methylcellulose-based colony formation assay the outgrowth of colony-forming units of granulocytic and macrophage lineages was decreased by about 40%, while the erythroid burst forming units were reduced by about 30%, at least with this clinical HHV-8 isolate used. Among herpesviruses, Epstein-Barr virus has been implicated in aplastic anemia and human cytomegalovirus as well as human herpesvirus 6 have been reported to be mainly responsible for delayed platelet engraftment in transplant patients. This study shows, for the first time, that HHV-8 may infect BM progenitors and may exert a myelosuppressive effect *in vitro*. HHV-8 is thus implicated as a novel causal agent of bone marrow failure, at least in the transplantation setting.

086
REAL TIME AND COMPETITIVE QUANTIFICATION OF BCR-ABL TRANSCRIPTS IN CML PATIENTS IN COMPLETE CYTOGENETIC REMISSION AFTER INTERFERON-ALPHA BASED THERAPY

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We measured by a competitive-quantitative polymerase chain reaction - capillary electrophoresis (PCR-CE) based assay, the levels of bcr-abl transcripts in 44 patients with chronic myeloid leukemia (CML) after interferon-alpha (IFN-α) therapy, who achieved a major (10 patients, MCR group) or complete (34 patients, CCR group) cytogenetic response. All 34 CCR patients had molecular evidence of residual disease detected on bone marrow samples at the time of best karyotypic response. The median number of bcr-abl transcripts of 34 evaluable patients of the CCR group at the time of complete cytogenetic remission was 4/μg RNA (range 3-4,600), while the median number of bcr-abl transcripts of 10 patients of MCR group at the time of best cytogenetic response was 4,490/μg RNA (range 600-23,900) (p = 0.000024).[6-7] In nine CCR and five MCR patients we were able to quantify the amount of bcr-abl transcript both at diagnosis and after interferon therapy: no statistical difference (p = 0.18) was found between the two groups at diagnosis (bcr-abl transcripts/μg RNA median value was 30,000 vs. 39,650, respectively). During IFN therapy, the two groups were evaluable at the time of major karyotypic conversion: at this point, there was a statistical difference of expression of bcr-abl transcript between the CCR group (17 patients) (median 2,700; range 76-40,000) and the MCR group (10 patients) (median 4,490; range 600-23,900), respectively (p = 0.046). No differences of bcr-abl amount of transcript were found in patients with CCR obtained either by IFN thera-

py alone (20 patients) vs. IFN plus autologous bone marrow transplantation (ABMT) (13 patients) ($p = 0.47$). We firstly demonstrated that although the CCR and MCR groups were clinically, cytogenetically and molecularly indistinguishable at diagnosis time, the two groups could be successfully recognized during interferon therapy based on the level of bcr-abl amount of transcript.

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087
ENGAGEMENT OF THE LEUKOCYTE-ASSOCIATED IG-LIKE RECEPTOR-1 PREVENTS PROLIFERATION AND INDUCES APOPTOSIS IN HUMAN ACUTE MYELOID LEUKEMIAS

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The leukocyte-associated Ig-like receptor-1(LAIR-1), previously termed p40, has been cloned and assigned to the immunoglobulin family.[1,2] LAIR-1 molecule is expressed on the majority of peripheral blood leukocytes, including cells of myelomonocytic origin. It inhibits the proliferation of T-lymphocytes in response to T-cell receptor-mediated signaling and of natural killer cells in response to interleukin-2.[3,4] Recently, we have reported that the engagement of LAIR-1 on CD34+ peripheral blood precursors interferes with their differentiation to monocytes and dendritic cells induced by granulocyte-monocyte colony-stimulating factor (GM-CSF).[5] Herein we provide evidence that oligomerization of LAIR-1 molecule, achieved with a specific monoclonal antibody, at the surface of acute myeloid leukemia cells, obtained from either peripheral blood or bone marrow, prevents proliferation in response to GM-CSF and leads to apoptosis. We analyzed 8 patients with the following diagnosis according to the FAB classification: 1/M0, 1/M1-M2, 1/M2, 2/M4, 1/M5, 1/M5b. Apoptosis is a form of programmed cell death (PCD) characterized by reduction of cell size, DNA fragmentation and by defined ultrastructural changes.[6] In our system, PCD was evident both by propidium iodide staining (DNA damage) and by electron microscopy (nuclear blebbing). Cells undergo PCD either during ontogeny, in which PCD is a way of remodeling tissues, or in pathologic conditions, such as viral infections and neoplasias, in which apoptosis avoids or delays the release of noxious contents from dying cells.[6] PCD is generally mediated by the activation of a series of pro-enzymes, the caspases, responsable for the cleavage of critical cellular substrates, leading to the above mentioned changes and cell death.[7,8] Interestingly, PCD elicited via LAIR-1 was not blocked by different caspase inhibitors, at variance with apoptosis induced via CD95/Fas, which was prevented by the caspase-1 and caspase-8 specific inhibitors. In addition, we found that the p65 subunit of the nuclear factor k-B (NF-kB), constitutively expressed in the nucleus of three myelomonocytic cell lines, was retained in the cytoplasm upon LAIR-1 engagement. As blocking of NF-kB activation has been shown to rescue sensitivity to anti-cancer drugs in solid tumors,[9] we suggest that LAIR-1 represent a possible target for pharmacologic approaches aimed at potentiating anti-leukemic therapy

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088
MOLECULAR ANALYSIS OF CHIMERISM AFTER ALLOGENEIC STEM CELL TRANSPLANTATION FOR HEMATOLOGIC MALIGNANCIES AS PREDICTIVE ASSAY FOR RELAPSE

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Disease relapse remains a major cause of failure after transplantation and may be due either to resistance of neoplastic cells to the conditioning regimen or to the inability of the immune system to mount an effective immune response against the tumor. Due to the variable balance between donor and host in the transplantation chimera, the detection of host-type hematopoiesis (mixed chimerism, MC) after transplantation could be of help in the recognition of patients at high risk of relapse. We studied the incidence of MC after peripheral blood stem cell (PBSC) or bone marrow (BM) transplantation by polymerase chain reaction (PCR) amplification of DNA hypervariable regions (mini and microsatellite). Sixty-six patients, with different hematologic malignancies (AML, ALL, MM, NHL, CML, CLL) were evaluated for chimerism status. Forty-two patients received PBSC, all from matched family donors, and 24 had BM transplantation either from related or unrelated matched donors. The chimeric status was assessed in serial samples at six-month intervals starting one month post-grafting. Our data show that the detection of full donor chimerism (FC) may be predictive of low relapse probability . In fact, patients who achieve a stable FC status show <5% probability of disease recurrence, while those with MC have a 50% probability of relapse at 48 months (p=0.0001). When the patients were stratified according to risk of relapse (high risk, N=47; low risk, N=19), the probability of having MC was 10% for the low risk group and 45% for the high risk (p=0.02). Moreover we found that mixed chimerism is associated with a lower incidence of acute and chronic GVHD (p=0.018). We conclude that patients showing persistent complete donor hematopoiesis have a low risk of relapse while the assessment of mixed chimerism identifies patients headed to relapse.

089
COMPOUND HETEROZYGOSITY FOR TWO DIFFERENT AMINO ACID SUBSTITUTION MUTATIONS IN THE THROMBOPOIETIN RECEPTOR (C-MPL GENE) IN CONGENITAL AMEGAKARYOCYTIC THROMBOCYTOPENIA

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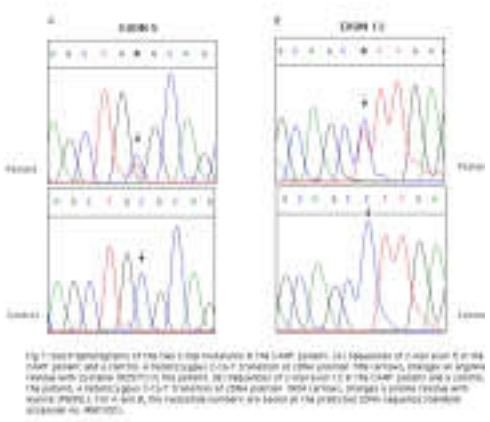
Congenital amegakaryocytic thrombocytopenia (CAMT) without physical anomalies is a rare disease presenting with isolated thrombocytopenia and megakaryocytopenia without serum thrombopoietin (TPO) deficit. The genetic transmission is autosomal recessive or X-linked. It should be considered as a myelodysplastic syndrome as almost half of the cases have the tendency to evolve into aplastic anemia. It could also be considered a preleukemic syndrome in view of its tendency to evolve into acute leukemia (Freedman *et al. 1990;* Alter *et al. 1998*). Recently, two heterozygous truncating mutations of the thrombopoietin receptor MPL, coded for by the c-mpl gene, were identified in a 10-year old Japanese patient with CAMT transmitted in an autosomal recessive manner. DNA sequence analysis showed a C-to-T transition at the cDNA nucleotide position 556 (Q186X) on exon 4 and a single nucleotide deletion of thymine at position 1,499 (1,499 delT) on exon 10 (Ihara *et al. 1999*). Both mutations were predicted to translate a prematurely terminated MPL protein lacking all intracellular domains essential for signal transduction. In a 2-year-old Italian child with CAMT transmitted in an autosomal recessive manner, the sequencing analysis of the full length coding sequence, the exon/intron junctions and a portion of the c-mpl promoter revealed 2 different point mutations. These are the first two MPL amino acids substituting mutations identified in human disorders. C-to-T transitions were detected on exons 5 and 12 at the 769 and 1,904 cDNA nucleotide positions, respectively. The mutation in exon 5 substitutes an arginine with a cysteine (R257C) in the extracellular domain, 11 amino acids distant from the WSXWS motif conserved in the cytokine receptor superfamily.



The mutation in exon 12 substitutes a proline with a leucine (P635L) in the last amino acid of the c-terminal intracellular domain, responsible for signal transduction. As in the Japanese family, the mutations were both transmitted from the parents. TPO plasma levels were greatly increased in the patient. The patient's 7-year old brother, who was a candidate donor for allografting, turned out to be an asymptomatic heterozygous carrier of P635L, and showed defective megakaryocyte colony formation from bone marrow progenitor cells. This observation suggests the need for adequate screening of apparently healthy sibling bone marrow donors. The present study also provides important confirmation that CAMT can be associated with c-mpl mutations.

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TREATMENT OF ACUTE MYELOGENOUS LEUKEMIA IN THE ELDERLY WITH DAUNOXOME PLUS ARA-C "3+7" SCHEDULE: THE GIMEMA EXPERIENCE

Vignetti M, Tafuri A, Fazi P, Amadori S, Camera S, Ferrara F, Fioritoni G, Mariani G, Pagano L, Piccaluga P, Petti MC, Russo D, Specchia G, Zaccaria A, Baccarani M, Mandelli F

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Since November 1998, some GIMEMA centers have started to utilize daunoxome (DnX) (Gilead/NexStar) for the treatment of acute myeloid leukemia (AML) patients aged more than 60 on a compassionate basis. This liposomal daunorubicin is associated with Ara-C in the classic "3 + 7" schedule: patients receive an induction treatment consisting of DnX 80 mg/m^2/day for 3 days and Ara-C 100 mg/m^2/day on days 1 to 7. Patients achieving complete remission (CR) are submitted to a consolidation course with the same drugs at the same dosages; patients in partial remission (PR) receive a second induction cycle, followed by the consolidation course for those achieving CR, and patients fully resistant after the first induction cycle receive palliative treatments only. The rationale for this program was mainly based on the following: (1) frequent overexpression of the multidrug resistance gene (MDR) product, the P-glycoprotein (P-gp), may contribute to the higher rate of treatment failure in elderly AML patients; (2) a higher tumor cell drug delivery , with improved pharmacokinetic and therapeutic indices, also resulting in a reduced toxicity profile, has been reported for liposomal daunorubicin. A total of 59 patients have so far been treated in 13 different centers, with 29 CR reported (55%) out of 53 evaluable cases, 4 cases being too early and 2 not evaluable for response. Five patients died during induction and 19 (36%) were resistant. Median age at diagnosis was 68 years (ranging from 61 to 78), with a median WBC count of 12.3 x 10^9/L (ranging from 0.4 to 450). FAB subtypes were M0 in 1 case, M1 in 15, M2 in 24, M4 in 9, M5 in 8 and M6 in 2. The hematological toxicity was acceptable, with a median time of 22 days (min 13, max 33) to achieve a PMN count >0.5 x 10E^9/L and 21 days for platelet count > 20 x 10E^9/L. The extra-hematopoietic toxicity consisted mainly of infections. Grade 4 WHO toxicities were cardiac arrhythmia (1 case), liver (1) and renal (2) function abnormalities, sepsis (3), pneumonitis (1). To date, 17 patients are in continuous CR after a median of 7 months (range 2 - 15), 11 have relapsed after a median of 4 months and 1 died in CR. The relatively high proportion of resistant cases (19 out of 53, 36%) prompted us to increase the dose of DnX to 320 mg/m^2 total dose, divided in 4 days and administered over 6 hours. So far the number of cases treated with this second schedule is too low to draw conclusions about its activity, but the overall results obtained with these DnX-based therapies seem to suggest that they a feasible approach in elderly patients with AML and further prospective studies should be planned.

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MOBILIZATION OF TYPE 1 AND TYPE 2 DENDRITIC CELLS IN NORMAL DONORS OF PERIPHERAL BLOOD STEM CELLS

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Dendritic cells type 2 (DC2) are able to induce naive T-cells to produce high levels of Th2-type cytokines, such as interleukin-4 (IL4) and IL10, which have been shown, in animal models, to protect against acute GVHD. We have recently shown that, after mobilization with G-CSF, the peripheral blood of healthy peripheral blood stem cell (PBSC) donors contained increased counts of circulating DC2, while counts of dendritic cells type 1 (DC1) were unchanged. It has therefore been hypothesized that DC2 in PBSC grafts might contribute to GVHD prevention by inducing donor T-cells to differentiate toward Th2 effector cells. We have devised a flow cytometric assay to identify and enumerate DC2/DC1 in peripheral blood and apheresis products obtained from normal donors of G-CSF mobilized PBSC. DC were initially gated as negative for cell specific lineage markers (CD3, CD14, CD16, CD19, CD20, CD34, CD56) (lin-) and positive for HLA-DR. DC1 and DC2 were then identified according to the expression of the adhesion molecule CD11c, which is positive on DC1, and of the alpha chain of the IL3 receptor (CD123), which is brightly expressed on DC2. Before G-CSF treatment median counts of DC2 and DC1 in peripheral blood of 13 normal donors were 6.75x10^6/L (range=1.84-16.9) and 12.2x10^6/L (range=4.95-20.5), respectively. On day 4 of G-CSF treatment median DC2 counts increased to 35.2x10^6/L(range=48.3-74.5) (p<0.01), while median DC1 counts were not significantly different (15.98x10^6/L, range=4.95-31.3). Mobilization of DC2 did not correlate with CD34+ cells nor with monocytes mobilization. Finally median counts of DC2 and DC1 in 14 PBSC harvests were 3.22x10^6/Kg of recipient's body weight (range=1.13-5.16) and 2.45x10^6/Kg (range 1.3-3.99), respectively. Two groups of donors were identified based only upon high or low numbers of DC2 (>3.2x10^6/Kg) vs <3.2x10^6/Kg) in the apheresis product, since they had comparable values of DC1, CD34+, CD14+, CD19+, CD3+, CD4+ and CD8+ cells. This observation will allow us to address the hypothesis whether DC2 may correlate with the immune recovery and/or clinical outcome of patients receiving an allogeneic PBSC transplant.

092
A NEW EFFECTIVE PURGING TECHNIQUE FOR AUTOLOGOUS TRANSPLANTATION IN MULTIPLE MYELOMA

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We recently described a two-step negative selection procedure whereby G-CSF mobilized peripheral blood stem cells (PBSC) used for autologous transplantation in multiple myeloma (MM), can be efficiently purged of contaminating neoplastic cells by anti CD19, CD56, and CD138 magnetic microbeads (Rambaldi *et al.*, Blood 1998). Fourty-five newly diagnosed MM patients received 3 cycles VAD followed by cyclophosphamide (CTX, 7 g/sm^2) + G-CSF (5 µg/kg/day) for stem cell collection. Thereafter they were randomized to receive autologous unmanipulated CPC (Arm A, 21 patients) versus highly purified plasma cell-purged (Arm B, 24 patients) to support tandem sequential transplants (TRX) conditioned with melphalan (200 mg/sm^2) and Melphalan (140 mg/sm^2) plus total body irradiation (TBI, 1,200 cGy) for the second transplant. Aims of this study were to evaluate the efficacy of *in vitro* purging on the neoplastic plasma cell fraction and the quality of the hematopoietic and lymphoid reconstitution after transplantation. The level of minimal residual disease (MRD) contamination of PBSC, before and after purging, was evaluated by qualitative PCR analysis performed with consensus oligonucleotide primers for the CDR3 region of rearranged heavy chain alleles and, in some patients, also by quantitative "real time" PCR. This approach allowed us to demonstrate that in all cases the unmanipulated aphereses contained a heavy plasma cell contamination as opposed to the purified stem cells obtained after *in vitro* purging which showed a remarkable (> three logs) reduction of the neoplastic cells. Two aphereses were sufficient to meet the minimum criteria of 5 x 10^6 CD34+ cells/kg to support each transplant and to have a back-up source of unmanipulated cells. The hematologic engraftment was rapid, time to neutrophils > 1,000 and to platelets > 50,000 was respectively +9 and +13, not different in the two arms. The immunologic reconstitution (as determined by enumeration of T-, B- and NK- cells) was comparable in both arms and no transplant-related mortality has been so far. These results suggest the lack of any significant hematologic or immunologic toxicity associated with transplantation of plasma cell-purged CPC. The evaluation of the clinical benefit of this procedure is still ongoing and requires an adequate period of follow-up.

093
REDUCED DIHYDROFOLATE REDUCTASE ACTIVITY IN THE ERYTHROBLASTS OF THE 5Q- SYNDROME

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The 5q- syndrome is a particular type of myelodysplastic syndrome (MDS) characterized by isolated interstitial deletion of the long arm of chromosome 5.[1-3] A striking number of genes encoding hematopoietic growth factors and growth factor receptors and also the functional human dihydrofolate reductase (DHFR) gene (region q11-q13) have been mapped on the long arm of chromosome 5.[4,5] We studied DHFR activity in bone marrow erythroblasts of patients with the 5q- syndrome to see whether there are differences in DHFR expression in comparison with normal controls and patients with other types of MDS. We carried out the cytochemical reaction on bone marrow imprints of 10 normal controls, 8 patients with MDS classified as having refractory anemia (RA) (male/female=5/3; medium age=48; range 30-58 years) and 4 patients with the 5q- syndrome (males/females=2/2; medium age=67; range 61-72 years) at the onset, not previously treated. Two cases had del(5) (q13q33); 1 case del(5) (q13q31), while in the other the breakpoint was not identified. Employing a Vickers M86 scanning and integrating microdensitometer the optical density (OD) of 100 erythroblasts for each normal control and patient was counted. A very weak perinuclear pattern of positivity was observed in the cytoplasm of the 5q- erythroblasts, whereas in normal and RA erythroblasts the intensity of the reaction was stronger. In pathologic erythroblasts of RA patients the OD was significantly higher (mean 97.3±4.4) than in normal erythroblasts (mean 75.9±3), whereas in the cases with the 5q- a significant decrease in enzyme activity (mean 45.7±1.7) was observed. These differences were independent from other biological parameters, i.e. the maturation stages of the erythroblasts, the progenitor growth *in vitro* and the apoptosis rate, as measured by TUNEL technique. Also the hypolobulate megakaryocytes observed in the bone marrow imprints from patients with 5q- syndrome showed a moderately lower DHFR level than the same normal cells. At present the cause of this enzyme reduction is not known; further studies are needed to determine the possible association between gene deletion and enzyme decrease. In conclusion, for the first time, reduced DHFR expression has been demonstrated in 5q- erythroblasts. This enzymatic abnormality could have an important role in the pathogenesis of the disease.

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IMMUNOTHERAPY POST-AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANT WITH INTERLEUKIN-2 PLUS LAK CELLS IN PATIENTS WITH HIGH RISK LEUKEMIA

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In patients with high risk leukemia autologous peripheral blood progenitor cell transplantation (PBPCT) is often followed by relapse and in chronic myeloid leukemia (CML) this approach can only prolong the survival without eradicating the Ph+ clone. In the early phase after PBPCT there is an important, although transient, peak of natural killer cells with increased cytotoxic activity; the use of interleukin-2 (IL-2) has been proposed in the early phase after trans-

plantation but the planned dose often has to be reduced because of poor hematologic tolerance. We report our preliminary experience of immunotherapy in 6 patients autotransplanted for high risk leukemia. Two patients were affected by acute leukemia (AL), one by Ph+ ALL, one by ANLL with bulky abdominal disease; 4 were affected by CML, 2 of them in accelerated phase and 2 with advanced chronic phase (after failure of interferon ± Ara-c); only one patient had no evaluable karyotypic markers before starting immunotherapy, but there was a residual measurable abdominal mass. The leukaphereses contained a median of 10% Ph+ cells (range 2%-100%), in the 5 cytogenetically evaluable patients after autoPBSCT. Four out of six patients achieved hematologic complete remission (HCR); two patients with CML in acute phase did not reached HCR, 1 patient with advanced chronic phase (ACP) CML obtained a transient karyotypic response and the other one did not achieve any karyotypic response after transplant. The patient with Ph+ ALL had 2.6% Ph+ cells in bone marrow, while the patient with ANLL, had an abdominal residual mass after PBSCT. Immunotherapy was started a median of 60 days post transplant (range 30-120) and consisted in administration of 4.5 MIU/4 days/week s.c. of IL-2 and 150 µg s.c. of GM-CSF, added on the first day/week. First LAK infusion was started on day +120 (median) (range 90-150). A median of 5 LAK reinfusions (range 2-9) was given to each patient, by aphereses containing $3.9x10^8$/Kg MNC (median). Cell suspensions before the reinfusion were volume-reduced by centrifugation, transferred to Lifecell culture bags and incubated at concentration of $1x10^7$ cells/mL for 24 hours in RPMI 1640, 10% ACD, 20% autologous plasma and human recombinant IL-2 at a concentration of 1000 IU/mL (Fig.1). Immunotherapy was performed on an outpatient basis; the only noteworthy side effects were fever and chills at the end of LAK reinfusion. No major organ toxicities were observed and only two patients showed moderate depression not requiring specific treatement. The main biological effects of immunotherapy consisted *in vivo* of a median 5 fold increase of NK activity, and a median 1.6 fold increase of LAK activity in peripheral blood and leukaphereses. To date all 6 patients are alive, with a median follow-up after diagnosis of 33 months (range 18-57), 2 of them (CML) stopped the immunotherapy after 3 months because of disease progression and required alternative treatment. The other two CML patients are still at high risk with minor karyotypic response, while the two patients with AL are in CCR; the patient with ANLL showed complete disappearance of the abdominal mass, while the patient with ALL had a reduction of Ph+ metaphases to 0%. In this preliminary experience this approach seems the feasible, with low toxic side effects and to be promising in terms of activity against very high risk AL or CML.

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PCR-BASED PRE-EMPTIVE THERAPY WITH CIDOFOVIR FOR CYTOMEGALOVIRUS REACTIVATION IN ALLOGENEIC STEM CELL TRANSPLANT RECIPIENTS: A PRELIMINARY SINGLE CENTER EXPERIENCE

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Cytomegalovirus (CMV) infections remain the most frequent infectious complications after allogeneic stem cell transplantation, in particular for patients receiving mismatched or unrelated transplants. In seropositive bone marrow transplantation(BMT) recipients, reactivation of latent CMV occurs in 42-78% of the cases, irrespective of donor serology. Pre-emptive therapy at the time of viral detection has been shown to notably reduce risk of progression to CMV disease. Ganciclovir has marked myelosuppressive activity and is therefore difficult to use early after BMT during engraftment; foscarnet is not myelosuppressive but has been reported to possess significant renal toxicity. Cidofovir (CDV) is a nucleoside analog with proven *in vitro* effects against CMV, wich has been successfully used in AIDS patients with CMV retinitis. We performed a prospective study to evaluate the efficacy and toxicity of CDV employed as PCR-based pre-emptive therapy in patients at high-risk for CMV infection submitted to allogeneic stem cell transplantation in our Unit. Nine patients with hematologic malignancies (acute myeloid leukemia n=5, chronic myeloid leukemia n=3, multiple myeloma n=1) undergoing allogeneic stem cell transplantation from an HLA-identical sibling donor (n = 3), a matched unrelated donor (n = 5) and from partially-matched (4/6) cord blood (n=1) entered the study. A conditioning regimen consisting of total body irradiation (1,320 cGy) and chemotherapy was employed in patients submitted to unrelated transplant (n=6) whereas the other patients (n=3) were given myeloablative chemotherapy. All patients received prophylactic iv acyclovir (500 mg/m^2 every 8h from day –5 to +30) and weekly intravenous immunoglobulin 400 mg/kg body weight from day –4 to day +30. Patients were monitored for CMV infection twice weekly between days 15 and 30 and then weekly by pp65 antigen assay and qualitative and quantitative blood/plasma CMV-PCR. The indication for treatment was positive qualitative and quantitative CMV-PCR. CDV was administered at a dosage of 5 mg/Kg once every week for two weeks followed in most cases by maintenance therapy (3mg/Kg) every two weeks. All patients received probenecid and hydration and their plasma level of creatinine and proteinuria were monitored daily. Success of treatment was defined as negative qualitative and quantitative CMV-PCR. The median time of onset of CMV infection was 62 days (range 42-97). In all cases the reactivation of CMV was associated with pancytopenia and in 4 cases it was concomitant with aGVHD occurrence. Clearance of viremia occurred in 5 patients (55%); only one patient received more than two weeks of therapy. Patients who not respond to CDV were treated with ganciclovir or an asscociation of ganciclovir/foscavir. Despite the change of therapy, none of these became CMV-PCR negative: one patient died of interstitial pneumonia and the remaining three patients were submitted to maintenance therapy. No patient developed signs of renal toxicity (defined as >1.5 x increase in serum creatinine or development of proteinuria). In our experience Cidofovir as first-line preemptive therapy was effective in 55% of cases as reported by others; no renal toxicity was observed and the administration protocol was feasible also in an outpatient setting. Moreover we suggest shifting to a conventional approach with ganciclovir/foscavir in case of persistence of CMV-PCR positivity after the second week of CDV administration.

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AUTOLOGOUS STEM CELL TRANSPLANTATION IN LYMPHOMA PATIENTS AFTER HEPATITIS B REACTIVATION: THE ROLE OF LAMIVUDINE

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In the recent past high-dose chemotherapy (CHT) followed by autologous stem cell transplantation (ASCT) has been increasingly used as consolidation treatment for high-risk non-Hodgkin's lymphomas (NHL). Among chronic carriers of hepatitis B virus receiving CHT for NHL, a reactivation of virus replication has often been observed and this may give rise to hepatitis, hepatic failure and death, and may prevent from performing further therapy including trasplantation procedures, from being performed. Herein we present our experience in four patients with NHL in whom, a hepatitis flare-up was observed after 2 (in three patients) and 6 (in one patient) cycles of standard-dose chemotherapy. The patients were affected by mantle cell, follicle center grade III, peripheral T-cell unspecified and diffuse large B-cell, respectively, were male, aged 39, 47, 52 and 62 years and were HCV and HIV negative. In all patients, pretreatment HBV serology was as follow: HbsAg positive, HbeAg negative, total anti-c Ab positive, IgM anti-c negative, anti-e Ab positive and anti-s Ab negative. Three of them were treated with the third generation F-MACHOP regimen (Infanti *et al., 1996*) whereas the oldest patient was treated with the classic CHOP regimen. HBV-DNA was negative before starting chemotherapy and became positive in all four patients at a median of 26 (19-34) days after the previous cycle of chemotherapy. After spontaneous recovery the patients were treated with lamivudine (Zeffix, Glaxo-Wellcome, 100 mg once daily) and this allowed the chemotherapy program to be resumed and completed without another reactivation of hepatitis B. In two patients high-dose chemotherapy and autologous stem cell tranplantation was also performed under lamivudine as a part of our program for high-risk NHL. The hematologic recovery after chemotherapy overlapped with that of other patients with NHL submitted to ASCT. Antiviral treatment was stopped 4 to 6 months after the last chemotherapygiven. During the follow-up period they were monitored with twice-monthly blood counts, transaminase levels and HBV-DNA: all these parameters remained normal/negative all throughout the period. Currently, patient B.S. is in complete remission (CR) 19 months from diagnosis and 13 months from the end of chemotherapy; patient D.F.A. is in CR 22 months from diagnosis and 12 months from the end of chemotherapy, patient V.E. is in CR 34 months from diagnosis and 26 months from trasplantation; patient C.G. is in CR 23 months after diagnosis and 6 months after transplantation. These data suggest a possible role for lamivudine in preventing hepatitis B reactivation during administration of chemotherapy to chronic carriers of hepatitis B virus. Moreover lamivudine enables the completion of both standard and hig-dose chemotherapy with autologous transplantation in patients with previous hepatitis B reactivation.

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SELF-RENEWAL POTENTIAL OF PURIFIED MURINE HEMATOPOIETIC STEM CELLS PURIFIED ACCORDING TO THE SCA1+ OR THE 15.1.1- PHENOTYPE IN NON-ABLATED W/WV MICE

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The biological properties of two marrow cell populations containing purified murine hematopoietic stem cells (HSC) (light density, wheat germ agglutinin (WGA)+ bone marrow cells further divided into either 15.1.1- or Sca1+ cells) were compared. Consistent with the hypothesis that Sca1+ cells contain more HSC than 15.1.1- cells, Sca1+ cells were less frequent in the bone marrow (0.05 % vs 0.1%, respectively) and engrafted more efficiently and at later time points non-ablated W/Wv mice (125 Sca1+ vs 1500 15.1.1-cells induced donor hematopoiesis in 100% of the host mice. In mice transplanted with 15-1.1- cells, donor cells were detected as early as at 1.5 months after the transplant and all of them were engrafted by 6 months. In contrast, some of the recipients of Sca.1+ cells did not express donor hematopoiesis until 6-12 months after the transplant). Other data, however, suggested that 15.1.1- cells are more immature than Sca1+ cells. In fact, Sca1+ cells are metabolically/cycling more active than 15.1.1- cells (rhodamine exclusion enrich for cells with engrafting potential the 15.1.1-, but not the Sca1+ fraction) and express detectable levels of differentiation-associated genes such as Gata1 and EpoR by RT-PCR). Furthermore, 15.1.1- cells generate more HSC *in vivo* than Sca1+ cells (10 million TBM of primary 15.1.1- recipients engraft 80% of the secondary transplanted animals vs only 12% of the animals engrafted when transplanted with similar TBM doses from primary Sca1+ recipients). To clarify the relationship between 15-1.1- and Sca1= cells, 15-1.1-/+ cells were further divided according to the Sca phenotype. Although all the Sca1+ cells were found to be 15.1.1-, 15.1.1- cells contained both Sca1+ and Sca1- cells (in a 1:10 ratio). However, all the engraftment activity was contained in the Sca1+ fraction of the 15-1.1- cells, confirming that Sca1+ cells are the cells that engraft the primary recipients. However, none of the 35 animals transplanted with the bone marrow from 7 different W/Wv mice engrafted with the 15.1.1- Sca1+ cells expressed C57BL hematopoiesis, confirming that Sca1+ cells are not able to generate HSC *in vivo*. These data suggest that the 15.1.1- fraction contain cell precursors capable of generating HSC *in vivo* but that are not Sca1+ and do not engraft primary animals.

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EVALUATION OF MINIMAL RESIDUAL DISEASE IN INV(16) POSITIVE ACUTE MYELOID LEUKEMIA BY QUALITATIVE AND QUANTITATIVE RT-PCR ASSAYS OF CBFß/MYH11 FUSION TRANSCRIPTS

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The pericentric inversion of inv(16)(p13q22), closely associated with FAB M4Eo acute myeloid leukemia (AML) subtype, is present in about 10% of adult AMLs and results in the transcription of a specific CBFβ/MYH11 fusion mRNA. RT-PCR for CBFβ/MYH11 transcript can be used for diagnostic purposes as well as for the detection of minimal residual disease (MRD) after conventional or transplantation treatments. Qualitative RT-PCR studies of MRD have so far produced conflicting results with some patients in long-term clinical remission being persistently positive; thus, qualitative RT-PCR seems of limited prognostic value in a consistent proportion of cases. We have retrospectively evaluated MRD in a large series (35 patients) of CBFβ/MYH11 positive AMLs employing both qualitative and quantitative (REAL TIME RT-PCR) approaches. In qualitative studies, carried out by *nested* RT-PCR assay, sequential bone marrow samples were positive in all patients in complete remission (CR) after induction and consolidation therapy; however, follow-up samples were found to be persistently negative in patients remaining in continuous CR (CCR) for more than 12 months; in contrast, the detection of CBFβ/MYH11 transcript after 1 year of CR was strongly associated with impending relapse. Thirteen patients were sequentially evaluated by quantitative REAL-TIME RT-PCR assay: the CBFβ/MYH11 transcript copy number was calculated as the normalized value of CBFβ/MYH11 transcript per the copy number of control transcript, ABL mRNA. A 2-3 log decline of fusion transcript copy number was observed after induction/consolidation therapy with a lower level observed in patients treated with high-dose ARA-C regimen; more important, after achieving CR, the mean copy number was lower in patients remaining in CCR compared to patients who subsequently relapsed. Moreover, in CCR patients the copy number fell under the detection threshold soon after the treatment protocol was completed and remained undectable over time, thus overlapping the results obtained with qualitative RT-PCR. In the seven patients who relapsed the copy number found in CR never declined under the detection threshold; at relapse, transcript levels were roughly equivalent to those observed at diagnosis. Two major conclusions can be drawn from the above results: i) patients remaining in CCR are persistently negative by RT-PCR assays in follow-up samples obtained 1 year after achieving CR and afterwards; ii) higher levels of CBFβ/MYH11 transcripts are present in CR samples from patients who subsequently relapse than from patients in CCR. This finding, if confirmed in a larger series of patients in a prospective study, should confer an important predictive value to quantitative PCR determinations of MRD in CR patients with inv(16) leukemia.

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CLONAL EVOLUTION OF BCR-ABL-TRANSDUCED HEMATOPOIETIC PROGENITORS IS CORRELATED WITH LEVEL OF BCR-ABL EXPRESSION

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The expression of bcr-abl chimeric gene, the molecular marker of chronic myeloid leukemia (CML) is known to deliver a strong proliferation signal that, in turn, permits the illegitimate expansion of clonal hematopoiesis over its normal counterpart. However, the steps involved in the progression of CML progenitors toward a fully transformed phenotype are still poorly understood. We investigated bcr-abl gene amplification as a pathway responsible for the escape of bcr-abl-transduced hematopoietic progenitors from cell proliferation regulatory controls. To this purpose, we sequentially measured bcr-abl expression levels in a 32D cell line transducing a temperature-sensitive p210 bcr-abl construct which retains the abl tyrosine kinase activity only at the permissive temperature of 33°C. To quantify the levels of bcr-abl transcript we developed a competitive PCR strategy fulfilling the requirements of sensitivity (it detects transcription levels of $1x10^6$ molecules/mg total RNA magnitude) and reproducibility (the standard error is repeatedly less than 10%). In the first 10 days following transfection and selection in G418-additioned medium, bcr-abl-transduced 32D cells showed a rapid increase of bcr-abl transcript approaching $5x10^8$ molecules/mg total RNA, and only at that point developed the factor-independence. In 5-8 weeks following the cloning, IL-3-independent bcr-abl-transduced 32D cells further increased bcr-abl expression levels up to approximately $1x10^9$ molecules/mg total RNA and developed resistance to radiation-induced apoptotic death. Our results are consistent with a role for the increase of bcr-abl transcription rate in clonal selection and evolution of bcr-abl-transduced cells and support our effort to build up a prognostic classification of CML patients on that basis.

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FUNCTIONAL ACTIVITY AND ONCOGENIC POTENTIAL OF FGFR3 MUTANTS OVEREXPRESSED IN MULTIPLE MYELOMA CELL LINES WITH T(4;14)

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The t(4;14)(*p*16.3;q32) chromosomal translocation is associated with approximately 20% of multiple myelomas (MM) and results in the deregulation of the FGFR3 and WHSC1/MMSET genes. FGFR3 codes for a tyrosine-kinase receptor involved in signaling transduction regulating cell growth and differentiation. Interestingly, FGFR3 point mutations known to be associated with autosomal dominant human

skeletal disorders have been found in some MM cell lines with the t(4;14); however, their pathogenetic role in MM is still controversial since they occur rarely in primary tumors, as we have previously reported. In the present study, we investigated the functional activity and oncogenic potential of FGFR3 mutants in MM cell lines KMS11 (Y373C), OPM2 (K650E), and the recently established KMS18, in which we identified the novel mutation G382D. This mutation occurs within the transmembrane domain in a highly conserved pentameric sequence thought to play an important role in receptor dimerization. FGFR3 is overexpressed, albeit at different levels, in the three cell lines; notably, the mutated FGFR3 allele is expressed in all of them almost exclusively. We demonstrated in serum-deprived cells, in the absence or presence of suramine (a competitor of FGFs), that KMS11 and OPM2 cell lines express phosphorylated FGFR3 at appreciable levels, indicating a constitutive activation of the mutated receptors. However, addition of a FGF ligand to serum-deprived KMS11 and OPM2 cells increased the level of receptor phosphorylation. Conversely, G382D in KMS18 appears not to be an activating mutation since the receptor is phosphorylated only in the presence of the ligand. We also examined the transforming activity of the three FGFR3 mutants by focus formation assay in NIH3T3 cells and found that Y373C and K650E but not G382D, are able to induce transformed foci. These data provide new insights into our understanding of the tumorigenic role of FGFR3 in MM.

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AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION IN UNRESPONSIVE MULTIPLE SCLEROSIS

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Multiple Sclerosis (MS) is characterized by inflammatory lesions in the central nervous system (CNS) white matter probably due to an autoimmune reaction to myelin. Active lesions show blood brain barrier (BBB) damage, which may be demonstrated by gadolinium (GOL) enhancement at magnetic resonance imaging (MRI); the lesions then persist in the CNS after recovery of the abnormal BBB permeability. We designed a study mainly directed to investigate MRI and laboratory changes following autologous peripheral blood progenitor cell (PBPC) transplantation in patients affected by advanced, poor prognosis and refractory MS. The study was approved by both the Italian Cooperative Group for BMT (GITMO) and the local ethical comittee; at present 15 Centers have joined the study, fullfilling the accreditation criteria adopted for Neurology and Radiology Depts and BMT Units. Patients were mobilized with CTX 4 g/m^2 plus filgrastim 5 µg/Kg and conditioned with BEAM followed by rabbit ATG (Thymoglobulin IMTIX-Sangstat, 5 mg/Kg on days +1 and +2). CyA 1 mg/Kg i.v. was added during the BEAM regimen to prevent cytokine release. Ten patients from 5 Centers were transplanted; the procedure was well tolerated and no serious adverse events were reported. All patients engrafted promptly and were discharged from the BMT Unit within two weeks after BMT. A marked inversion of circulating CD4+/CD8+ ratio and a low CD19+ count were shown; 7/10 patients showed, a few months after PBPC transplantation, an improvement of 1 or 0.5 points of the EDSS scale and at least 10 points of Scripps scale, with a median follow-up of 8 months; the other 3 cases remained stable. The number of Gd enhancing lesions started to decrease after the mobilization treatment and 2-4 months after transplantation dropped to zero. These preliminary data indicate that PBPC transplantation can be effective in removing inflammatory activity in MS, as judged by MRI parameters, and could therefore become a possible therapy for severe unresponsive rapidly progressing MS. The duration of the anti-inflammatory response and the effects on the enlarging and new areas appearing on T2-weighted images are still to be clarified.

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ABERRANT METHYLATION OF DEATH-ASSOCIATED PROTEIN KINASE IN B-CELL NEOPLASIA

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B-cell neoplasias form a heterogeneous group of lymphoproliferative disorders derived from the clonal expansion of B-cells arrested at different stages of maturation. Many advances have been made in the understanding of the pathogenesis of B-cell neoplasias, and growing evidence implicates deregulation of programmed cell death (apoptosis) in the pathogenesis of these malignancies. Death-associated protein kinase (DAP-kinase) is a newly discovered calcium/calmodulin-dependent serine/threonine kinase required for apoptosis induced by interferon gamma, tumor necrosis factor-alpha and FAS. Expression of DAP-kinase in human tumors may be reduced or absent as a consequence of promoter hypermethylation, thus increasing the pro-apoptotic threshold of the tumor clone. On this basis, the aim of our study was to evaluate the involvement of DAP-kinase hypermethylation throughout the spectrum of B-cell neoplasias recognized by the WHO classification. Toward this aim, a panel of 201 B-cell tumors representative of the clinicopathologic spectrum of the disease was analyzed by methylation-specific polymerase chain reaction. Overall, DAP-kinase hypermethylation was detected in 76/179 (42%) mature B-cell neoplasms and in 3/22 (14%) precursor B-cell acute leukemias. With respect to mature B-cell neoplasia, the frequency of DAP-kinase hypermethylation varied markedly in different clinico-pathologic categories of the disease. The highest frequency of DAP-kinase hypermethylation was observed in follicular lymphoma (8/10; 80%) and in low-grade MALT-lymphoma (8/11; 73%). Conversely, among other indolent lymphoproliferative disorders, DAP-

kinase hypermethylation was consistently absent in lymphoplasmocytoid lymphoma (0/9) and was restricted to a minority of B-cell chronic lymphocytic leukemias (6/22; 27%) and hairy cell leukemias (3/11; 27%). Also, DAP-kinase was restricted to 4/18 (22%) mantle cell lymphoma. Among aggressive B-cell lymphomas, DAP-kinase hypermethylation occurred in 34/55 (62%) B-lineage diffuse large cell lymphomas (B-DLCL) and in 13/27 (48%) Burkitt's lymphomas. DAP-kinase hypermethylation occurred throughout the clinico-pathologic spectrum of B-DLCL, including systemic nodal B-DLCL (61%), extranodal B-DLCL (40%), CD5+ B-DLCL (83%), primary splenic B-DLCL (70%), CD30+ anaplastic B-DLCL (67%) and primary central nervous system lymphoma (75%). Finally, DAP-kinase hypermethylation was consistently absent in multiple myeloma and plasma cell leukemia (0/16). The implications of these data are threefold. First, inactivation of DAP-kinase by promoter hypermethylation represents a frequent lesion in B-cell malignancies and conceivably represents a major event in the development and/or progression of these diseases. Second, the frequency of DAP-kinase hypermethylation is heterogeneous among the different subsets of mature B-cell neoplasia, corroborating the existence of multiple pathogenetic pathways in these tumors. In particular, it is notable that the highest frequency of DAP-kinase hypermethylation clusters with follicular lymphoma, a tumor in which apoptosis deregulation is thought to play a predominant role. Finally, the identification of DAP-kinase hypermethylation as a mechanism of altered apoptotic control in B-cell neoplasia may provide a novel target for molecular therapy.

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CANINE CD34+LIN- HEMATOPOIETIC CELLS TRANSDUCED WITH RETROVIRAL VECTORS GIVE RISE TO LONG-TERM MONO-MYELOID AND LYMPHOID HEMATOPOIESIS

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In vitro studies have suggested roles for both human and murine CD34-Lin- and CD34+ Lin- progenitor cells in hematopoietic recovery after transplantation, consistent with the concept that CD34- stem cells exist. Using a canine autologous transplant model, we evaluated short- and long-term repopulating abilities of canine CD34+Lin- and CD34-Lin- marrow cells. Marrow cells were immunomagnetically enriched for depleted of CD34+ cells, and further purified by fluorescence activated cell sorting (FACS) to select CD34+Lin- (CD34+CD3-CD14-) and CD34-Lin- (CD34-CD3-CD14-) cell populations. Mean cell yields of CD34+Lin- cells and CD34-Lin- cells were 3.25 x10^6 (range 2.7-4 x 10^6) and 11x10^6 (range 4.2-18.4 x 10^6), respectively, with mean purities of 98% (range 97%-99%) and 99% (range 98%-100%), respectively. Separately, the two cell populations were retrovirally transduced with the vectors LN and LNY, respectively, by co-cultivation on irradiated PG13 based packaging cells. LN and LNY carried the neomycin (neo) resistance gene and short sequence differences allowed cells marked with each of the vectors to be distinguished by PCR using the same set of primers. Three dogs were transplanted with both CD34+Lin- cells (cell doses infused: 4.3x10^6, 2.9x10^6 and 4x10^6) and CD34-Lin- cells (cell doses infused: 5x10^6, 2.3x10^6 and 2x10^6) after myeloablative total body irradiation (920 cGy). Two additional dogs were given only CD34-Lin- cells (cell doses infused: 1.9x10^6 and 6.5x10^6). At transplant, dogs also received non-transduced cryopreserved autologous bone marrow or enriched CD34+ cells to ensure rapid hematopoietic recovery. Transduction efficiencies, by standard CFU-C assays, ranged from 6% to 18% in the CD34+Lin- cells. No neoresistant CFU-C formation was observed with CD34-Lin- cells. All dogs readily engrafted. DNA for molecular studies was extracted weekly after transplant from white blood cells. To evaluate the presence of the neo gene in mono-myeloid and lymphoid lineages, in two dogs given both CD34+Lin- and CD34-Lin- cells, DNA was extracted from monocytes, granulocytes and T-cells, which were purified by FACS at 7.5 and 9 months post-transplant, respectively. LN was detected in white blood cells from all dogs infused with transduced CD34+Lin- cells. Gene transfer levels were up to 10% with a median follow-up of 12 months (range 10-13 months). LN was also detected in all sorted mono-myeloid and lymphoid populations. The LNY vector was not detected in blood cells from the dogs infused with CD34-Lin- cells with a median follow-up of 11 months. Our study showed that highly purified CD34+Lin- cells were successfully transduced with retroviral vectors and contributed to short- and long-term engraftment of both mono-myeloid and lymphoid lineages. A CD34- stem cell was not detected with this experimental design.

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FLUORESCENCE *IN SITU* HYBRIDIZATION FOR DETECTION AND MONITORING TRISOMY 12 IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA: A STUDY OF PERIPHERAL BLOOD IN 17 PATIENTS

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Trisomy 12 has been described as a common abnormality in B-cell chronic lymphocytic leukemia (B-CLL), but the clinical significance of +12 in B-CLL is still not clear.[1] With interphase fluorescence *in situ* hybridization (FISH)[2] it is possible to detect the trisomic clone in different hemopoietic tissues[3] and monitor it over time.[4] Few sequential cytogenetic studies have been done and the data reported are conflicting.[5,6,7] The aim of this study was to evaluate the sequential changes of trisomic clones in patients with B-CLL during the course of their disease and following chemotherapy. In this study peripheral blood lymphocytes from 17 patients with B-CLL (13 males / 4 females; mean age 59.7 years, range 36-82) were sequentially evaluated by FISH at diag-

nosis and after treatments over a mean interval of 31 months (2 - 72). The diagnosis of CLL was made according to N.C.I. criteria. According to Binet classification 4 patients were in stage A, 9 in stage B and 4 in stage C. All patients received the following treatment: chlorambucil (CLB) or combination regimen including anthracyclines (CHOP-like) in 6 cases; CLB, CHOP in 5 cases; CLB, fludarabine (FAMP) in 3 cases; CLB, CHOP, FAMP in 3 cases. FISH was performed on the peripheral blood of the above patients using published methods[8] with a direct labeled alphoid chromosome 12 specific probe (Figure 1). For each sample, at least 300 nuclei were scored using a fluorescence microscope. A trisomy 12 level of >2% was interpreted as clonal abnormality. Data for FISH were corrected for denominator of calculated CLL cells (%lymphocytes x %CD5+CD23+ cells). Wilcoxon signed rank tests were used to test for differences in absolute number of leukemic cells evaluated during the course of the disease in every case. All patients showed peripheral blood lymphocytosis higher than $5x10^9$/L (mean 61.4; range 5.0 - 156.0); the mean percentage of CD5+ CD23+ cells in peripheral blood was 80% (37 -98); the mean absolute number of CD5+CD23+ cells was 54,688/mm^3. Trisomy 12 was detected by FISH in 8/17 patients.

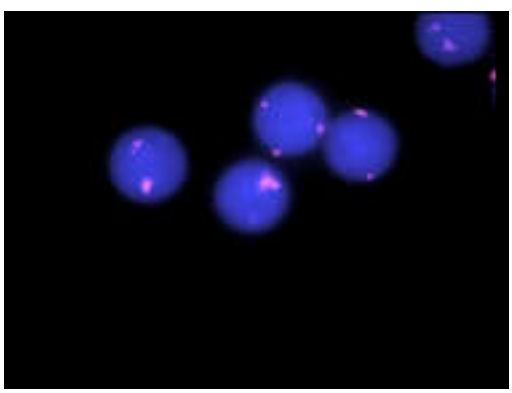

Figure 1. Directly labelled fluorescent α-satellite chromosome 12 specific probe hybridized to lymphocytes from a patient with B-CLL showing three fluorescent signals in one cell and two fluorescent signals in the other cells.

The mean corrected percentage was 24.6% (2.4 - 85.4). Five of the eight patients with cytogenetic evidence of trisomy 12 had atypical morphology. The mean absolute number of trisomic cells was 10,422/mm^3 (1,714 - 25,369) in the group of trisomy 12 positive patients and 229.3/mm^3(0 - 1,195) in the group of trisomy 12 negative patients. After the treatments, a significant decrease of the CD5+CD23+ cell absolute number (Wilcoxon signed rank test, $p < 0.1$) was observed in all patients but a significant decrease of the absolute number of trisomic cells was observed only in 10/17 cases. A significant increase of trisomic cell absolute number (Wilcoxon signed rank test, $p < 0.1$) was observed in the remaining 7/17 cases, two of them were trisomy 12 positive patients at diagnosis (Table 1). There was no significant association between increase in trisomic clone and poor prognosis, but the speed of increase of trisomic clone seems to be closely correlated with disease progression. In conclusion, the prognostic relevance of trisomy 12 is still controversial. The expansion of the trisomy 12 clone after treatment, observed in some cases, suggests a possible proliferative advantage of cells with trisomy 12 over the normal cells; in these cases the evidence of disease progression is generally quick. In other cases, trisomic clones appear to remain stable during the course of the disease.

Table 1: Change in absolute number CD5+CD23+ cells and +12 cells (peripheral blood) for 17 patients with B-CLL undergoing sequential immunophenotypic / FISH analysis.

Name	*CD5$^+$CD23$^+$/mmc*		*Δ*	*m.*	*p**
	Diagnosis	*Follow-up*			
B.N.	26208	9126	7	-17082	
B.D.	29640	371	72	-29269	
C.S.	7434	9118	62	1684	
D.M.	143450	74226	21	-69224	
F.G.	28800	12880	55	-15920	
R.A.	57285	33608	10	-23677	
S.A.	71440	6761	58	-64679	
					<0.1
D.F.C.	24820	22392	8	-2428	
D.N.F.	25920	5190	29	-20730	
G.G.	89580	2534	5	-87046	
G.Gius.	5149	2465	6	-2684	
M.C.	22601	323	39	-22278	
M.M.	106700	1423	42	-105277	
P.L.	152880	152046	2	-834	
R.V.	47880	1225	66	-46665	
R.A.	88065	7969	42	-80096	
V.A.	1850	27	12	-1823	
					<0.1
Name	*+12/mmc*		*Δ*	*m.*	*p**
	Diagnosis	*Follow-up*			
B.N.	509	2646	7	2137	
B.D.	0	7	72	7	
C.S.	0	137	62	137	
D.M.	14300	28948	21	14648	
F.G.	288	1932	55	1644	
R.A.	0	638	10	638	
S.A.	1714	2561	58	847	
					<0.1
D.F.C.	2730	2194	8	-536	
D.N.F.	22153	3299	29	-18854	
G.G.	25369	631	5	-24738	
G.Gius.	72	47	6	-25	
M.C.	11730	17	39	-11713	
M.M.	1195	0	42	-1195	
P.L.	0	0	2	0	
R.V.	1264	0	66	-1264	
R.A.	4120	0	42	-4120	
V.A.	0	0	12	0	
					<0.1

*m. = months; *Wilcoxon signed rank test.*

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MULTIPLE MYELOMA: DIFFERENT IMMUNOPHENOTYPIC AND BIOLOGICAL CHARACTERISTICS OF PLASMA CELLS FROM BONE MARROW AND LEUKAPHERESIS PRODUCTS IN PATIENTS UNDERGOING AUTOLOGOUS STEM CELL TRANSPLANTATION

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In multiple myeloma (MM) patients, reinfusion of peripheral blood progenitor cells (PBPC) has been widely used to support high-dose chemotherapy. PBPC mobilization obtained by chemotherapy and G-CSF induces plasma cell (PC) release into peripheral blood, and therefore leukapheresis collections contain both PBPC and PC. In 88 MM patients at diagnosis we evaluated, by cytofluorimetric analysis, the number and phenotypic characteristics of PC from bone marrow and leukapheresis products. Moreover, we analyzed the S-phase of the 2 different PC populations. PC were identified by the high expression of surface CD38. Median BMPC were 19% (2.5-73), median PC from leukapheresis 1.2% (0.1-19). The phenotypic characteristics of PC were studied using the following monoclonal antibodies: CD19, CD40, CD28, CD44, CD45, CD56, CD138, cytoplasmic Ig, and the results expressed as percentage of co-expression of these antigens on the PC surface, i.e. on CD38++ cells. No statistical difference was observed in the expression of CD40 and CD28 between BMPC and PC from leukapheresis (p=0.2 and p=0.6, respectively). Most PC from bone marrow and leukapheresis express CD40 and are negative for CD28. BMPC express CD138, CD56, while they are negative for CD19, CD45; PC from leukapheresis express significantly lower CD138, CD56, CD45 than BMPC (p<0.0001), and significantly higher CD19 (p<0.0001). Interestingly, all BMPC are phenotypically monoclonal, while PC from leukapheresis are predominantly polyclonal. Moreover, the S-phase of PC from leukapheresis is significantly higher than the S-phase of BMPC (17.9% vs 1.9%; p<0.0001). Our phenotypic and biological data suggest that PC released into PB are probably reactive PC, not belonging to the tumor clone.

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MYELODYSPLASTIC SYNDROME PATIENTS WITH NORMAL KARYOTYPE: PROGNOSTIC RELEVANCE OF FISH ANALYSIS IN 99 PATIENTS

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The biologic and clinical relevance of cytogenetic analysis in myelodysplastic syndromes (MDS) is already established and cytogenetics has, therefore, been introduced by the International Prognostic Scoring System (IPSS) as one of the three variables defining prognosis in MDS. At diagnosis, 30 to 50% of MDS patients have, as assessed by conventional cytogenetic analysis (CCA), normal karyotypes and are classified, according to IPSS criteria, in the low-risk cytogenetic group. However, MDS patients with a normal karyotype appear quite heterogeneous from a biological point of view and the definition of outcome in this group of patients is sometimes quite unpredictable. For these reasons, further studies are warranted in order to identify, among MDS patient with normal karyotypes, those who are at high risk of progression and have a worse prognosis. In comparison to CCA, FISH analysis is a more sensitive technique which allows the identification of smaller clones and/or the evaluation of non-dividing cells and of cryptic deletions which are undetectable by CCA. We therefore analyzed by FISH 99 patients (46 RA, 7 RARS, 32 RAEB, 8 CMML, 6 RAEB-t) with normal karyotypes for the occurrence of abnormalities which are most frequently observed in MDS i.e. +8, -5/del(5)(q31), -7/del(7)(q22-q32), del(17)(p13). In thirteen patients (13%), one FISH abnormality was detected in 15-33% of cells. Five patients had trisomy 8, 4 had del(5)(q31), 2 had del(7)(q32), 1 had monosomy 7 and 1 had del(17)(p13). FISH abnormalities were more frequently observed among patients with more than 5% and more than 20% bone marrow blasts (p=0.02). FISH abnormalities were also associated with a higher rate of evolution into AML (8/13 vs 13/86, p=0.0014) and were predictive for a worse prognosis (p=0.0033). Cox's analysis confirmed the prognostic relevance of FISH analysis (p=0.0034) along with that of bone marrow blasts (p=0.01), LDH (0.026), white blood cell count (p=0.013) and age (0.017). On the whole, our data demonstrate that 10 to 15% of MDS patients with a normal karyotype have, if analyzed with a more sensitive technique, clones of cells with cytogenetic abnormalities not detected by CCA. The presence of FISH abnormalities identifies a subset of MDS patients with a normal karyotype characterized by bad prognosis which deserves separate evaluation. Additional probes could be evaluated to define the prognosis better of MDS patients with normal karyotype.

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NEED OF ACCURATE MOLECULAR DIAGNOSIS TO ASSESS THE DONOR ORIGIN OF LEUKEMIA RELAPSE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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We report the case of a 49-year old man who underwent allogeneic transplantation from his HLA-identical sister because of BCR-ABL+ acute lymphocytic leukemia. After having achieved a complete hematologic and molecular remission, two years later an overt leukaemia relapse occurred with an immunophenotype similar but not identical to that documented at diagnosis. Conventional and fluorescence *in situ* hybridization (FISH) karyotyping revealed the presence of multiple chromosome abnormalities and most surprisingly, a female karyotype in all the 25 metaphases analyzed. PCR amplification of the Y chromosome-specific DYS14 sequence was positive in DNA samples isolated at diagnosis but negative at the moment of relapse. Moreover, the molecular evaluation of hematopoietic chimerism performed by the YNZ-22 VNTR demonstrated that at the time of relapse, a consistent proportion of hematopoietic cells was of donor origin. However, molecular cloning and sequencing of the CDRIII region of the immunoglobulin heavy chain (IgH) gene rearrangement in leukemic blasts at diagnosis and relapse allowed demonstration of their identity, thus formally proving the origin of both leukemic clones to be the patient. This case strongly emphasizes the need for accurate and extensive molecular characterization to prove the donor origin of a leukemia relapse after allogeneic transplantation.

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MEGAKARYOCYTIC COMMITMENT OF NORMAL AND MYELODISPLASTIC CD34+ PROGENITORS: ROLE OF TRANSFORMING GROWTH FACTOR-BETA AND P15INK4B

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Transforming growth factor-beta (TGF-β) plays a key role in the regulation of megakaryocytopoiesis. In epithelial cells TGF-β effect is mediated by the induction of the cyclin-dependent kinase inhibitor p15INK4B (p15). Recently, we demonstrated that p15 upregulation occurs during megakaryocytic differentiation of normal CD34+ progenitor cells; on the other hand myelodysplastic syndromes (MDS) are characterized by a low level of p15 expression due the abnormal methylation of p15 gene promoter. In order to clarify the role of TGF-β and of p15 in megakaryocytopoiesis, CD34+ cells, isolated from bone marrow of normal donors and of patients with MDS, were cultured with Interleukin-6 and thrombopoietin to induce megakaryocytic commitment; normal CD34+ cells were cultured with and without neutralizing anti-TGF-β antibody. At different times of culture we analyzed, by flow cytometry, the expression of several phenotypic markers of megakaryocytic differentiation (CD41, CD62, SDF-1 receptor); moreover cells were sorted on the basis of CD41 expression and p15 mRNA was evaluated by semiquantitative RT-PCR in CD41+ and CD41- subfractions. In addition, in MDS-derived cultures, DNA methylation pattern in CpG islands of p15 gene promoter was determined by methylation-specific PCR. We found that the inhibition of TGF-β by neutralizing antibody produced a reduced expression of CD41, CD62 and SDF-1 receptor in normal CD34+ cells; these findings were associated with a significant reduction of p15 expression in CD41+ but not in CD41- cells. Although CD34+ cells isolated from MDS patients showed, after 7 days of culture, a phenotypic profile similar to that of normal cells, their proliferative ability was significantly lower than that of normal cells, and no viable cells were harvested after 14 days of culture. Furthermore, p15, absent or expressed at very low level at baseline, was not upregulated during megakaryocytic differentiation of MDS CD34+ cells. Accordingly, we found abnormal methylation of p15 gene at baseline and throughout the culture time. These data show that p15 is induced during normal megakaryocytic differentiation by TGF-β and suggest that the absence of p15 may have a role in the disorders of megakaryocytopoiesis occurring in MDS.

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ANALYSIS OF LYMPH NODE TISSUES BY MULTISPECTRAL IMAGING AUTOFLUORESCENCE MICROSCOPY. A CHANCE FOR THE DEVELOPMENT OF ADVANCED DIAGNOSTIC METHODS

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Although histochemical and immunohistochemical methods are the standard procedures in diagnosis of lymphoproliferative disorders, useful improvements in diagnostics can be obtained with the introduction of new analytical techniques. We used microspectrofluorometry and multispectral imaging autofluorescence microscopy (MIAM) techniques to analyze lymph node biopsies from patients with lymphadenopathy of different origins. The aim of the research was to study autofluorescence properties of lymph node tissues and, applying this knowledge, to develop advanced diagnostic methods. Multispectral imaging consists in acquiring monochrome images, related to the fluorescence of one or more endogenous chromophores emitting in a narrow band of wavelengths. Then monochrome images from the blue, green and red bands of the visible range can be combined together in a single multicolor image. Using a suitable computing function, the contrast between the different structures can be enhanced. The results obtained show that the principal contributions to the autofluorescence of lymph node sec-

tions came from extracellular collagen and elastin and intracellular NAD(P)H in the blue band, and from flavins in the green band. The fluorescence emission due to the connective stroma is from 2 (neoplastic sections) to 10 (hyperplasia) times higher than that due to the cells. Neoplastic cells generally show a more intense autofluorescence compared to normal cells. Clear examples are Reed-Sternberg cells in Hodgkin's lymphoma and neoplastic cells in lymph node metastasis. The comparison between autofluorescence imaging of non-neoplastic and neoplastic samples clearly shows in the dramatic changes in connective stroma organization in the latter. These findings show that the organization of connective stroma could be regarded, after proper modelling, as one of the key pieces of information for autofluorescence image analysis in the diagnosis of lymph node diseases. MIAM offers the advantage of avoiding sample chemical manipulations, such as the use of exogenous markers, and obtaining real time results performing the analysis immediately upon specimen resection. A comparison between fluorescence imaging and standard histochemical microscopy gave a satisfactory validation of the proposed technique, demonstrating the possibility of improving upon current diagnostic procedures for malignant lymph node alterations.

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CLINICAL SIGNIFICANCE OF LUNG RESISTANCE PROTEIN IN ACUTE MYELOID LEUKEMIA

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Several mechanisms of multidrug resistance have previously been studied for their clinical relevance in acute myeloid leukemia (AML).[1-3] Expression of the lung resistance protein (LRP) was recently shown to correlate with lower response rates and shorter survival.[4,5] In order to compare the clinical relevance of drug resistance factors, we studied LRP, P-glycoprotein (JSB-1), multidrug resistance protein (MRP1) and bcl-2 in addition to age, WBC and karyotype in 85 *de novo* AML patients (median age 56 years, 42 females and 43 males). All the patients were treated between 1997 and 1999 by intensive chemotherapy regimens based on EORTC/GIMEMA AML-10, AML-13 and GIMEMA AIDA protocols. We measured the expression of JSB-1, LRP and MRP by flow cytometry as percentages of positivity. The thresholds were set at > 10% for MRP or LRP and at > 20% for JSB-1. Bcl-2 was measured as mean fluorescence intensity (MFI) ratio (MFI sample: MFI negative control) and the cut-off level was fixed > 10. LRP positivity was associated with higher WBC count (p=0.010), CD7 (p=0.014) or CD11b expression (p=0.019), poor risk karyotype (p=0.010) and increased expression of JSB-1 (P=0.001), MRP1 (p=0.007) and bcl-2 (p=0.018). Poor response to induction chemotherapy was associated with both karyotype (p<0.001) and LRP or JSB-1 positivity (p=0.001), less significantly with MRP1 (p= 0.020) and age (p=0.050). Age (P=0.012), JSB-1 (P=0.013) and LRP (p=0.028) retained their predictive value in the multivariate logistic regression analysis. Moreover, LRP positivity was significantly correlated with both a higher relapse rate (45.5% vs 15.4%; P=0.016) and a shorter time to relapse (p=0.003; Figure 1A). With regard to disease free survival (CCR), a shorter complete remission (CR) duration was associated with bcl-2 expression (p=0.02), with karyotype (p=0.004), but mainly with LRP positivity (p=0.0002; Figure 1B). A shorter overall survival (OS) was correlated with age (p=0.001), bcl-2 (p=0.002), MRP1 (p=0.006), JSB-1 (p=0.012), karyotype (p=0.00002) and LRP (p=0.00003). In the multivariate Cox regression analysis, karyotype (p=0.015), bcl-2 (p=0.032) and LRP (p=0.001) retained their prognostic significance with regard to CCR, while age (p=0.02), bcl-2 (p=0.03) and LRP (p=0.002) were prognostically independent factors for OS. Furthermore, within the group of patients with good prognosis (patients <60 years with good or intermediate karyotype), both overall survival (p=0.0019) and CR duration (p=0.00010) were shorter in the presence of LRP expression. In conclusion, our data confirm the multifactorial nature of drug resistance, the frequent coexpressions of drug resistance factors[6,7] (LRP, MRP, JSB-1 and bcl-2) and the strong independent prognostic significance of LRP in AML.

Fig.1A Time to relapse(Kaplan-Meier) by LRP

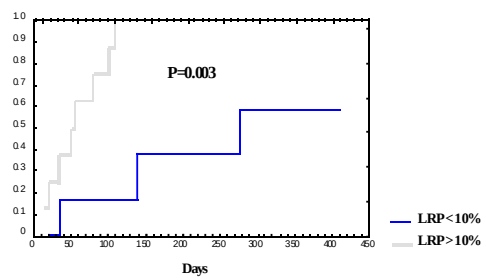
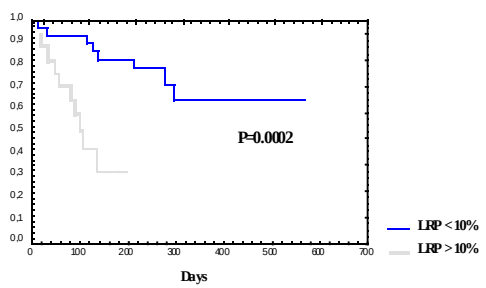


Fig.1B CCR (Kaplan-Meier) by LRP



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THE EVOLUTION OF INDICATIONS AND TECHNOLOGIES IN AUTOLOGOUS TRANSPLANTATION. A REPORT ON ONE THOUSAND AUTOGRAFTS OVER THE LAST TWENTY-YEARS IN THE HEMATOLOGY DEPARTMENT, "LA SAPIENZA" OF ROME

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A total of one thousand patients – 362 AML, 75 ALL, 6 AUL, 110 CML, 26 CLL, 241 NHL, 59 HD, 116 MM and 5 MDS – received a hematopoietic cell reinfusion after high-dose treatment at our Center between 1981 and 1999. The first was performed in 1981 in a patient affected by AML in relapse. After myeloablative treatment and bone marrow reinfusion, complete hematopoietic recovery and remission were reached, but the patient died after 8 months because of disease recurrence. Since 1981 goals, definition, technologies, and indications slowly evolved becoming more or less different, with only the name remaining unchanged - autograft. The review of our experience, besides its historical value, might offer information about the procedure and its role in the treatment of hematologic malignancies. Three main steps may be identified: (1) The change in disease phase. Before 1986, considering AML and CML, about one-half of transplanted cases were in *advanced phase* of disease. In the following years, discouraging results prompted performance of the procedure earlier in the history of the disease. Today only in experimental trials should autografting be performed in patients not in remission or with a chemoresistant disease. (2) Type of disease. Most cases autografted at our Center have AL or NHL. Major indications in AML are first or second complete remission (CR) in patients without HLA identical siblings. The role of autografting in AML has been widely investigated in the last 15 years, in particular in 1st CR with regard to its efficacy as compared to "standard" chemotherapy, but no definitive conclusion can be drawn. Poor results are obtained in ALL and, after the initial experience in the late '80s, autografting is no longer performed in this disease. CML represents a particular disease, in which autografts were extensively carried out: up to 20 cases per year between 1988 and 1992. The emerging role of α-interferon (IFN) and the need to enroll patients in large co-operative IFN trials caused the discontinuation of this strategy. However the 10-year follow up of the CML cohort of patients autografted between 1988 and 1992, recently analyzed, shows encouraging results and prospective studies evaluating the role of autografting in this disease should be planned. (3) *New technologies*. Autografting in NHL is strictly linked to the emerging availability of growth factors for clinical use which allows the collection of peripheral blood stem cells (PBSC). The very low transplant-related mortality associated with the reinfusion of PBSC prompted widespread use of this procedure in these patients, in particular when standard treatment failed. In recent years, other lymphoproliferative diseases, such as MM and CLL, started to be treated with high dose therapies, and the number of cases autografted in our Center in the last five years has progressively increased. Finally, the new millennium started with the application of high dose treatments for non-malignant diseases, such as multiple sclerosis. The main conclusion, above many others, is that autografting must now to be considered only in the context of a total treatment strategy for any given disease.

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INTERPHASE MONITORING OF DEL13Q14 IN MULTIPLE MYELOMA AFTER IMMUNOMAGNETIC POSITIVE SELECTION OF PERIPHERAL BLOOD PROGENITOR CELLS

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Multiple myeloma (MM) is mainly composed of terminally dfferentiated B-cells with low proliferative activity; karyotype analysis may not, therefore, be very informative, representing in most cases normal dividing bone marrow cells. Interphase cytogenetics is thus particularly suitable for the detection of specific chromosomal abnormalities in MM, such as aneuploidies or deletions of known chromosomal bands. The prognostic relevance of del13q14 in MM has recently been established and the availability of commercial probes for this region makes it worth applying this technique widely to detect 13q14 deletions, or monosomy 13 in MM patients. We report here a case in which interphase cytogenetics allowed the detection at onset and the monitoring of this abnormality in the marrow and progenitor cells of a patient whose cells were collected after positive immunomagnetic selection. A 50-year old female was diagnosed as having stage IIIA,IgG-κ MM in August, 1999. At diagnosis, FISH analysis, using a DNA probe containing the entire RB1 gene directly labeled with cyanine Cy3, evidenced the presence of 13q14 deletion in more than 50% of examined nuclei, on marrow samples showing 60% of neoplastic plasma cell infiltration. At this time, the karyotype appeared normal. The patient entered a high dose chemotherapy program, including 2 VMD (vincristine, mitoxantrone and dexamethasone) and 3 cyles of peripheral blood progenitor cells (PBPC) mobilizing chemotherapy (cyclophosphamide 7 g/m^2, modified EDAP and Dexa-BEAM). Pre-transplant re-evaluation showed only a partial response, with persistence of a significant proportion (at least 30%) of bone marrow plasma cells with 13q14 deletion, as detected by FISH. Part of the CD34+ PBPC collected were positively selected by an immunomagnetic device (CliniMACS). Interphase cytogenetics on a sample of the selected cell population showed only normal nuclei, according to the simultaneous flow cytometry evaluation of CD34+ cells that gave a recovery of 75% with a purity of 98%. Only selected "del 13q14 free" autologous CD34+PBPC (6.2x10e6/Kg) were infused after conditioning therapy with melphalan 200mg/m^2. At the time of writing this abstract, the patient is still experiencing the aplastic phase of the transplant. Interphase cytogenetics data on marrow samples after complete hematologic recovery will be available for the Meeting. Our data sug-

gest that FISH monitoring of del13q14 is easily feasible in MM patients who undergo high dose chemotherapy followed by PBPC autologous transplantation and may represent another useful method for evaluating, in selected cases, the efficacy of an *in vitro* purging procedure, such as positive PBPC immunomagnetic selection.

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PERTURBATION OF LYMPHOCYTE MITOCHONDRIAL FUNCTION AND IMBALANCE BETWEEN BCL-2 FAMILY MEMBERS AS MOLECULAR MECHANISMS OF G-CSF-INDUCED IMMUNE DYSFUNCTION

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Sera from healthy subjects receiving recombinant human granulocyte colony-stimulating factor (rHuG-CSF) to mobilize CD34+ peripheral blood progenitors (PBPC) have been shown render allogeneic lymphocytes unresponsive to mitogenic challenge (Rutella *et al.*, Exp Hematol: 26:1024, 1998; Rutella *et al.*, Exp Hematol, in press). In the present investigation, the effects of rHuG-CSF on the early stages of lymphocyte activation-induced apoptosis were evaluated. Peripheral blood mononuclear cells (PBMC) from normal untreated donors were stimulated with phytohemagglutinin (PHA) in the presence of serum collected prior to (preG) or after (postG) rHuG-CSF administration. Mitochondrial function, i.e., incorporation of 3,3'-dihexyloxacarbocyanine iodide [DiOC6(3)] and generation of reactive oxygen species (ROS) as well as the expression of Bcl-2 family members were evaluated by multiparameter flow cytometry. $CD4^{+}DiOC6(3)^{low}$ and $CD8+DiOC6(3)^{low}$ T-lymphocytes increased and reached 32% (range 27-38) and 20% (range 15-23) of circulating T-cells, respectively, on day 4 of rHuG-CSF administration. Hypergeneration of ROS could be demonstrated in 65% (58-82) of CD4+ T-lymphocytes and in 0.4% (0.2-0.8) of circulating CD8+ T-cells. When PBMC were challenged with PHA in the presence of postG-serum, both collapse of mitochondrial transmembrane potential and hypergeneration of ROS were induced, and lymphocytes displayed fragmentation of genomic DNA. Interestingly, the neutralization of surface CD95 abrogated the perturbation of lymphocyte mitochondrial transmembrane potential, suggesting that the CD95 signaling pathway might be involved. Moreover, bax protein was overexpressed in postG (MFI = 180, range 168-186) compared with preG cultures (MFI = 75, range 68-80; $P<0.01$), while no differences in bcl-2 and bcl-xl staining intensity were observed. We hypothesize that soluble immunoregulatory mediators contained in postG-serum might signal through CD95 surface receptors leading to Bax overexpression and to the subsequent collapse of lymphocyte mitochondrial transmembrane potential. Whether immune dysfunction will favourably impact on incidence and severity of acute graft versus host disease (GVHD) after allogeneic PBPC transplantation by enhancing apoptosis of alloreactive T-cells remains to be determined.

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GRAFT FAILURE IN CLASS 3 YOUNG AND ADULT THALASSEMIC PATIENTS

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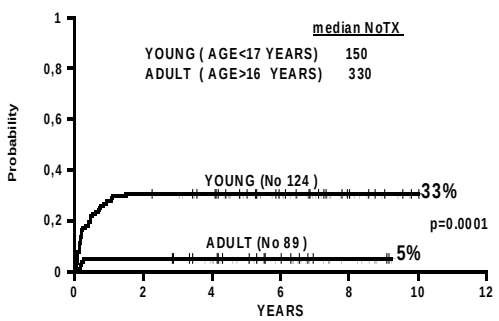
Allogeneic bone marrow transplantation (BMT) is the only available curative treatment for thalassemia. Graft failure and autologous reconstitution are the most frequent causes of BMT failure in transplanted thalassemic patients. A retrospective analyses of the results obtained in 222 consecutive thalassemic patients aged less than 16 years, all prepared for the transplant with busulphan (BU) 14 mg/Kg and cyclophosphamide (CY) 200mg/Kg, showed that the class 3 patients had a non-rejection mortality of 47% and a rejection rate of 12%. We therefore adopted a preparative regimen with doses of CY reduced to 120-160 mg/Kg, with the purpose of decreasing lethal toxicites due to CY. The transplant option was, at this time of our experience, also extended to patients older than 16 years, who constitute the group of adult thalassemic patients. In the group of class3 patients less than 16 years old (young), the new regimens reduced the non-rejection mortality rate to 20%, but were followed by an increased probability of rejection (from 12% to 30%). In the Class 3 adult patients the same regimens were followed by a non-rejection mortality rate of 35% with a rejection rate as low as 4%. Statistical analyses of the rejection rate in the two groups, young and adult, prepared for the transplant with the same regimens, demonstrated that the rejection rate was inversely proportional to the age of the patients and to the number of red blood cell transfusions received before the transplant. The second condition is obviously related to the first as shown in Figure 1.

	BU14; CY120-160		BU14; CY200	
AGE - NoTX	>16 - 330	<17 - 150	>16 - 180	<17 - 150
REJECTION	5%	33%	10%	25%

TAB.1 AGE transplant, median received before the : years, median. NoTX: number rbc transfusions
BU= busulphan mg/kg. CY=cyclophosphamide



When the young and the adult Class 3 patients were prepared for the transplant with BU14 and CY 200, the differences in the rejection rate were cancelled. The effect of age or of the pre-transplant number of transfusions no longer affected the rejection rate, as shown in Table 1.

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MOLECULAR EPIDEMIOLOGY OF HUMAN HERPESVIRUS-8 INFECTION IN DISEASES OTHER THAN KAPOSI'S SARCOMA BASED ON THE ANALYSIS OF THE HYPERVARIABLE ORF-K1 GENE

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Human herpesvirus-8 (HHV-8) is the predicted infectious cause of all clinico-epidemiological forms of Kaposi's sarcoma (KS), and is also linked to rare lymphoproliferative diseases, namely primary effusion lymphoma (PEL), multicentric Castleman's disease (CD) of plasma cell type, and plasmacytic proliferations, often in association with human immunodeficiency virus (HIV) infection and in transplant patients. The HHV-8 genome contains, at the left end, the orf K1 gene, encoding a transmembrane protein, which exhibits much more sequence variability than the rest of the viral genome. The variability in this gene among different isolates has been so far exploited to study the molecular epidemiology of HHV-8 obtained from individuals with KS in different parts of the world, demonstrating the existence of four major molecular subtypes of the K1 gene (called A, B, C, D). Information on the distribution of HHV-8 genotypes in HHV-8 associated diseases other than KS are scarce. We report here the sequencing studies of the orf-K1 gene in a series of 6 patients with rare HIV negative, HHV-8 associated, lymphoid disorders, and in 2 patients who developed HHV-8 associated complications after autologous peripheral blood stem cell transplantation (APBSCT). Genotype C was found in 2 cases of Castleman's disease, in 1 case of reactive lymphadenopathy with giant lymph node hyperplasia and increased vascularity, and in 1 patient who developed fever, cutaneous rash and hepatitis, due to HHV-8 active infection, after APBSCT. Genotype A was found in 1 case of Castleman's disease, in 2 cases of PEL, and in 1 patient who developed fever and bone marrow failure, due to HHV-8 active infection, after APBSCT. These data, together with those we already reported (AIDS 1999; 13: 1165), suggest that HHV-8 strains show no apparent association with a particular disease but appear to be geographically related. In fact, HHV-8 B strains seem to predominate in Africa, while strains A and C are found in Europe and the USA, both in the malignant diseases (KS, PEL) and in non-malignant illnesses (acute inflammatory post-transplant complications), associated with HHV-8 infection. We also exploited the high level of variability of the orf-K1 gene of the HHV-8 genome to assess the genetic relatedness of the HHV-8 strains identified in the post-transplant KS lesions which developed, simultaneously, 20 months after transplantation, in 2 recipients of twin kidneys from the same cadaver donor. The 100% identity of nucleotide and amino acid sequences of the most variable region of the HHV-8 genome in these 2 patients provided the first unequivocal, molecular evidence of organ-related transmission of HHV-8 in the setting of transplantation.

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WILMS' TUMOR GENE IS A MOLECULAR MARKER FOR ACUTE LEUKEMIAS AND MYELODYSPLASTIC SYNDROMES

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The Wilms' tumor gene (WT1) is a tumor marker for acute and chronic leukemia cells, regardless of the disease subtype or the presence or absence of specific fusion transcripts. Thus, the WT1 assay makes it possible to assess the effectiveness of treatment rapidly and to evaluate the degree of eradication of leukemic cells in individual patients. Moreover, molecular relapse can be evaluated by monitoring WT1 expression levels using RT-PCR assay in bone marrow or peripheral blood samples before clinical relapse becomes apparent. In the present study we retrospectively evaluated a heterogeneous group of acute leukemias characterized by t(12,21), t(8,21), inv(16), t(15,17), acute leukemias without specific molecular markers and myelodysplastic syndromes. In all cases we found an intense expression of WT1 at diagnosis, no detectable levels during remission and a gradual increase of the specific signal before clinical relapse. Moreover, in some subtypes of acute myeloid leukemia (AML) such as FAB M2 in which qualitative RT-PCR studies seem of limited prognostic value observing a number of patients in long term clinical remission persistently positive for the AML/ETO fusion mRNA, WT1 analysis allowed identification of molecular remission by the disappearance of the specific signal and prediction of clinical relapse by the gradual increase of the level of expression. In addition we evaluated a number of patients with myelodysplastic syndromes and in all cases we detected elevated levels of expression at diagnosis. The expression significantly increased from refractory anemia (RA) to RA with excess blasts in transformation (RAEB-t). We never detected WT1 in normal peripheral blood or bone marrow. So we can conclude that WT1 analysis may be considered a useful method for the detection of leukemic cells: it can be used for monitoring minimal residual disease and may have clinical significance in the prediction of relapse of specific subtypes of AML and in acute leukemia without a specific tumor marker. Moreover, monitoring of WT1 expression levels allows continuous assessment of the disease progression of myelodysplastic disease as well as the prediction of the evolution from mild forms of myelodysplastic syndrome to RAEB-T or overt AML.

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EVALUATION OF THE HEMATOPOIETIC ACTIVITY OF THE C-TERMINAL REGION OF OSTEOGENIC GROWTH PEPTIDE

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Osteogenic growth peptide (OGP) is a positively charged 14-amino acid mitogen identical to the C-terminus of histone H4. Besides its regulatory role in osteogenesis, it has recently been shown that OGP increases blood and bone marrow cellularity and enhances engraftment of bone marrow trasplants in mice. It has also been demonstrated that the C-terminal truncated pentapeptide OGP(10-14) is a naturally occurring form that shares several properties with the full length polypeptide. The present study examined the possible effects of OGP(10-14) on human hematopoietic precursors obtained from bone marrow, umbilical cord blood and peripheral blood stem cells (PBSC). CD34+ cells were subjected to clonogenic assays on methyl-cellulose supplemented with stem cell factor (SCF), interleukin-3 (IL-3), GM-CSF, erythropoietin and with or without OGP(10-14) 10^{-8} M. After 14 days, colony counts revealed an increase in CFU-GM bone marrow derived cultures, an increase in CFU-GM and BFU-E cord blood derived cultures whereas no relevant changes were observed in cultures derived from PBSC and CD34+ purified cells. The latter were also tested for their expression of the receptors for SCF, IL-3 and GM-CSF showing that OGP(10-14) does not induce any alteration of their responsiveness. In order to evaluate whether OGP acts directly or through stromal cells we observed the effects of the pentapeptide on human bone marrow mononuclear cells in long-term cultures on fibroblast feeder-layers. After 5 weeks, non-adherent and adherent cells were removed from the cultures and subjected to classic clonogenic assays which allowed us to quantify long-term culture-initiating cells. Overall, our results suggest that OGP(10-14) is a positive regulator of hematopoietic stem cells. This regulatory role seems to be carried out without any evident change of the stromal compartment and without excessive exhaustion of hematopoietic precursors.

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DISTRIBUTION OF PROMOTER HYPERMETHYLATION OF THE O6-METHYLGUANINE-DNA METHYLTRANSFERASE THROUGHOUT THE SPECTRUM OF B-CELL NEOPLASIA

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B-cell neoplasia represents a heterogeneous group of diseases originating from B-cells at different stages of maturation. The pathogenesis of B-cell neoplasia is a highly complex process involving activation of proto-oncogenes and disruption of tumor suppressor genes. Aberrant promoter methylation is an acquired epigenetic alteration, alternative to genetic lesions, causing inappropriate gene silencing. Hypermethylation of the tumor suppressor gene O6-methylguanine DNA methyltransferase (MGMT) has been recently reported in several human cancers. MGMT encodes a DNA repair protein that removes alkyls from the O6 position of guanine and its loss of expression in MGMT-/- knockout mice favors lymphomagenesis. On this basis, the aim of our study was to define the involvement and frequency of MGMT promoter hypermethylation throughout the spectrum of B-cell neoplasia recognized by the WHO classification. Toward this aim, 226 B-cell neoplasms representative of most clinico-pathologic categories of the disease were subjected to methylation specific-PCR of the MGMT promoter CpG island. Overall, MGMT promoter hypermethylation was detected in 55/206 (26.6%) mature B-cell neoplasms and in 7/20 (35%) precursor B-cell acute leukemias. With respect to mature B-cell neoplasia, MGMT hypermethylation was not randomly distributed, but rather, displayed preferential associations with specific disease categories. Among clinically aggressive B-cell non-Hodgkin's lymphomas (B-NHL), MGMT hypermethylation occurred frequently in B-lineage diffuse large cell lymphoma (B-DLCL) (40/106; 37.7%), whereas it was rare in sporadic Burkitt's lymphoma (2/15; 13.3%) and was consistently absent in mantle cell lymphoma (0/17). The frequency of MGMT hypermethylation was overall similar throughout the clinico-pathologic spectrum of B-DLCL occurring *de novo*, including systemic B-DLCL (28/70; 40%), primary splenic B-DLCL (4/10; 40%), primary extranodal B-DLCL (4/9; 44.4%), CD5+ B-DLCL (2/6; 33.3%) and CD30+ anaplastic B-DLCL (1/5; 20%). Conversely, MGMT hypermethylation was restricted to 1 single case of B-DLCL transformed from a previous follicular phase (16.6%). Among indolent NHL, MGMT hypermethylation was overall rare, occurring in 4/33 (12.1%) cases, including 2/11 (18.2%) B-cell chronic lymphocytic leukemia/small lymphocytic lymphomas, 2/15 (13.3%) follicular lymphomas and 0/7 lymphoplasmacytoid lymphomas. Also, MGMT hypermethylation was a sporadic event in multiple myeloma (2/16; 12.5%). Finally, MGMT hypermethylation occurred in 4/10 (40%) hairy cell leukemia and in 3/9 (33.3%) MALT-NHL. Among precursor B-cell neoplasms, MGMT hypermethylation occurred in 7/20 B-lineage acute lymphoblastic leukemias independent of the genotypic variant of the disease. The implications of these data are threefold. First, MGMT hypermethylation is frequently implicated in B-cell neoplasia and may represent a major event in the pathogenesis of selected types of both mature and precursor B-cell tumors. Second, the marked difference in MGMT hypermethylation observed in B-DLCL as compared to in Burkitt's lymphoma corroborates the notion that normal germinal center B-cells may be targeted by multiple distinct molecular pathways leading to different types of clinically aggressive B-NHL. Finally, because MGMT status affects tumor cell resistance to the genotoxic effects of alkylating agents, our

results prompt studies aimed at defining the prognostic value of MGMT hypermethylation in B-cell neoplasia.

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CORRELATION BETWEEN NUMBER OF HUMAN CD34+ CELLS INFUSED AND PERCENTAGE OF ENGRAFTMENT IN NOD/SCID MICE

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Hemotopoietic stem cell xenotransplantation in NOD/SCID mice is a useful experimental model for studying hematopoiesis *in vivo*. In previously irradiated NOD/SCID mice we assessed engraftment of human CD34+ cells which were purified through positive selection (Clini-MACS system)and administered in escalating doses of 37,500, 75,000, 150,000, 300,000, and 600,000 cells. After obtaining informed consent, human CD34+ cells were taken from a healthy donor after G-CSF stimulation. Twenty-five NOD/SCID mice received 3.5 Gy in a single dose and CD34+ cells were injected 24 hours later. Post-transplant mortality due to infections occurred early in mice receiving fewest stem cells. Seventeen of the 25 mice were sacrificed after 12 weeks. Bone-marrow and spleen cells were analyzed by cytofluorimetry (FACS) using a human anti-CD45 FITC antibody. Engraftment was always achieved in spleen and bone marrow. The engraftment rate of human myeloid cells correlated with the number of CD34+ cells. In bone marrow, where the engaftment was always best, it reached 65% in the 5 mice receiving the maximum number of CD34+ cells. These results show this experimental model is highly predictive of human CD34+ cell engraftment and that the engrafment rate correlates with the number of infused cells. This model might be a valid approach to the study of human hematopoietic stem cell engraftment in animal models.

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PROMPT RESPONSE OF GATA-1 LOW MICE TO *IN VIVO* PERTURBATION OF ERYTHROPOIESIS SHEDS A LIGHT ON COMPETITION BETWEEN ERYTHROID AND MEGAKARYOCYTIC LINEAGES

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Mice carrying a targeted mutation of GATA-1 upstream region including a DNase I hypersensitive site and distal ("testis") promoter (McDevitt MA *et al.*, PNAS 1997; 94:6781) are characterized by the selective loss of GATA-1 expression in megakaryocytic (Mk) cells, while erythroid (E) cells express GATA-1 at levels that, apparently, are enough to allow normal erythroid development. We took advantage of this GATA-1low mouse model to investigate some of the mechanisms underlying commitment to E and Mk lineages and, particularly, to learn more about the supposed competition between E and Mk development at the level of a common precursor cell. Adult GATA-1low male and double-hemizogyte females were found to be severely thrombocytopenic (platelets <10% of controls), while their Htc was slightly higher than normal (48.9% vs 46.5%). The spleens of GATA-1low mice were >3-fold larger than littermates, due to a conspicuous tissue engulfment by both Mks at different stages of maturation and E cells, as revealed by histologic and immunohistochemical analyses. Conversely, bone marrow (BM) cellularity was about 1/3 that of controls, and >20% were Mks. The absolute number of E progenitors was greatly increased (CFU-E: 6.8- and 2.5-fold the controls, in the spleen and BM, respectively; BFU-E: 2- and 1.2-fold, respectively), while Mk progenitors (CFU-Mk) were less than one-third of controls. In further experiments, we tested the capacity of GATA-1low mice to respond to perturbations of erythropoiesis that were obtained by either phenylhydrazine (PHZ) treatment to induce a severe anemia or repeated administration of rhEpo to induce a polyglobulic state. GATA-1low mice were found to recover faster than controls from PHZ-induced anemia as shown by discrepancies in both reticulocyte counts at day 2 (80% for GATA-1low vs 25% for normal mice) and hematocrit values at day 5 (48% vs 40%). Moreover, response to rhEPO administration (10 U/mouse) was also more effective in GATA-1low than normal mice yielding, at day 6, hematocrit values of 68% and 55%, respectively, which were accompanied by a parallel increase in reticulocyte counts. Evaluation of erythroid progenitors in the spleen of PHZ-treated animals revealed that these cells were augmented approximately 5-fold in both normal and GATA-1low mice as compared to in untreated normal mice. However, the number of bipotent E/Mk precursors - identified by the co-expression of E (Ter-119) and Mk (4A5) markers by FACS analysis (Vannucchi AM *et al.*, Blood 2000; 95:2559) - was found to be strikingly higher (about 5-fold) in GATA-1low than in normal mice. Finally, the expression of β-globin gene in the spleen was higher in GATA-1low than in normal mice, and greatly increased after PHZ. On the whole, these data support the concept that impaired Mk maturation as imposed by the targeted disruption of GATA-1 gene in GATA-1low mice may favor erythropoiesis downstream of the bipotent E/Mk precursor. We believe that perturbations of erythropoiesis in GATA-1low mice may represent a valuable tool for investigating mechanisms underlying commitment of the bipotent E/Mk precursor and provide useful information on competition between E and Mk lineages that, in the course of some dyserythropoietic processes, appears to undergo alternative regulation.

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FLUDARABINE AND ANTI-CD95 APOPTOSIS INDUCTION IN CHRONIC LYMPHOCYTIC LEUKEMIA B-CELLS FOLLOWING CD40 TRIGGERING

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Chronic lymphocytic leukemia (CLL) is characterized by the relentless accumulation of B-cells refractory to activation signals and arrested in G0/G1 phase of the cell cycle. The finding of low or negative CD95 expression on these cells suggests the hypothesis of deregulated apoptosis in the disease. However, CD40-CD40L interaction may rescue CLL B-cells from their anergic state and upregulate different co-stimulatory antigens (CD80/CD86, CD70 and CD95). In this study we were interested in investigating whether CD40 triggering could also modulate CLL B-cell sensitivity to apoptosis induction by anti-CD95 agonistic monoclonal antibody (moAb) or fludarabine. Apoptosis was determined in cytofluorographic analysis by cell staining with propidium iodide (PI) and, in selected cases also with the APO2.7 moAb. Despite purified B cells from all the 16 CLL cases studied showing high CD95 expression, after CD40-CD40L interaction, we detected anti-CD95 mediated apoptosis in only 4 out of the 16 cases. Fludarabine-induced apoptosis, following CD40 triggering was, on the other hand, clearly modified. According to the results obtained, we divided the 16 patients into two groups. The first included 11 patients showing low sensitivity to fludarabine *in vitro* under resting conditions but a significantly increased apoptosis after activation. Contrariwise, B-cells from the second group of patients (4 out of 16) were highly sensitive to fludarabine under resting conditions and became more resistant to this drug after activation. Different sensitivities to fludarabine apoptosis induction within the two groups of patients were not related to specific phenotypic features or disease stage. In order to understand mechanisms regulating apoptosis better, we tried to identify the caspases mediating fludarabine apoptosis induction. From our data it appears that a specific pattern of caspases is involved in this phenomenon and this pattern is shared by the two groups. Caspase inhibitors 1 and 6, in fact, strongly blocked fludarabine apoptosis of all CLL B-cells studied (10 out of 16), but caspase inhibitors 2 and 5 did not. Furthermore caspase inhibitors 3 and 8 blocked apoptosis in only 4 out of 10 cases. However, after CD40 triggering of CLL B-cells, fludarabine apoptosis inhibition could no longer take place by the use of the same inhibitors used under resting conditions. The last finding suggests that two distinct apoptotic pathways may be activated depending on the resting or activated state of these cells. Moreover, the above reported data on the possibility that a low *in vitro* sensitivity to fludarabine apoptosis induction of CLL B-cells might be overcome by CD40 triggering needs to be better elucidated in view to designing novel therapeutic strategies.

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HETEROGENEOUS SUSCEPTIBILITY OF DIFFERENT, FRESHLY ISOLATED B-CELL NON HODGKIN'S LYMPHOMA CELLS TO RITUXIMAB AND COMPLEMENT

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Rituximab is a chimeric anti-CD20 monoclonal antibody which is currently used for the the treatment of B-cell Non-Hodgkin's lymphomas (B-NHL). Its mechanism of action relies upon complement-mediated lysis and antibody-dependent cellular cytotoxicity. We have recently shown that in follicular lymphoma cell lines, the complement inhibitors CD55 and CD59 play an important role in regulating anti CD20 and complement mediated lysis (Golay *et al.*, Blood: in press). In order to generate *in vitro* assays capable of predicting the *in vivo* response to rituximab, we investigated this drug's ability to lyse *in vitro* freshly isolated lymphoma cells obtained from patients with different types of B-NHL including follicular, diffuse large B and mantle cell lymphomas as well as B-cell chronic lymphocytic (B-CLL) and prolymphocytic (PLL) leukemias and hairy cell leukemias. A threshold level of CD20 expression was confirmed to be crucial for complement-mediated lysis since negligible cytotoxicity was observed for all the B-CLL cases showing a mean CD20 fluorescence intensity lower than 100, as evaluated by conventional flow cytometry on an arbitrary log fluorescence scale. Contrariwise, B-CLL cases expressing higher levels of CD20 (mean fluorescence intensity over 100) could be lysed by Rituximab and complement to variable degrees (15-70%). Furthermore, B-CLL and PLL cases, partially susceptible *in vitro* to rituximab-mediated lysis, showed a significant increased lysis when anti CD55 monoclonal antibody was added to the assay. Different follicular lymphoma patients showed a variable susceptibility to rituximab (60-95%) and interestingly the lysis of the most resistant follicular lymphoma cells could be increased significantly with blocking anti CD55 and/or CD59 antibodies thus confirming the crucial role of these inhibitors in complement-mediated cytotoxicity. Finally, neoplastic B-cells obtained from mantle cell lymphomas and hairy cell leukemias showed a dramatic and reproducible *in vitro* susceptibility to rituximab. These data suggest that the level of CD20 expression as well as that of complement inhibitory molecules is predictive of *in vitro* response of neoplastic cells to rituximab and may indicate strategies to identify patients likely to have a good clinical response to this biological therapy.

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DETECTION OF ADHESION MOLECULES ON LYMPHOBLASTS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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In view of the relevance of adhesion molecule expression for the mechanisms of homing, trafficking and spread of malignant cells,[1-7] we investigated the expression of surface adhesion molecules on lymphoblasts from 57 acute lymphoid leukemia (ALL) cases and correlated the adhesive phenotype with the immunologic typing, prognostic factors at diagnosis and clinical follow-up. All cases expressed adhesion molecules at high rates. Beta1 integrin was consistently found on blasts from most ALL cases; among integrins of the Beta2 family, LFA-1 was detected in 58% of cases, in the virtual absence of other alpha chains. CD54 and CD58 were expressed in variable proportions of ALL cases and CD44 was found on blasts in the majority of ALL, whereas the CD62L selectin was detected in 24% of cases. We next examined the adhesive phenotype in the 57 ALL cases classified according to lineage and stage of maturation (Table 1). An overall phenotypic evaluation indicates that B-lineage ALL cases at the three maturational stages consistently displayed the same phenotype; one exception was provided by the high incidence of cases expressing CD18/CD11b in ALL at the pro-B stage (CD10-, CyIg-) vs. pre-B and hybrid phenotypes. In contrast, significant phenotypic differences emerged when B-lineage ALL cases (n=45) were compared with the hybrid cases (n=9). A statistically significant difference between these two groups was observed for CD18, overexpressed in the B-lineage compared to hybrid cases (p=0.004). Moreover, we found that integrins of the Beta2 family, namely CD11a (LFA-1), were expressed in a lower proportion of hybrid vs. B-lineage cases (p=0.054). The same held true when CD44 expression on hybrid ALL blasts was compared with that of B-lineage leukemic cells (p=0.051).

Table 1. Percentage of adhesion molecule expression in ALL cases according to B-lineage subset and hybrid phenotype.[a,b]

	B-LINEAGE			*Hybrid (n=9)*	*p[c]*
	CD10- CyIg- (n=5)	*CD10+CyIg- (n=23)*	*CD10+CyIg+ (n=17)*		
CD29	80	100	100	100	
CD49d	60	96	100	100	
CD18	80	78	59	11	=0.004
CD11a	80	52	70	22	=0.054
CD11b	40	4	6	0	
CD11c	0	0	6	0	
CD54	20	17	29	11	
CD58	60	61	70	67	
CD44	80	83	100	56	=0.051
CD62L	40	13	18	22	

[a]ALL subsets defined according to surface phenotype; [b]positivity defined as >30% blasts expressing any given marker; [c]statistical difference between hybrid and B-lineage ALL.

We did not find a significant correlation between adhesion molecule expression and well established risk factors (age, white blood cell count, central nervous system involvement, chromosomal changes), with the exception of splenomegaly, that was significantly associated with CD18 expression. In the follow-up, no evidence of significant correlation between adhesive phenotype and adverse events, such as leukemic relapse and death, was found. However we report a case of a child with ALL characterized by unusual kinetics of adhesion molecule expression by lymphoblasts during treatment induction.[8] Concomitantly with an impressive reduction of hepatosplenomegaly and a transient blast increase in peripheral blood, just a few hours after chemotherapy, the adhesive phenotype of malignant cells changed with a loss of CD18 and a high, significant expression of CD44 V isoform V5, and CD62L selectin. Although expression of adhesion molecules on lymphoblasts does not seem (in the current follow-up) to bear relevance for the clinical aspects of the disease and for prognosis, our data indicate an intraclonal phenotypic heterogeneity that may vary during the course of treatment, and perhaps, by affecting leukemic cell homing, may act on the response to therapy and on the clinical behavior: of course, to draw definitive conclusions, in relation to the relatively good prognosis in childhood ALL, a very long follow-up is needed.

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IDENTIFICATION, ISOLATION, CHARACTERIZATION AND GROWTH FACTOR REQUIREMENT OF PRIMITIVE CIRCULATING CD34+CD105+ PRECURSORS

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A CD105+ subset of circulating CD34 cells was identified, isolated by a double immunomagnetic selection method and then extensively characterized. Confirming our previous reports, these cells had a phenotypic profile consistent with that of primitive precursors, showing higher Thy-1, AC133, and lower CD38, c-kit than the CD34+/CD105- counter-

part. Functional assays revealed that CD34+/CD105+ cells had very low direct cloning efficiency but retained a considerable number of extended long-term culture-initiating cells (ELTC-IC) while the CD34+/CD105- counterpart did not. CD34+/CD105+ but not CD34+/CD105- generated a considerable number of C7+/CD3-/TCR-α/β/γ/δ- lymphocyte precursors in stroma-free liquid cultures. A wide number of growth factor combinations was tested for their ability to maintain stable hematopoietic function of isolated $CD34^+/CD105^+$ over a 8 day culture period. The sole IL-15, VEGF, Flt3-ligand plus EPO combination was found to be capable of maintaining $CD34^+/CD105^+$ cells in a quiescent status and with an unaltered frequency of ELTC-IC in these cells. Finally, neutralization of autocrine TGF-β1 in the above mentioned cultures impaired the hematopoietic potential of these cells through a cell-cycle and p15 independent mechanism which was associated with bcl-2 modulation and x upregulation, as described previously for circulating cycling CD34+/ CD105+.

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IMMUNOGENIC CHARACTERIZATION OF CD86+ ACUTE MYELOID LEUKEMIA BLAST CELLS AND BLAST-DERIVED DENDRITIC CELLS

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CD86 (B7-2) is a co-stimulatory molecule constitutively expressed on professional antigen presenting cells (APC), which can bind to specific T-cell receptors (i.e. CD28 and CTLA-4) and delivers the second signal required for T-cell responses. Immunophenotypic analysis of acute myelogenous leukemia (AML) blasts revealed that 43 out of 78 (55%) consecutive AML cases contained >15% (range: 16-82%) CD86+ blast cells, while only 2/78 AML were CD80+. Samples enriched in CD86+ blasts induced an allogeneic T-cell proliferative response in primary mixed lymphocyte culture (MLC), but failed to elicit an allo-cytotoxic response in 7/7 experiments. In kinetics experiments (n=5 experiments) these cells were incubated in liquid culture with recombinant GM-CSF and recombinant interleukin-4 ± recombinant tumor necrosis factor-alpha for 3, 5 and 7 days and tested for dendritic cell (DC) differentiation. After 3 days a high proportion of cells showing dendritic morphology by electron microscopy and CD80+, CD86+, CD40+, CD83+, CD11c++ and HLA-DR++ expression by flow cytometry were observed. FISH analysis performed in two cases demonstrated the same chromosomal abnormality in both AML blasts and AML-dendritic cells (AML-DC). However, although AML-DC were able to stimulate allogeneic T-cell proliferative responses potently, they failed to induce T-cell cytotoxicity, even in the presence of exogenous recombinant interleukin-12. These data suggest that human AML blasts that express CD86 constitutively stimulate T-cell proliferation and are committed to the macrophage/DC lineage. However, since neither CD86+ blasts nor blast-derived DC activate cytotoxic T-cells more information on potentially suppressive soluble or cellular factors are warranted in order to use AML-DC in anti-tumor vaccine strategies.

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THE BIOMOLECULAR PATHWAYS EVOKED BY INTERFERON-ALPHA IN CLONAL HEMATOPOIETIC PROGENITORS OF CHRONIC MYELOID LEUKEMIA

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Although interferon-alpha (IFN-α) treatment has changed the natural course and prognosis of chronic myeloid leukemia (CML), the mechanism involved in its selective activity on clonal hematopoietic progenitors remain largely unknown. We sought to determine whether IFN-α evokes the molecular pathways devoted to the control of cell cycle checkpoints, and in particular of the G1-S checkpoint, which is abrogated by the expression of the bcr-abl rearranged gene. To this purpose we developed a competitive PCR strategy, fulfilling the requirements of sensitivity and reproducibility needed for measuring transcriptional induction of two "rare" genes: Gadd45 and p21WAF1/CIP1. These genes co-operate at the G1-S boundary in inducing cell cycle arrest and DNA repair. Using this method we measured gene induction by IFN-α on 32D cell clones transducing a temperature-sensitive p210 bcr-abl construct at the temperature (33°C) permissive for abl tyrosine kinase activity. Transcriptional induction of both genes was already significant (their transcript levels increased approximately 2.5 fold compared to the steady state levels) following 24 hr treatment with IFN-α at the dose (1,000 U/mL) capable of reducing cell proliferation, measured in semisolid assay, to about one third and further rose (more than 6 fold compared to untreated controls) after 4 more days. Accordingly, the fraction of cells residing in the G1 phase of cell cycle was significantly enlarged. In addition, we investigated p53-dependence of gene induction by IFN-α by assessing the transcription rate of both gadd45 and p21 WAF1/CIP1 in 32D cell clones co-expressing the p210 bcr-abl chimera and the human papilloma virus (HPV) 16 E6 gene product, which induces loss of p53 function. Our results validate the hypothesis of IFN-α working in concert with- or in consequence of p53 transcriptional activation on bcr-abl-transduced hematopoietic progenitor cell cycling status.

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ALLOGENEIC TRANSPLANTATION OF HEMATOPOIETIC CELLS IN MULTIPLE MYELOMA: DURABLE MOLECULAR REMISSIONS ARE FREQUENTLY ACHIEVED

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On behalf of the Myeloma Subcommittee of EBMT Chronic Leukemia Working Party

Concern about the use of allogeneic transplantation for myeloma patients has recently been mitigated by some

European data showing a reduction in treatment-related mortality (TRM) during the last 5 years. A pilot study previously showed that clinical (CR) and molecular remissions (MR) may be achieved after allografting with G-CSF mobilized peripheral blood cells (BCT)(*Corradini et al. J Clin Oncol 1999*). We have now started an EBMT study in which all patients achieving CR after transplant, undergo PCR-based monitoring of minimal residual disease (MRD). Tumor markers were derived from the rearrangement of IgH variable region genes for each patient. Fifty retrospective patients have been enrolled so far; the analysis is still ongoing and PCR monitoring has been performed on 34 patients. Eleven patients received marrow cells, 7 T-cell depleted marrow, and 16 BCT. The patients' main characteristics were as follows: 16 males/18 females, median age 43 years (range 29-54); disease status at transplant was: 6 CR, 19 partial remission, and 9 refractory disease. The conditioning regimen included total body irradiation (TBI) in 19 patients, Bu/Cy in 5, and Bu/Melphalan in 10 patients. GVHD prophylaxis consisted of CyA alone or CyA and MTX. There was no grade 3-4 acute GVHD; grade 1-2 acute GVHD was scored in 8 patients receiving BMT, 4 T-cell depleted BMT, and 9 BCT. Extensive chronic GVHD was more frequent after BCT. MR were distributed as follows: 45% after BMT, 43% after T-cell depleted BMT, and 80% after BCT. Median molecular follow-up was 38 months (range 6-120). Overall 21 of 34 (62%) patients achieved MR; 6 have relapsed so far, and only one was in the PCR negative group. MR did not correlate with the use of TBI or GVHD occurrence and grading. In conclusion, this is the largest study showing, on a multicenter basis, that MR are frequent after allografting; patients achieving MR have a significantly lower relapse rate (p=0.03).

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CRYPTIC ABL TRANSLOCATION TO 8P AND RECOMBINATION WITH ETV6 IN ACUTE MYELOID LEUKEMIA WITH EOSINOPHILIA AND A T(8;12)(P12-21;P13) RECIPROCAL TRANSLOCATION

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The BCR-ABL fusion gene, resulting from the t(9;22), typically induces phosphorylation and activation of the ABL tyrosine kinase. Another mechanism of activation of the ABL gene, due to its fusion with ETV6 gene (12p13), was reported in one case of atypical chronic myeloid leukemia (CML) with eosinophilia and a t(9;12;14), in one acute myeloid leukemia with a t(12;14), and in one acute acute lymphocytic leukemia without karyotypic information. We observed a new case of AML in a 29-year old man who showed a t(8;12)(p12;p13) reciprocal translocation as an isolated anomaly in 100 % of bone marrow cells. An increasing leukocytosis (from $6.7x10^9$/L to $31x10^9$/L) and eosinophilia (from 5.5% to 15%) was noticed in this patient during the fifteen months preceding diagnosis. FISH on the derivative 8p showed that the YAC761A2 (Genes Chromosomes Cancer 22: 186, 1998; kindly provided by M. Chaffanet) was retained. 12p13 was investigated using the following locus specific probes from telomere to centromere: 4H9A-170G6-407G6-543P15-433J6, and a panel of cosmids for ETV6 (5'-179A6-50F4-132B11-242E1-184C4-148B6-3'). The breakpoint was narrowed between cos184C4, translocated to the der(8), and cos148B6, kept on der(12), in the region between the 5th and the 8th exon of ETV6. Starting from these results 5' and 3' RACE-PCR was performed with primers specific for the ETV6 gene. We identified in the 3' RACE a chimeric product resulting from a fusion between ETV6 exon 5 and ABL exon 1b. The breakpoint was exactly located at the ETV6 intron 5 donor site resulting in its elimination and therefore in an abnormally spliced product. RT-PCR done with primers specific for ETV6 exon 4 and ABL exon 2 confirmed the existence of a chimeric transcript resulting from the splicing between ETV6 exon 4 and ABL exon 2. FISH with a D-BCR/ABL probe (Oncor) was helpful in localizing the ETV6-ABL fusion gene to the derivative 8p. Results were as follows: two green spots, corresponding to BCR gene, on the two chromosomes 22, and three red spots, corresponding to ABL gene, on both chromosomes 9 and on the der(8). We conclude that the ETV6-ABL fusion protein might be related to eosinophilia in myeloid malignancies. The ETV6-ABL rearrangement occurs as a cryptic event in the presence of either normal chromosomes 9 or a complex karyotypic change. The AIRC *(Associazione Italiana per la Ricerca sul Cancro)* is kindly acknowledged.

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LEVELS OF IL-4, IL-10 AND INF-γ IN THE SERUM AND IN THE PBMC CULTURE SUPERNATANTS FROM 31 PATIENTS WITH HEMATOLOGIC MALIGNANCIES

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Objectives. We studied the production of TH1-type versus TH2-type cytokines by lymphocytes of patients (pts) with hematological malignancies: these cytokines may play a role in disease progression. Interleukin (IL-)4 and IL-10 are cytokines produced by TH2-type whereas interferon (IFN-)γ is produced by TH1-type lymphocytes. The major antineoplastic activity is played by host's TH1-type cells. A shift from TH1-type cytokines towards TH2-type is considered an evidence and a possible cause of cancer progression. *Methods.* We studied the levels of IL-4, IL-10 and IFN-γ in the serum and in the culture supernatants from PHA- or anti-CD3 monoclonal antibody (MoAb)-stimulated peripheral blood mononuclear cells (PBMC)] in 36 pts (mean age 57.3 years, range 23-83) with hematological malignancies [14 non-Hodgkin's lymphoma (NHL), 6 Hodgkin's lymphoma (HL), 10 multiple myeloma, 4 hairy cell leukemia (HCL), 1 chronic myelogenous leukemia (CML) and 1 chronic lymphocytic leukemia (CLL)]. Ten age-sex-matched normal subjects were used as controls. The pts with NHL, HL and HCL were divided in two groups: those with active disease (AD) and those in clinical complete remission (CR); the pts with myeloma were all AD whereas the pts with CML and CLL were in CR. Results. Serum levels of IL-4 and IFN-γ were in the same range in pts (either AD or CR) and in control group. Serum levels of IL-10 were significantly higher in pts with NHL, HL and HCL with AD as compared either to pts with NHL, HL and HCL in CR, myeloma, CML,

CLL or controls. The levels of IFN-gamma in culture supernatants from PHA- or anti-CD3 MoAb-stimulated PBMC were in the same range in controls and in all pts except that with HCL (AD), in whom the levels were higher. The levels of IL-4 were higher in culture supernatants from PBMC of pts with NHL, HL, HCL (AD) and myeloma than those of pts in CR and controls. The levels of IL-10 were higher in culture supernatants from PBMC of pts with AD in comparison with those of pts in CR and controls. The culture supernatants from PBMC of pts with HCL in CR had lower levels of IL-10 than that of pt with AD, even if those levels were higher than controls. *Conclusions*. These results suggest that in hematologic malignancies, especially with AD, there is a shift from TH1-type to TH2-type cytokine production, which may play a role in disease progression. Only PBMC from pt. with HCL (AD) were able to release high amounts of IFN-γ. *Work supported by M.U.R.S.T. National Research Projects, Project No. 9906041835.*

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THALIDOMIDE FOR THE TREATMENT OF MULTIPLE MYELOMA AND OTHER HEMATOLOGIC MALIGNANCIES

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Thalidomide is an immunomodulating agent with recently discovered anti-angiogenesis effects. Since angiogenesis has been found to be important for tumor growth and progression, thalidomide has been recently employed for the treatment of several neoplastic diseases, including multiple myeloma (MM) and other hematologic malignancies. We are currently evaluating the therapeutic role of thalidomide in patients with MM, myelofibrosis (MF) and myelodysplastic syndromes (MDS). Ten patients with advanced, resistant or relapsed MM (at least 3 lines of previously received chemotherapy, including high dose chemotherapy with autologous stem cell support in one case) have been so far treated at our Institution with thalidomide alone, at the dose of 200 mg/d p.o. Five patients have completed at least 8 weeks of treatment and are evaluable for response. Two patients showed progression of the disease, while in a further two subjects no significant modification of clinical and laboratory parameters occurred. In the last patient, a severely transfusion-dependent 54-year old female, completely resistant to 3 lines of treatment, including high-dose mobilizing chemotherapy, a dramatic response was seen: total proteins and M-component reduced from 11.3 to 7.8 g/dL and from 5.6 to 2.9 g/dL, respectively, while bone pain significantly decreased and no further transfusional support was required, with Hb levels constantly maintained above 9 g/dL. However, bone marrow plasma cell infiltration (about 90% at baseline) did not change significantly and no reduction of bone marrow vessel density or serum levels of vascular endothelial growth factor (VEGF) were found during treatment. After 2 to 5 weeks of therapy with thalidomide at the same dose, no significant modifications of clinical or laboratory parameters have been seen in 6 patients, two with MF and four with MDS (two sideroblastic and two refractory anemias). No relevant side effects occurred. In a single patient constipation required reduction of the dose to 100 mg/d. Increases up to 300 mg/d or more of thalidomide were not tolerated in a further three, non-responsive patients, who complained of various gastro-intestinal and neurologic symptoms. Our preliminary experience confirms that thalidomide, at relatively low dose, may be highly effective in selected patients with MM. As far as MF and MDS are concerned, results on larger numbers of patients with longer follow-up will be presented at the meeting.

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HD-MACHOP RADIOTHERAPY + AUTOLOGOUS STEM CELL TRANSPLANTATION AS FRONT LINE THERAPY FOR PATIENTS WITH HIGH-INTERMEDIATE AND HIGH RISK NON-HODGKIN'S LYMPHOMA

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Patients with non-Hodgkin's lymphoma (NHL) and high-intermediate (HI) or high (H) risk disease according to the International Prognostic Index (IPI) have a poor outcome, with a 5-year projected survival of 40 % and 30 %, respectively. Most of the cases fail to respond to traditional CHOP or CHOP-like regimens and are not able to be submitted to a front line intensive treatment based on induction therapy followed by consolidation with autologous stem cells transplantation (ASCT). Herein we report the preliminary results of an ongoing prospective, single center clinical trial for patients with age-adjusted HI and H risk NHL, based on the administration of an intensified third generation induction regimen (HD-MACHOP); radiotherapy (RT) to bulky or localized residual disease, peripheral blood stem cell collection (PBSC) and ASCT. The induction therapy with HD-MACHOP consists of HD-MACHOP/A (prednisone 60 mg/m^2 day 1 to 14, vincristine 2 mg day 1, cyclophosphamide 800 mg/m^2 day 2, adryamycyne 60 mg/m^2 day 2, cytosine-arabinoside 1,000 mg/m^2 day 2 and 3, methotrexate 1500 mg/m^2 day 3) alternated to HD-MACHOP/B (prednisone 60 mg/m2 day 1 to 14, vincristine 2 mg day 1, iphosphamide 800 mg/m^2 day 2, adryamicine 60 mg/m^2 day 2, cytosine-arabinoside 2,000 mg/m^2 day 2 and 3, methotrexate 500 mg/m^2 day 3) to be repeated every 21 days for a maximum of 6 courses. Patients in complete (CR) or partial remission (PR) underego PBSC collection with G-CSF (16 g/kg) two to three months after the last HD-MACHOP course. For patients who fail the first PBSC collection a second or third attempt from bone marrow is allowed. ASCT is performed using BAVC as conditioning regimen. Inclusion criteria are: age less then 60 years old, absence of previous treatment, HIV negativity, absence of other important diseases. Precursor B- and T-cell NHL are excluded. At present 13 patients (9 HI and 4 H risk), median age of 38 years (range 20-55 years), have been registered in the study; 10 patients have completed induction treatment, 2 patients underwent RT, 9 patients underwent PBSC collection, 5 patients have been autotransplanted and 3 patients are still in treatment with the induction therapy. After HD-MACHOP RT 5/10 patients achieved a CR, 4 patients a PR and 1 patient was considered a nonresponder due to an early relapse. Stem cell collection was succesfully

in 6/9 patients and the median number of CD 34 + cells was 2.4 x10e^6/kg (range 1.1-3.2). Four out of 6 patients needed more then one collection. After a median follow-up from diagnosis of 13 months (range 3-19 months), 12/13 patients are alive; 3 patients are not evaluable for the remission status because they are still in induction treatment, 6 patients are in continuous CR and 3 patients are in PR. During HD-MACHOP therapy, grade III-IV anemia, thrombocytopenia and neutropenia were observed in 1/10, 3/10 and 6/10 patients, respectively. Two patients required red blood cell transfusion; none developed grade III-IV infection. Extra-hematologic toxicity was mild and consisted in grade III mucositis in 3 patients. These preliminary results underline the feasibility and efficacy of this intensive therapeutic program and stimulate further accrual of patients.

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EFFECTS OF ARSENIC TRIOXIDE AND ATRA ON SHORT-TERM CULTURES OF NON-M3 LEUKEMIC BLASTS

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Recently, a remarkable efficacy of arsenic trioxide (AS_2O_3) has been demonstrated in patients with acute promyelocytic leukemia (APL). The molecular mechanism by which AS_2O_3 exerts its activity is largely unknown even if the induction of apoptosis seems to be involved. The efficacy of ATRA in the treatment of APL is well known; it has the capacity to induce terminal differentiation and plays an important role in inducing apoptosis. Based on these data, we studied the biological effects of AS_2O_3 and ATRA, *in vitro*, on myeloid blasts from patients with non-M3 acute mieloid leukemia (AML). The aim of this study was to evaluate the inhibition of cellular growth, the induction of apoptosis and a possible synergistic activity between AS_2O_3 and ATRA. *Material and Methods*. Cellular cultures: the mononuclear cell population ($1x10^6$ cells/mL) from peripheral blood of patients affected by AML non-M3, obtained by centrifugation on density gradient (Ficoll), were cultured in RPMI-1640 supplemented with FCS heat-inactivated, glutamine, penicillin and streptomycin, at 37°C, in a humid atmosphere and 5% CO_2 in air. After 24h the culture medium was removed; ATRA and AS_2O_3 were added, using different concentrations (10^{-5}M-10^{-9}M). After incubation with the drugs, some parameters were analyzed to evaluate the inhibition of cellular proliferation and to evaluate the percentage of cells in the different phases of cell cycle. The parameters studied were: 1) the number of cultured viable cells, using trypan blue 2% staining and a Burker chamber; 2) the morphologic features of apoptotic cells stained with May-Grünwald-Giemsa and analyzed microscopically; 3) cycling cells using Ki-67 and BrDU with immuno-cytochemical methods; 4) nuclear DNA fragmentation using the TUNEL method on cytospin obtained from cultured cells. *Results*: after 48h of treatment with AS_2O_3 we observed, in comparison with control cultures, an inhibition of proliferation with all the concentration used in our study. The percentage of inhibition was: 13%, 26%, 31%, 45%, 73% respectively for 10^{-9}M, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M of AS_2O_3 concentrations. The inhibition of myeloid blasts growth with ATRA, after 48h of treatment, was: 19% at 10^{-9}M, 29% at 10^{-8}M, 35% at 10-7M, 45% at 10^{-6}M, 61% at 10^{-5}M. The results of Ki-67 and BrDU studies showed a decrease of positive cells in cultures treated with both drugs. The results so far obtained are insufficient to assert that the inhibition observed is related to apoptotic events. Studies of apoptosis with the TUNEL method and the evaluation of a possible synergistic action between AS_2O_3 and ATRA are in progress. If synergistic effects of the two drugs will be demonstrated in the planned experiments, they would represent a background for adjuvant therapies against leukaemia.

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PREDICTIVE VALUE OF QUANTITATIVE REAL-TIME EVALUATION OF MOLECULAR RESPONSE IN CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH α-INTERFERON

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A substantial minority of patients with chronic myeloid leukemia (CML) achieve a complete cytogenetic response (CCR) to treatment with interferon-α(IFN), defined as the disappearance of Philadelphia chromosome-positive metaphases. We used a competitive RT-PCR[1] and Real-Time TaqMan to quantify BCR-ABL[2-6] transcripts in 323 bone marrow and peripheral blood specimens collected from 44 patients who had achieved CCR with IFN.[2] The median duration of observation was 2.4 years (range 1.2-12.2). Total ABL, GAPDH, β2microglobulin transcripts were quantified as internal control and expressed as BCR-ABL transcripts/microRNA and as BCR-ABL/ABL, BCR-ABL/GAPDH, BCR-ABL/β2 microglobulin ratios. All 44 patients had evidence of residual disease. The actual level of minimal residual disease correlates with the probability of

cytogenetic response. The median number BCR-ABL/μRNA at the time of maximal response for each patients was 4 (range 3-4,600) and was significantly lower in patients who remained in CCR than in those who had a major karyotypic response (4,490 versus 4, $p<0.0001$). Our findings show that the level of residual disease falls with time in complete responders to α-IFN. We used the same analysis on 118 samples (17 bone marrow, and 101 peripheral blood) of 18 CML patients in accelerated or blastic phase, who received tyrosine kinase inhibitor therapy (STI 571). We found that BCR-ABL transcript levels fell with time in STI 571 responders. Our competitive RT-PCR and Real-Time TaqMan assays both proved reliable and sensitive methods for monitoring CML patients receiving different types of treatment,[7] and in particular predicting α-IFN response.

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AGE-RELATED CHANGES IN CD50 AND CD62L ADHESION RECEPTOR EXPRESSION ON NAIVE (CD45RA+) AND MEMORY (CD45RO+) T-LYMPHOCYTES

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Several changes occur in the immune system with advancing age, leading to decreased immune responsiveness. Naive and memory peripheral blood T-cell subpopulations are defined by the mutually exclusive expression of the two isoforms of CD45 leukocyte antigen, namely CD45RA and CD45RO. A decrease in the proportion of CD45RA+ naive T-cells concomitant to an expansion of CD45RO+ memory T-lymphocytes is the hallmark of *senescence*; in addition these subsets show functional anomalies that could be partly due to altered expression of surface receptors, and in particular cell adhesion molecules (CAMs), mediating important cellular functions. In the present study we used a three-color flow cytometry method to quantify the proportion, the absolute number and the density expression or median fluorescence intensity (MFI) of CD50 (ICAM-3) and CD62L (L-selectin homing receptor) adhesion receptors on CD45RA+ and CD45RO+ peripheral blood CD3+ T-cell subsets from 10 healthy elderly subjects and 10 young controls. Both elderly (range 70-100 years) and young (range 20-49 years) subjects were selected on the basis of clinical evaluation and of biochemical and hematologic parameters, as recommended by the SENIEUR Protocol for gerontological studies. Our aim was to investigate age-dependent changes in the expression pattern of these CAMs on naive and memory lymphocytes which might contribute to the remodelling of the immune system in the elderly. The percentage of CD45RA+ T-cells expressing CD50 is not significantly modified in elderly (94.81 + 5.05) compared to young individuals (97.77 + 3.19). In contrast, the percentage of CD45RA- T-cells exhibiting CD50 is lower in elderly than young donors (91.90 + 6.37 vs 98.28 + 2.17; $p<0.01$). The percentage of CD45RO- T-lymphocytes expressing CD62L is decreased in the elderly (53.26 + 18.86 vs 80.79 + 11.03; $p<0.001$), whereas the proportion of CD45RO+ T-lymphocytes expressing CD62L is substantially comparable between the two age groups (63.55 + 15.67 vs 54.75 + 12.29). The absolute number of CD50+ naive T-cells from aged individuals is decreased (251.91 + 141.94 vs 621.80 + 238.02; $p<0.001$) whereas memory peripheral blood T-lymphocytes expressing CD50 are substantially unchanged (863.81 + 260.92 vs 802.69 + 139.61). The absolute numbers of naive and memory peripheral blood T-lymphocytes exhibiting CD62L are respectively decreased (190.77 + 133.41) and increased (515.06 + 146.84) in elderly donors compared to young controls (601.27 + 129.14 and 351.83 + 195.04; $p<0.001$ and 0.05, respectively). Concerning CAM density expression at a per cell level, CD50 MFI values of naive as well as memory T-cell subpopulations from aged subjects are increased compared to those in young donors (14.97 + 1.98 vs 9.83 + 1.20 and 13.97 + 2.01 vs 11.60 + 1.34; $p<0.001$ and 0.01 respectively). CD62L is also overexpressed in both naive (8.45 + 1.62 vs 6.75 + 1.43; $p<0.05$) and memory (10.37 + 2.51 vs 5.43 + 1.14; $p<0.001$) T-cell subsets in the elderly. Naïve end memory T-lymphocytes from aged donors express CD50 and CD62L at increased intensity levels and in different percentages compared to young subjects. This upregulation could be interpreted, rather than as an age-related anomaly, as a compensatory mechanism to a decreased responsiveness and a greater requirement for activation signals.

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VASCULAR ENDOTHELIAL GROWTH FACTOR ISOFORMS 121 AND 165 ARE EXPRESSED ON B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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The possibility of a role for angiogenesis in B-cell chronic lymphocytic leukemia (CLL) was based on evidence of either increased microvessel density in the bone marrow or high circulating levels of vascular endothelial growth factor (VEGF) in the serum. On this background we analyzed, by flow cytometry, the expression of VEGF on leukemic cells of 11 B-CLL patients using a monoclonal antibody directed against 121 and 165 isoforms. For this purpose a monoclonal antibody anti-VEGF whose specificity covered 121 and 165 isoforms (Clone, 26503; mouse IgG 2b; R&D Systems Inc.) was used. All experiments were carried out in double staining according to previsiously reported methods. In all instances diagnosis of typical B-cell CLL relied on either cytomorphologic or immunologic analysis which substantiated using a panel of monoclonal antibodies including CD5,CD23,CD22,FMC7,CD79b and light chain SmIg. All patients tested displayed a positive reaction for VEGF. The percentage of leukemic cells reactive to VEGF ranged between 37.2% and 97% (median, 62.5%). With respect to the antigen density a different pattern could be observed from patient to patient. Mean fluorescence intensity (MFI) ranged between 99 and 156. Interestingly, MFI of patients with a progressive pattern of disease was higher,altough a statistical significance was not reached, than MFI of patients with stable disease at the time of sampling(median MFI,138.5 vs 109; p=0.170). A potential implication of cellular VEGF expression in the mechanisms underlying the progression of disease can be drawn from our observation.

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ARSENIC TRIOXIDE INDUCES APOPTOSIS AND CELL CYCLE ARREST IN HERPESVIRUS INFECTED PRIMARY EFFUSION LYMPHOMA CELL LINES

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The compound arsenic trioxide is an effective drug in the treatment of acute promyelocytic leukemia, inducing apoptosis in leukemic cells *in vivo* and *in vitro*. Recent studies reported that arsenic trioxide induces apoptosis or cell cycle arrest in lymphoid neoplasias, including multiple myeloma, chronic lymphocytic leukemia and human T-cell lymphotropic virus-I (HTLV-I) associated adult T-cell leukemia. In this study we analyzed the effect of arsenic treatment on BCBL-1 and HBL-6 cell lines, which had been derived from patients with primary effusion lymphomas, infected with human herpesvirus-8 (HHV-8) and HHV-8/Epstein-Barr virus (EBV) respectively. Apoptosis was measured with propidium iodide (PI) incorporation and cell cycle analysis by flow cytometry and confirmed by DNA fragmentation and cell morphology after Giemsa staining. BCBL-1 showed 65% of apoptotic cells after treatment for 3 days with 1μM of arsenic trioxide. Lower apoptosis (about 20%) but an arrest in G_0/G_1 phase were observed in HBL-6 cells at the same concentration of arsenic trioxide. It has been reported that apoptosis induced by arsenic trioxide is frequently mediated by a downregulation of anti-apoptotic bcl-2 expression. To verify wether this event is associated with apoptosis in BCBL-1 cells, we analyzed bcl-2 expression by flow cytometry and immunostaining, but very low levels of protein expression were observed in both untreated and treated cells. HHV-8 genome is characterized by the presence of several homologs of cellular genes, including the anti-apoptotic gene coding for v-bcl-2. Thus, we further tested, by semiquantitative RT-PCR, viral bcl-2 expression but no downregulation by arsenic trioxide was observed in treated cells. We are currently studying the expression level of other anti-apoptotic viral genes and other apoptotic pathways. The dramatic induction of apoptotic events by arsenic trioxide in HHV-8 infected BCBL-1 cell lines is interesting, considering that among high grade B-cell lymphomas (such as Burkitt's lymphoma) arsenic trioxide-induced apoptosis has been not observed. This study suggests that the use of arsenic trioxide could be considered for the treatment of selected patients with primary effusion lymphomas resistant to standard chemotherapy.

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IDIOPATHIC THROMBOCYTOPENIC PURPURA AND SPLENECTOMY: 109 CASES FROM A SINGLE CENTER

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Chronic idiopathic thrombocytopenic purpura (ITP)is characterized by thrombocytopenia due to platelet sequestration. First line therapy with steroids induces complete (CR) or partial (PR) response in 70-80% of cases. However, immunosuppressive therapy fails to be effective in the majority of the patients over time. Splenectomy is effective in about 70-80% of cases. In the present study we analyzed 109 patients with ITP, 36 males and 73 females with a median age at diagnosis of 34 ± 16 years (range 6-67), who underwent laparotomic splenectomy. One hundred and two patients underwent splenectomy after one or more therapeutic lines, while 7 patients had been untreated. The mean number of platelets at the time of splenectomy was 36 ± 31 x 10^9/L, with a mean interval between diagnosis and splenectomy of 22 ± 30 months. ninety-four of the 109 (86%) patients had a favorable response (CR+ PR) after splenectomy; 15(14%) patients were refractory and then underwent second line therapy, which was effective in 10 cases. Twenty-two of the 94 (23%) patients relapsed 33 ± 62 months (range 2-252) after splenectomy. Eighteen of 22 patients underwent further treatments which were effective in 12 cases. The mean follow-up after splenectomy was 176±81 (range 10-433) months. No infective complications were documented. Our experience confirms the efficacy and safety of

splenectomy in chronic ITP, even though a different timing of other therapeutic approaches should be considered for refractory or relapsed patients.

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REAL-TIME QUANTIFICATION OF MINIMAL RESIDUAL DISEASE IN INV(16) POSITIVE ACUTE MYELOID LEUKEMIA CAN PREDICT CLINICAL RELAPSE AND IDENTIFY PATIENTS IN DURABLE REMISSION

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In acute myeloid leukemia (AML) patients with inv(16)(p13;q22) or t(16;16)(p13;q22), RT-PCR can detect the presence of the CBFβ-MYH11 fusion transcript.[1-4] Favorable clinical outcomes have been reported despite the presence of CBFβ-MYH11 transcripts as minimal residual disease (MRD).[5] To investigate the prognostic value of CBFβ-MYH11 fusion transcript quantification, we used real-time RT-PCR to study eighteen AML patients with inv(16)(p13;q22) at diagnosis and after induction and consolidation therapy. Real-time RT-PCR is a fluorometric-based technique that allows simple and rapid quantification of a target sequence during the extension phase of PCR amplification. With respect to end-point quantitative competitor methods, real-time RT-PCR has the advantage that it provides absolute quantification of the target sequence, expanding the dynamic range of quantification over 10^5 times. It eliminates the need for post-PCR processing, and reduces labor and carry-over contamination. Abl is used as the reference gene. In this study, we used real-time quantitative RT-PCR to evaluate CBFβ-MYH11 transcripts in 18 AML patients with inv(16)(p13;q22) at diagnosis and after induction and consolidation chemotherapy. All patients showed (≥) 10^2 transcripts at diagnosis. Thirteen of the 18 patients had a 2- to 4-log decrease following induction chemotherapy. This reduction corresponds to what is generally thought to occur in AML patients who achieve complete remission (CR) after induction.[6-7] After consolidation, all 13 of these patients had complete disappearance of the transcript and attained long-term CR. The five patients who had a decrease of less than 2-log after induction all displayed an increase in the number of transcripts after consolidation. In all cases, this was followed by relapse. Two of these 5 patients died after relapse, while the other three achieved a 2- to 4-log decrease in transcript number after re-induction. These three patients all achieved long-term CR. We conclude that real-time RT-PCR quantification during CR seems to be more predictive of cure or relapse than qualitative assessment.

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SILENCING OF SOMATIC MUTATION OCCURS PRIOR TO SWITCHING TO MULTIPLE ISOTYPES IN HAIRY CELL LEUKEMIA

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Hairy cell leukemia appears to derive from a mature B-cell which expresses markers associated with activation. Analysis of Ig variable region genes has revealed somatic mutation in the majority of cases, consistent with an origin from a memory B-cell. One unusual feature of hairy cells is the frequent expression of multiple Ig heavy chain isotypes, with dominance of IgG3, but only a single light chain type. The origin and clonal relationship of these isotype variants has been unclear. Analysis of RNA transcripts of VHDJh-constant region sequences can be used to investigate the genes encoding the variants. We have investigated 5 cases of typical HCL, all expressing multiple Ig isotypes at the cell surface. The tumor-derived VHDJH-Cm sequences were identified and found to be somatically mutated (range 1.4-6.5%), with a low level of intraclonal heterogeneity. In 2/5 cases, IgD-derived clonal sequences with identical mutational patterns were obtained. In all cases, IgG3-derived clonal sequences were identified, with additional IgG subclasses in 3/5. IgA-derived clonal sequences were detected in 4/5. In 3 cases, full length VHDJh-constant region sequences were

obtained for all the isotype variants. These showed identical mutational patterns, indicating that somatic mutation had been silenced at the IgM stage. Expression of Ig protein generally correlated with detection of transcripts, except for IgA, in which 3 cases with detectable transcripts did not express IgA. These findings indicate that the multiple isotypes of HCL are clonally related and that the process of isotype switching occurs after somatic mutation. Switching to IgG3 appears to be common, but IgG1, IgG2 and IgA1 also arise, consistent with a continuing influence of the switching process in the tumor clone.

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COLLECTION AND ENGRAFTMENT CHARACTERISTICS OF PERIPHERAL BLOOD STEM CELLS MOBILIZED WITH HIDOSE CYCLOPHOSPHAMIDE OR DHAP REGIMEN PLUS G-CSF IN NON-HODGKIN'S LYMPHOMA PATIENTS

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High dose chemotherapy plus autologous stem cell transplantation plays an important role in achieving long-term remission in certain groups of patients with non-Hodgkin's lymphoma (NHL). Peripheral blood stem cells (PBSC) are increasingly used world-wide for autotransplantation in malignancies and are now even proposed for allografts. The optimal protocol for mobilization of PBSC remains unknown. Not all chemotherapies are capable of PBSC mobilization which is also relevant to various factors such as the type of disease, prior treatments or individual factors (age, stage, etc). Both different combination chemotherapy and single high-dose chemotherapy have been used, also depending on the type of disease. Our study analyzes the possible factors influencing mobilization and hematopoietic stem cell harvest and engraftment in 36 NHL patients submitted to PBSC transplantation. There were 19 males, 17 females with NHL (21 follicular and 15 large cells). Their median age was 37 years (range 17-60); 32 (89%) had stage III-IV disease; 24 (66%) had bone marrow involvement. Systemic B symptoms were present in 21 (58.3%). The patients had received a median of 2 previous chemotherapy regimes. Mobilization chemotherapy was the DHAP regimen in 24 (66%) and cyclophosphamide (CPM) (5 g/sm^2) in 12 (23%). Median time from mobilization to harvest was 11.5 days. At the time of PBSC mobilization 20 patients (58.8%) were considered responsive (in complete remission, in partial remission or in sensitive relapse) and 14 (41.2%) not responsive (in relapse or refractory to therapy). Regression analysis showed that, in contrast with other parameters analyzed (histology, disease status, bone marrow involvement, mobilization regimen) only preapheresis blood CD34+ cell count and number of previous chemotherapy regimens were predictive of the total amount of CD34+ cells in the apheresis product (r=0.4 and –0.5 respectively). In addition, we found a poor correlation between WBC count in the peripheral blood and number of CD34+ cells in the peripheral blood or in the apheresis component. WBC count in the blood was found to have correlation with the number of mononuclear cells collected (r=0.4). Otherwise age was predictive of the total amount of CFU-GM collected (r=-0.5). Type of mobilizing regimen, histology, disease status, bone marrow involvement did not correlate with either MNC, or CD34+ cells or CFU-GM collected. Overall engraftment occurred within a median of 10 days to ANC >0.5x10^9/L and 12 days to PLT >30x10^9/L. No statistically significant difference in ANC recovery was noted in the two mobilized groups. The median number of days to PLT >30˙10^9/L was 13 for the DHAP group and 10 for the CPM group, with a statistically significant difference (p=0.022), not evident in long-term recovery. Patients receiving more than 5x10^6/kg CD34+ cells had even more rapid haematopoietic reconstitution with a significant reduction in hospital stay and transfusion requirements. The mobilizing regimen (CPM vs. DHAP) did not influence the achievement of the threshold CD34+ cell yield for optimal engraftment (Table 1).

Table 1. Collected cells.

	DHAP		*CPM*
CMN x10^8/kg	3.5	P=ns	2.9
CD34+ cells x10^6/kg	4.9	P=ns	7.06
CFU-GM x10^4/kg	263.8	P=ns	266.1

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COMBINATION CHEMOTHERAPY WITH FLUDARABINE, CYTARABINE AND TOPOTECAN (FLAT) FOR ACUTE MYELOID LEUKEMIA IN THE ELDERLY: A PILOT STUDY

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The higher incidence of adverse prognostic factors and the reduced capacity to withstand the intensity of chemotherapy negatively affect the clinical results in elderly patients with acute myeloid leukemia (AML). An important question relates to the choice of drugs or to the doses applied in remission-induction treatment. Despite patient selection, standard induction with an anthracycline plus Ara-C results in only 40-60% of complete remission (CR), due to toxic deaths and chemoresistance.[1] Thus effective and less toxic combinations were developed with the aim of extending the chance of CR to the majority of patients. The FLAG regimen combining high-dose Ara-C, a drug not effluxed by multiple drug resistance (MDR) membrane pump, plus fludarabine which increases the rate of accumulation of Ara-CTP within leukemic cells, is active in high-risk AML[2] and may constitute a valid option in the elderly.[3] There are no definitive data about a greater therapeutic efficacy of FLAG plus idarubicin[4] or mitoxantrone,[5] while encouraging results were reported with intermediate-dose Ara-C plus topotecan in high risk AML and myelodysplastic syndromes (MDS).[6] Thus we opened a phase II study with the combination of fludarabine, Ara-C and topotecan (FLAT) as induction treatment for elderly AML patients. Fludarabine 15 mg/m^2/d was given for 4 days i.v. in 0.5 hours, followed 4 hours later by Ara-C 2 g/m^2 i.v. over 4 hours and topotecan 1.25 mg/m^2 i.v. over 4 hours. After a single induction course, patients in CR were designed to receive consolidation with both idarubicin 10 mg/m^2 and etoposide 175 mg/m^2 for two days, while those with resistant disease underwent salvage therapy . Myeloid growth factors were given after chemotherapy only in the case of febrile neutropenia. Thirteen out of 14 elderly non-M3 AML patients consecutively referred to our Department from July 1999 entered the study. Patients characteristics are reported in Table 1. Noteworthy, due to associated diseases or poor PS, only 7 of them fulfilled the commonly applied eligibility criteria for a clinical trial. After one course of FLAT, CR was achieved in 9 patients (69%). Among the 4 patients with resistant disease, one was salvaged with ICE therapy, so that the final CR rate was 10/13 (77%). Neither preceding MDS nor the cytogenetic pattern was significantly related to CR achievement. Although very preliminary, these results are encouraging and appear to be mainly related to the negligible toxicity of the regimen: no patient died during induction and the planned post-induction treatment, either as consolidation or salvage therapy, could be applied to all patients. No documented infections were recorded, 7 patients experienced fever of unknown origin (median 2 days) while none experienced hepatic or renal toxicity. Moreover, no patient had alopecia. The mean time from the end of therapy to neutrophils >0.5 and platelets>20 x 10^9/L was 18.5 and 17.6 days, respectively. Transfusion support was constantly required with a mean of 9 red blood cell and 21 platelet units from random donor. In April 2000, after a median follow-up of 4 months, 2 patients are dead and 11 alive, 9 in continuous CR (one death in CR after consolidation). This induction regimen appears effective, well tolerated and suitable for most elderly AML patients: an adequate follow-up is needed to draw any conclusion about the impact on CR duration and survival.

Table 1. Patient characteristics.

N° patients	13
Male/Female	6/7
Median age (range)(years)	70 (60-76)
PS>2 (WHO)	3
Concomitant diseases	
Heart failure	3
Bilirubin >4n	1
Interstitial pneumonia	1
Perianal abscess	1
De novo AML	7
MDS/AML	5
t-AML	1
FAB subtype:	
M1/M2	2
M4/M5	11
Cytogenetic (12 performed)	
Low risk	1
Intermediate	5
High Risk	6

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COMPETITIVE POLYMERASE CHAIN REACTION OF GENOMIC DNA AS A METHOD TO DETECT THE AMPLIFICATION OF BCR-ABL GENE OF CHRONIC MYELOID LEUKEMIA

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The chimeric product of bcr-abl rearranged gene is critical in the pathogenesis of chronic myeloid leukemia (CML), yet its role in the progression of the disease remains unclear. So

far, increased bcr-abl expression levels, possibly due to gene amplification, precede the clonal evolution of CML hematopoietic progenitors toward a fully transformed phenotype and might be, altogether, involved in their resistance to interferon-α or tyrosine kinase inhibitors. The aim of the study presented here was that of developing a competitive polymerase chain reaction (PCR) strategy useful for monitoring the bcr-abl expression levels at different stages of CML. The competitive PCR technique is based upon coamplification of the sample template (target) together with increasing amounts of a DNA fragment (competitor) sharing with the target the primer recognition sites, but differing in size. A competitor for the quantification of both a2b2 and a2b3 alternative splicing forms of the bcr-abl chimera was obtained by cloning within the a2b2 PCR product sequence a 37 bp DNA fragment present in a commercially available molecular weight marker preparation. The PCR reactions were performed both on genomic DNA and reverse transcription (RT) products in microcapillary tubes using a Rapid Cycle DNA Amplification instrument (Idaho Technology). In preliminary experiments carried out on bcr-abl-transduced clones of the 32D hematopoietic cell line, we established, by mean of titration assays, the accuracy and reproducibility of our competitive strategy carried out on both genomic and reverse transcribed DNA. Competitive PCR of genomic DNA had a sufficient sensitivity to detect a single copy of the bcr-abl rearranged gene thus, it enabled us to measure the bcr-abl gene amplification precisely, when present. Competitive PCR carried out on RT products was highly sensitive and reproducible. We are presently attempting to correlate the bcr-abl genomic copy number and its transcription level in clinical samples and, on this basis, to build up a molecular prognostic classification of CML patients.

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EXPANSION OF CYTOTOXIC EFFECTORS WITH LYTIC ACTIVITY AGAINST AUTOLOGOUS BLASTS FROM ACUTE MYELOID LEUKEMIA PATIENTS IN COMPLETE HEMATOLOGICAL REMISSION

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The role of cytotoxic cells in the defense against leukemia is supported by numerous pieces of evidence. Many studies indicate that natural killer (NK) cell activity is reduced in patients with a variety of hematologic malignancies and that impaired cytotoxic functions are commonly seen in patients with leukemia and pre-leukemic disorders. The present study was designed to investigate: 1) whether, and if so, to what extent cytotoxic lymphocytes could be expanded *in vitro* from acute myeloid leukemia (AML) patients in chemotherapy-induced remission; 2) the integrity of the signal transduction machinery of this expanded lymphocyte population, and 3) whether these cells were cytotoxic against allogeneic and autologous blasts. Effector cells were generated and expanded from the peripheral blood of 6 AML patients in complete hematologic remission. Ficoll density gradient separated peripheral blood mononuclear cells were allowed to adhere to plastic for 2 hours and then cultured for 10 days at 37 °C with irradiated feeder cells. Four normal donors served as controls. At the end of the 10-day culture period, we obtained an average 4-fold increase in the total cell number and an average 19-fold increase in the number of CD3-CD16+/CD56+ NK cells, which represented 50-90% of this expanded population, while the remaining cells were CD3+. No monocytes or B-cells were observed. These results are comparable to those obtained with normal donors. In this cell population, we then investigated the expression of the zeta chain, which is associated to the CD16 or CD3-TCR complex, and of the tyrosine kinases of the SYK/ZAP-70 (SYK and ZAP-70) and src (LCK) families. By Western blot analysis on total cell lysates, we could show that the cytotoxic cell population expanded from AML patients in remission (n. 4) contained a signal transduction apparatus that was apparently preserved and, with regard to the investigated proteins, comparable to that of normal donors. We then tested the cytotoxic potential of the expanded cells from 2 patients and 2 donors in a standard ^{51}Cr release cytotoxic assay against the target cell lines K562, Raji and HL-60, as well as against autologous and allogeneic AML blasts. Patients' cells showed a cytolytic activity against the cell lines and allogeneic blasts comparable to that of normal donors. In addition, we demonstrated that the expanded cytotoxic cells exerted a lytic effect also against autologous AML blasts. Taken together, these findings document for the first time the expansion of cytotoxic effectors with autologous killing capacity from AML patients in remission. Further studies are underway in order to characterize this effector cell population better and to investigate the possibility of utilizing these cytolytic effectors for the management of minimal residual disease.

POSTERS

001
EXTREMELY HIGH EFFICIENCY OF GENE TRANSFER INTO HUMAN HEMATOPOIETIC CELLS USING A HIV-1 DERIVED LENTIVIRAL VECTOR

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Major limitations to the gene therapy of hematogic neoplasias derive from the relatively small proportion of cells that are successfully transduced with the therapeutic gene. Recently, in addition to the amphotropic Moloney murine leukemia virus (Mo-MLV)-derived vectors, several different lentivirus constructs have been engineered with the main purpose of targeting non dividing cells. In order to compare the efficiency of these two types of vectors, we have utilized the HIV-1 derived pRRLsinhPGK-GFPpre Transfer vector developed by L. Naldini (Nat Biotech 1997; 15:871), containing the EGFP gene under the control of human phospho-glycerokinase promoter. This construct was transiently transfected into the 293T epithelial cell line together with the packaging vector pCMV* 8.74 and the vesicular stomatitis virus (VSV) env encoding plasmid pMD.G for VSV pseudotyping. The infection protocol involved 4 cycles of spin infections, each for 45 minutes, over a two day period. With this procedure, we have achieved transfer of the EGFP gene into more than 98% of THP1, U937, and BJAB cells and into over 90% of Jurkat, CEM and K562 cell lines. This percentage of positivity was stably acquired since it was maintained for up to 25 days in continuous culture. By comparison, the efficiency with the Mo-MLV retroviral vector was much lower (3-30%). Of particular interest is the high efficiency of transfer using the HIV-1 based virus into several myeloid cell lines (70 to 98%) which are poorly infected by the Mo-MLV derived retrovirus (1-3%). We are currently investigating the relative efficiency of the two systems for gene transfer into normal human proliferating and resting T-lymphocytes, B-lymphocytes, monocytes, and dendritic cells of different origins. These data will be presented.

002
ALLOGENEIC TRANSPLANTATION FOR HIGH-RISK PATIENTS: REDUCED INTENSITY CHEMOTHERAPY WITH THIOTEPA, FLUDARABINE AND CYCLOPHOSPHAMIDE ALLOWS STABLE ENGRAFTMENT WITH LOW TOXICITY

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Allogeneic transplantation of hematopoietic cells (HSCT) represents not only a way to restore marrow function after high-dose chemoradiotherapy, but also a form of adoptive immunotherapy. Allografting efficacy, however, is frequently hampered by its toxicity. This is particularly true when candidates are over 45 years old, heavily pretreated, or affected by other comorbidities. In order to decrease treatment-related mortality (TRM), and to further enhance the graft-versus-tumor effect, we have developed a strategy in which reduced intensity chemotherapy (thiotepa 15 to 5 mg/kg, fludarabine 60 mg/m^2, cyclophosphamide 60 mg/kg) is associated with low-dose GVHD prophylaxis (cyclosporin A 1 mg/kg, methotrexate 10 mg/ms d+1, 8 mg/m^2 d+3, d+6), and early reinfusions of engineered(Tk+) donor CD3 lymphocytes. Cyclosporin is rapidly tapered at day +90: patients with molecular disease receive 1 x 10^7 CD3+/kg; patients with clinical disease receive 1 x 10^8 CD3+/kg. We have conducted a pilot study enrolling 22 patients: 9 with non-Hodgkin's lymphoma (NHL), 1 with Hodgkin's lymphoma (HD), 1 with acute lymphocytic leukemia (ALL), 5 with refractory anemia with excess blasts in transformation (RAEB-t), 1 with acute myeloid leukemia (AML), 3 breast and 2 renal cancers. Median age was 51 years (range 33-65), disease early/phase advanced in 7/15 patients. Patients were considered at high-risk of TRM because they were old, heavily pre-treated (9 relapsed after ABMT), and/or with concomitant organ dysfunction. Nine patients received bone marrow and 13 peripheral blood progenitor cell grafts. Twenty-one patients are evaluable so far. All patients engrafted: the median time to achieve 500 neutrophils was 13 days, median time to 20,000 platelets was 19 days; chimerism at day +30 and +90 was full donor in all patients achieving marrow remission. The median follow-up is 221 days (range 36-550); acute GVHD>2 was scored in 1 patient, WHO organ toxicity >2 was scored in 3 patients. Two patients died of disease progression and 1 of TRM (4.5%). Nineteen patients have a follow-up longer than 60 days: 13 had a disease response, including 2 molecular remissions. Five patients received donor lymphocytes for relapse or disease persistence: 1 minimal and 1 complete response were observed. In conclusion: i) short-term TRM was low in a cohort of high-risk patients; ii)early reinfusion of donor lymphocytes is feasible also for patients receiving T-repleted grafts; iii) antitumor activity is transient for patients with chemorefractory disease.

003
MOLECULAR AND PHENOTYPIC HETEROGENEITY OF AIDS-RELATED DIFFUSE LARGE CELL LYMPHOMA

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AIDS-related diffuse large cell lymphoma (AIDS-DLCL) may be segregated into two distinct morpho-phenotypic categories: large non cleaved cell lymphoma displaying the typical phenotype of germinal center B-cells, i.e. BCL-6$^+$/CD138-/LMP-1-, and immunoblastic plasmacytoid lym-

phoma displaying the post-germinal center B-cell phenotype, i.e. BCL-6-/CD138+/LMP-1+. In order to refine the pathogenesis and histogenesis of the disease, the usage and antigen selection process of immunoglobulin variable (IgV) genes were investigated in AIDS-DLCL representative of the two morpho-phenotypic categories. Further, mutations of the BCL-6 gene, which are accumulated during germinal center transit of B-cells, were investigated and correlated to IgV mutations. The tumor panel comprised 16 AIDS-DLCL, including 8 systemic cases and 8 cases primarily localized to the central nervous system. VH and BCL-6 genes were amplified by PCR using multiple independent strategies and PCR products were subjected to DNA sequencing. Evidence of antigen selection of IgV genes was analyzed by the Chang-Casali method. AIDS-DLCL utilized all of the most common VH genes, with no specific bias for a given IgV family. Independent of the expression pattern of BCL-6, CD138 and LMP-1, all VH genes utilized by AIDS-DLCL were found to carry somatic mutations at a frequency ranging from 2.2% to 21.2%. Evidence of antigen selection was restricted to 2/16 AIDS-DLCL (one BCL-6-/CD138+/LMP-1+ and one BCL-6+/CD138-/LMP-1-). Ongoing mutational activity of IgV genes was restricted to 1/16 AIDS-DLCL, displaying the BCL-6+/CD138–/LMP-1– phenotype. Notably, all AIDS-DLCL of the BCL-6-/CD138+/LMP-1+ phenotype carried VH genes harboring "crippling" mutations, i.e. stop-codon mutations within originally functional rearrangements. The BCL-6 gene was found to carry somatic mutations in 8/16 (50%) AIDS-DLCL. As expected, BCL-6 mutations displayed a mutation pattern similar to that of IgV genes, although occurring with a frequency approximately 10-fold lower than that of IgV. In particular, BCL-6 mutations in AIDS-DLCL were found to be strictly correlated with cases displaying a rate of IgV mutation >6%. These data reinforce the hypothesis that mutations of IgV and BCL-6 are caused by a similar mechanisms acting with different efficiency on the two loci. The implications of these data are threefold. First, all AIDS-DLCL derive from germinal center-related B-cells, independent of their differentiation stage, morpho-phenotypic profile, and site of origin. Second, lack of ongoing IgV mutations suggests that BCL-6+/CD138-/LMP-1- AIDS-DLCL are represented by B cell centrocytes, as opposed to centroblasts, whereas BCL-6-/CD138+/LMP-1+ AIDS-DLCL derive from post-germinal center B-cells. Third, the presence of "crippling" mutations in AIDS-DLCL of the BCL-6-/CD138+/LMP-1+ phenotype suggests a cellular origin from pre-apoptotic germinal center B-cells that have lost the ability to express antigen receptors. In this context, LMP-1 expression may play a role in the rescue from apoptosis of "crippled" neoplastic B-cells.

004
PURIFICATION AND AMPLIFICATION OF HUMAN MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSCs) are pluripotent progenitor cells retaining the capability to differentiate into various mesenchymal subtypes (fibroblasts, chondroblasts, osteoblasts, adipocytes, smooth muscle cells), as a consequence of both cell-to-cell interactions and modulation by soluble growth factors. MSCs may play an important role in a wide range of clinical events including restoration of damaged tissues, such as bone regeneration in patients affected by skeletal defects. MSCs have also been shown to be effective support for *in vitro* proliferation of hematopoietic stem cells and are under investigation for their ability to restore the damaged marrow microenvironment during hematopoietic stem cell transplantation. Human marrow stromal cells cultures are composed of a heterogeneous mixture of cells at various stages of differentiation and with distinct differentiation potentials. We optimized a method for purification and expansion of MSCs contained in marrow samples collected from healthy donors. Samples (n=6) were centrifuged at 2000 rpm for 10' and buffy coats were removed manually[1] or MNCs were separated by standard Ficoll density gradient[2] (n=2). In both conditions cells were then resuspended in IMDM with 10% FCS, seeded at concentrations of 10^5, $5x10^5$ and 10^6 cells/cm^2 in T75 flasks and finally incubated at 37°C in fully humidified atmosphere with 5% of CO_2. At confluence (90% of flask surface), adherent cell layers were detached by trypsin-EDTA, washed twice in HBSS, resuspended in 10% FCS IMDM and analyzed by flow cytometry for the expression of CD45 and CD14 antigens, in order to evaluate non-MSC contamination. Cells were then subcultured at a concentration of 20,000 cells/cm^2 in T75 flasks. In our experience, buffy coat was more effective in terms of both time to reach confluence and speed of disappearence of the CD45+/14+ component: in fact, all buffy coat derived primary cultures achieved confluence in two weeks, regardless of their initial cell concentration. On the other hand the lower concentration (10^5/cm^2 of Ficoll-derived cells was often ineffective in providing a confluent cell layer. Moreover, after first seeding, expression of CD45/14 was 0.87% and 19.74% from buffy coat and Ficoll-derived cells, respectively; after second seeding it was 0.64% and 11.23%. Osteogenic differentiation potential was analyzed after the second culture through the addition of 100 µg/ML ascorbic acid, 10nM dexamethasone, 10 mM beta-glycerophosphate in the culture (10,000 cells/cm^2 in T25 flasks); after 3-4 weeks, intense mineralization was observed with Alizarin red S staining. Finally we established co-cultures of irradiated MSC- derived stromal cells with either CD34 and AC133 highly purified hematopoietic cells, with or without addition of serum cell factor and thrombopoietin for two weeks. The presence of growth factors was necessary to maintain cellular vitality in culture and we observed a higher proliferation rate and a more sustained persistence of non-myeloid antigens (GPA and CD41) in the stroma+ culture as compared to the stroma-free culture.

005
THERAPY WITH POLYCLONAL IG M ENRICHED IMMUNOGLOBULINS IN HEMATOLOGIC FEBRILE NEUTROPENIC PATIENTS

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A common complication following chemotherapy for hematologic malignancies is febrile neutropenia, which is difficult to control. Although empirical antibiotic therapy

reduces the rate of early deaths associated with infection, neutropenia, particularly if prolonged, remains the most important risk factor in this group of patients. Moreover the risk increases depending on the condition of the patient's cellular and humoral immune system. Our aim was to evaluate the effectiveness of therapy with human polyclonal IgM enriched immunoglobulins (Pentaglobin Biotest) in neutropenic patients with acute myeloid leukemia and sepsis syndrome. This therapy was considered to be the most suitable because of the rapid onset of its antiendotoxic effects and because it contains antibodies against the most frequent sepsis pathogens. As a result of induction or consolidation chemotherapy, neutropenia (WBC<500/mL) developed, and the treatment was given to 24 patients. Their average age was 42 years (ranging from 29 to 61); twenty were male and four were female. All patients were treated with antimicrobial prophylaxis (ciprofloxacin 500mg/day) and developed a fever; 13 patients received Pentaglobin 300 ml/ day for 3 days during a first line empirical combined antibiotics (ceftazidime 3 g/day and amikacin 1 gr/day) while 11 patients received the treatment during second line empirical therapy (imipenem 3 gr/day) and liposomial amphotericin B from 3 to 5 mg/Kg/ day. No complications occurred during its administration, with the fever disappearing in 21 patients after a median time of 4 days (range 2-10 days). Only 3 patients, who received Pentaglobin during empirical second line therapy, died in aplasia as a consequence of recurring septic events. Microbiological isolation from 37 blood cultures showed 17 cases of *Staphylococcus epidermidis*, 9 *Pseudomonas Aeuriginosa*, 6 *E. Coli*, 1 *Serratia*, while 4 were negative. Knowing the severity of sepsis syndrome which can involve all organs and tissues leading to multiple organ failure and the severe impairment of the host defence, it is evident that any therapeutic support against bacterial invasion must be adopted as soon as possible. We have shown that the prompt treatment with human polyclonal IgM-enriched immunoglobulins offers a very promising therapeutic addition to current antibacterial strategies. However, further controlled randomized trials are needed to evaluate the real efficacy of the polyclonal Ig M enriched immunoglobulins.

006
ANGIOTROPIC LYMPHOMA MANIFESTED BY FEVER AND INTERSTITIAL LUNG DISEASE

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Angiotropic lymphoma is a rare malignancy characterized by neoplastic proliferation of lymphoid cells within the lumen of arteries, small veins and capillaries involving lung, central nervous system and skin while apparently sparing bone marrow and lymph nodes. More than one hundred cases have been reported in the literature. In February 2000 a 68-year old man was admitted to our hospital because of dyspnea and fever preceded by severe chills. These symptoms began four months previously when, in another hospital, high LDH (3043 IU/mL) and interstitial lung disease had been found. A two-month course of corticosteroids (0.5 mg/kg/day) was prescribed with a transient result. When we saw the patient for the first time, fever and pancytopnenia were present. Bone marrow examination revealed no infiltration; on CT scan enlargement of lymph nodes was not observed but micronodular interstitial lung disease was getting worse. Repeated transbronchial lung biopsies were performed. Histologic examination showed the presence of large, atypical lymphoid cells filling alveolar capillaries. Immunohistochemical studies were negative for cytokeratin, EMA, vimentin, S100, HCG, CD30, and T-cell markers while they were positive for LCA, and L26 thus demostrating the B-cell origin of the cells. No other site of involvement was found. A diagnosis of angiotropic lymphoma, also called intravascular lymphomatosis, was made. CHOP regimen therapy was started and a rapid clinical improvment was obtained. We report this case to increase the awareness of the possibility that fever and interstitial lung disease can be the initial manifestation of this rare disorder.

007
MOLECULAR HETEROGENEITY OF POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS: CLUES TO THE CLINICO-PATHOLOGIC DIVERSITY OF THE DISEASE.

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Post-transplant lymphoproliferative disorders (PTLD) are a heterogeneous group of lymphoproliferations that arise in immunosuppressed transplant recipients. PTLD comprise a histologic spectrum ranging from polyclonal hyperplasia to frank lymphoma or multiple myeloma. The precise molecular events involved in the pathogenesis of PTLD are poorly understood. Also, the histogenetic derivation of PTLD has not been addressed in detail and the precise lymphoid cell subset giving rise to these neoplasms is not known. In this study, we investigated a panel (n=29) of PTLD for several molecular and phenotypic markers of known pathogenetic and histogenetic relevance for lymphoid neoplasia. The molecular alterations investigated in this study included lesions of BCL-6, c-MYC, p53, O6-methylguanine-DNA-methyltransferase (MGMT), and death-associated protein kinase (DAP-Kinase). In parallel, the PTLD panel was subjected to molecular characterization of EBV genes. The phenotypic markers applied to the study of PTLD histogenesis included expression of BCL-6 and of CD138/syndecan-1, which segregate the germinal center (GC) stage of B-cell differentiation (BCL-6+) from later stages of maturation (CD138/syndecan-1+). With respect to pathogenesis, the most frequent lesion of PTLD was represented by hypermethylation of DAP-kinase and MGMT promoters. Hypermethylation of DAP-kinase occurred in 64% B-cell PTLD, but apparently in none of the PTLD with T-cell phenotype, whereas hypermethylation of MGMT occurred in 67% of PTLD, independent of the disease phenotype. Other genetic lesions (rearrangements of BCL-2 and c-MYC, mutations of p53 and infection by HHV-8) were consistently negative in all tested cases. With respect to viral infection, 62% of samples demonstrated the presence of EBV. A wild type LMP-1 gene was detected in all but one

case, which harbored the EBV/LMP-1 variant. Analysis of EBNA-2 and EBNA-3C genes revealed the presence of the EBV type-1 strain in all PTLD tested. Sequence analysis of EBNA-1 gene revealed a single prototype EBNA-1 sequence in all but one case. In one sample, multiple prototype and variant EBNA-1 sequences were detected, suggesting multiple infection of tumor clone. Expression of the EBV transforming antigen LMP-1 was detected in 80% of PTLD. Concerning PTLD histogenesis, mutations of BCL-6, regarded as a marker of B-cell transit through the GC, were observed in 37% of cases. However, at variance with other GC-related lymphomas arising in the setting of immunodeficiency, namely AIDS-related lymphomas, all PTLD tested failed to express the BCL-6 and CD138/syndecan-1. The implications of these data are threefold. First, our results provide the first evidence that hypermethylation of DAP-kinase and MGMT are involved in the pathogenesis of PTLD. Second, the presence of BCL-6 mutations suggests that a fraction of PTLD derives from GC-related B-cells. Third, the combination of BCL-6 mutations coupled to absent expression of both BCL-6 and CD138/syndecan-1 suggests that PTLD conceivably reflect a post-GC stage of B-cell differentiation which has not yet undergone preterminal maturation.

008
DETECTION AND CHARACTERIZATION OF A 6_p12-21 AMPLIFICATION IN THE HUMAN U937-1 CELL LINE

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The human cell line U937 was derived from the pleural effusion of a patient with histiocytic lymphoma. Cytogenetically, three different sublines, U937-1, U937-2, and U937-3, have been identified. We used molecular cytogenetic tools to study chromosomal rearrangements of the U937-1 subline which shows a complex karyotype with a modal chromosome number of 57 (range 41-60), and numerous derivative chromosomes and markers. Comparative genomic hybridization, performed according to the method described by Kallioniemi *et al.*(Science, 258: 818, 1992), showed gains and losses of several chromosomal regions. Among them a high level of amplification on chromosome 6p, at band 6p12-p21.3 was evidenced. This event was not detected on karyotype in which only a normal 6 and an add(6)(p21) could be demonstrated. Focusing on 6p, painting and FISH with pacs (kindly provided by P. Marynen, University of Leuven) specifically selected for the 6p11-p21 (telomere-160J11-280E11-524E15-132J3-50J22-1106P1-895C5-1043E3-1158N7-904F15-381E2-centromere) were performed. The add(6)(p21) resulted from a t(2;6) unbalanced translocation. In addition, three other 6p aberrations emerged in distinct subclones. One was a der (6), karyotypically described as an acrocentric marker chromosome. One was a der(6)t(6;?)(p21;?) due to duplication of 6p21.1-21.3. Finally, chromosome 6 material was found in a small marker. The amplicon was present only in the first and second of these three subclones identified by FISH. The amplification was restricted to the region flanked by pac 280E11 and pac 381E2. Structural rearrangements involving the 6p region of the U937-1 amplicon are known in human leukemias and lymphomas. Corresponding specific 6p overexpressed genes, however, have not yet been identified.

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009
LEUKEMIC CONTAMINATION OF PERIPHERAL BLOOD STEM CELL HARVESTS IN "HIGH RISK" ACUTE PROMYELOCYTIC LEUKEMIA

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Recently, a combined analysis of the GIMEMA and PETHEMA trials on acute promyelocytic leukemia (APL) reported that WBC and platelets counts at diagnosis were the only variables with independent prognostic value of relapse-free survival.[1] The resulting predictive model was able to identify a group patients (WBC > $10x10^9$/L) at high risk of relapse. We investigated leukemic contamination in peripheral blood stem cell (PBSC) harvests in comparison with bone marrow (BM) samples simultanously collected by means of a RT-PCR (sensitivity level 10^{-4})of PML/RARα fusion genes in seven pts presenting high risk features at diagnosis. From January 1993 to March 2000 , 25 patients with APL were evaluated by means of cytogenetic and molecular analysis at diagnosis in our hospital. Seven patients (4 M3 and 3 M3v) showed a WBC count >$10x10^9$/L. Four were females and three males. Median age was 52 years (range 23- 68 years). One case was secondary to rheumatoid arthritis. PBSC collections were performed in complete remission (CR) (2nd CR in two cases) after the first consolidation course of AIDA protocol followed by the administration of G-CSF 5 ug/kg/die. A median of 2 aphereses (range 1-3) were performed using a CS3000 (Baxter) cell separator until the target dose of >$3x10^6$/kg CD34+ cells was obtained. In all cases RT-PCR analysis showed no residual leukemic contamination in PBSC harvests. Persistence of fusion transcripts in BM was revealed in pts in 2nd CR. These patients were submitted to autologous PBSC transplantation. One relapsed at + 15 months and subsequently died, another one is in persisting molecular remission at+ 69 months. A 69-year old patient relapsed after 24 months and was treated with As_2O_3, the remaining patients are in molecular 1st CR at +3, +31, +39 and +43 months. In our experience, the apheresis products in "high risk" APL cases have no leukemic contamination even if collected after only 2 courses of chemotherapy. We think that PBSC collection in first CR could be used in a setting of autologous transplantation for treating relapse in patients older than 60 years or those lacking a suitable donor.

REFERENCE

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010
MASSIVE EXPANSION AND PROLIFERATION OF HUMAN HEMATOPOIETIC PROGENITORS ARE UNCOUPLED WITH SUSTAINED ERK ACTIVITY

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Extracellular signal-regulated kinases (ERKs) are protein-serine/threonine kinases belonging to the mitogen-activated protein kinase (MAPK) superfamily. Activation of ERK may induce the cell to opposite behaviors such as proliferation or differentiation and apoptosis depending on the duration of activation, on the characteristics of the stimuli and those of the cells involved. Thus, it is mandatary to investigate these kinases in primay cells when they are carrying out their specific functions. In this paper, we investigated by immunoenzymatic assays the levels of ERK activity in massive proliferating and expanding hematopoietic progenitors (HP). The levels of ERK activity were then investigated after treatment of expanding HP by a stimulus which reduces the self-renewal potential and induces differentiation. To perform our experiments we utilized a stroma-free cell culture system in which the combined use of FLT3 ligand (FL), stem cell factor (SCF) and thrombopoietin (TPO) is able to induce massive expansion of HP taken from cord blood including blast cell colony-forming units (CFU-Bl), long-term culture-initiating cells (LTC-IC) and *in vivo* repopulating stem cells, defined as NOD/SCID mice repopulating cells (SRC). In this system, the addition of interleukin-3 (IL-3) to FL, SCF and TPO results in a still impressive output of the HP which, however, is short lasting and in a rapid decrease of more primitive progenitors due to the prevalence of differentiation. To detect ERK immunoenzymatic activity we recovered HP from FL, SCF and TPO stimulated LTC after four weeks of culture. Some samples were recovered 16 hours after IL-3 addition to FL, SCF and TPO-stimulated LTC. CD34+ and CD34- fractions of the samples were separated and cells lysis were performed. We selectively immunoprecipitated p44/42 MAP kinase from 245 μg of cell lysates. Our results showed that ERK activity was undetectable in the compartment of expanding, massive proliferating and self-renewing HP. Conversely, after treatment of LTC by IL-3, sustained (still detectable 16 hours after the stimulus) and high levels of ERK activity were seen both in the CD34+ and CD34- fractions. We found 21 units in the CD34- fraction, 58 units in the CD34+ fraction, and 95 units in the leukemic cell line HL-60 after GM-CSF stimulation. Our results add novel information on the physiology of HP. Furthermore, they may explain the undetectable or very low levels of hematopoietic toxicity recently achieved by novel treatments of cancer using ERK inhibitors. We suggest that our approach may be useful for investigating the activity of different signaling proteins in HP and that of their inhibitors.

011
MUTATIONS OF THE FAS GENE ARE NOT INVOLVED IN AIDS-RELATED LYMPHOMA

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Non-Hodgkin's lymphomas (NHL) are a frequent complication of HIV infection and a major source of morbidity and mortality among patients affected by AIDS. AIDS-related NHL are a heterogeneous group of malignancies that derive from B-cells and display an aggressive clinical behavior. The disease pathogenesis is heterogeneous depending on the specific clinico-pathologic category of the lymphoma. Because AIDS-NHL frequently express the EBV encoded protein LMP-1, which increases cellular levels of BCL-2, it has been speculated that inhibition of apoptosis may play a major role in AIDS-NHL development. Recent studies have shown that loss of function mutations in the FAS (APO-1, CD95) death receptor are involved in the pathogenesis of some types of B-cell malignancies of the immunocompetent host, although this has not been tested in AIDS-NHL. Intriguingly, autoimmune phenomena, which frequently precede and/or accompany development of AIDS-NHL, are favored by FAS mutations both in humans and in mice. In order to determine the possible implication of FAS disruption in AIDS-NHL pathogenesis, we analyzed the status of the FAS gene throughout the clinico-pathologic spectrum of the disease. A panel of 49 patients with AIDS-NHL, including AIDS-Burkitt's lymphomas (n=10), AIDS-Burkitt-like lymphomas (n=6), AIDS-diffuse large cell lymphomas (n=17), AIDS-primary effusion lymphomas (n=12) and AIDS-primary central nervous system lymphomas (n=4), was examined for FAS mutations. All nine exons and flanking intronic sequences of the FAS gene were analyzed by PCR-single strand conformation polymorphism and DNA direct sequencing. With one exception, all cases of AIDS-NHL scored negative for FAS mutations. The single mutated case was found in a primary sample of AIDS-primary central nervous system lymphoma, that showed a 18 bp deletion localized in exon 9 which encodes the death domain region of the FAS receptor. This frameshift mutation causes the introduction of a proximal stop codon. Two previously unreported polymorphisms were found at codon 19 within exon 2 and at codon 163 within exon 6. In order to investigate whether FAS inactivation in AIDS-NHL may occur through mechanisms other than gene mutation, we tested the status of FAS protein expression in selected cases. Expression of FAS scored absent in 75% of tested cases. Overall, these results suggest that genetic alterations of the FAS gene are not involved in AIDS-NHL pathogenesis. The absence of FAS mutations in AIDS-diffuse large cell lymphoma corroborates the notion that the pathogenesis of this lymphoma differs markedly from that of diffuse large cell lymphoma of immunocompetent hosts, in which FAS mutations are observed at a certain frequency. Finally, because most AIDS-NHL fail to express FAS in the presence of an intact FAS locus, it is possible that epigenetic alterations may cause downregulation of the FAS molecule in these lymphomas.

012
QUANTIFICATION OF ANGIOGENESIS IN ACUTE MYELOID LEUKEMIA: A MULTIPARAMETRIC COMPUTERIZED IMAGE ANALYSIS MODEL

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There is considerable experimental evidence to indicate that tumor growth is dependent on angiogenesis and its key role in solid tumor growth is well established. In contrast, few and partial data are available for hematologic malignancies, especially concerning bone marrow neovascularization in acute leukemia. The purpose of the current study is to propose a multiparametric quantification model for investigating the role of angiogenesis in acute myeloid leukemia (AML), evaluating the association between intratumoral microvessel density (IMD) and angiogenic factors. A series of 6 paraffin-embedded bone marrow biopsies from adult patients with AML, were treated with immunohistochemistry using the APAAP method, with the aim of evaluating the expression of endothelial cell markers (von Willebrand factor) and endothelial growth factors (VEGF); quantification analysis of the stained sections was performed by multiparametric computerized image analysis. The results of this preliminary study confirm the data of literature concerning the direct correlation between the expression of IMD and VEGF and their significance as prognostic factors, but this automatic system of analysis has been suggested as a more objective method of assessing angiogenic expression and moreover it collects many other significant observations. The main advantage of this system is the additional morphometric parameters that can be detected: the number of vessels within a certain dimension range, the vessel lumen area, the vessel lumen perimeter and the percentage of immunostained area per microscopic field. All these parameters were measured in this study and they seem to be related to leukemia progression. This aspect offers promising perspectives for their use, in a multiparameter prognostic system, in guiding therapeutic choices, in monitoring leukemia response during treatment and in predicting leukemia relapse. It is necessary to confirm the prognostic value of these observations in a prospective, controlled multicenter study.

013
EXPRESSION OF CYCLINS AND CYCLIN-DEPENDENT KINASE INHIBITORS IN HODGKIN AND REED-STERNBERG CELLS

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The cell cycle of mammalian cells is controlled by cyclins, cyclin-dependent kinases (CDK) and inhibitor proteins (CDIs). Moreover, recently the role played by tumor suppressors such as retinoblastoma protein (Rb) and p53 was partially clarified. Hodgkin's disease consists of a few neoplastic cells, Hodgkin (H)and Reed-Sternberg (R-S) cells and these are reported to express p53 and p21 frequently. To clarify the relationship between cyclins and p21 or p53, we used immunohistochemistry to study fresh lymph nodes from 5 patients with Hodgkin's lymphoma. With the APAAP immunostaining system we measured cyclins of the mitotic phase (A and B1 and cyclin-dependent kinase p34), cyclins of the G1 phase (D1, D3 and E), CDIs (p16 and p21) and tumor suppressors (Rb and p53). We used the following antibodies: anti-cyclin B1, E and A (Calbiochem); anti-cyclin D1, D3, anti-p34, anti-p16, anti-p21, anti-Rb (NeoMarkers); anti-p53 (DAKO). We observed that all cases expressed cyclin A, B1 and p34. p34 and its regulatory subunit, cyclin B, control mitotic phase, and cyclin A is also involved in mitotic phase. Only one case out of five expressed the cyclins of the G1 phase. All cases expressed p21 and only two cases expressed p16. Finally all cases expressed Rb and p53. The expression of p21 has been reported to be significantly correlated with the expression of p53 in H and R-S cells. We observed in our cases that the number of H and R-S cells positive for p21 was lower than those positive for p53. In particular the median p21 positive cells was 13% and the median p53 positive cells was 62%. In our set of patients inhibitors of G1 phase, p16 and p21, were present respectively in two and in all patients but at a low rate of neoplastic cells. It is of note that Rb was expressed in all patients and at a high rate on H and R-S cells. Rb protein could be a potent tumor suppressor, but its phosphorylation allows progression to division. In conclusion our results confirm that H and R-S cells are indeed in cell cycle in particular in the later phases and the suppressor power of p21 is expressed in a small proportion of neoplastic cells.

014
PREVALENCE OF POLYMORPHISM IN METHYLENETETRAHYDROFOLATE REDUCTASE GENE IN ADULT PATIENTS AFFECTED BY ACUTE LYMPHOBLASTIC LEUKEMIA

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A recent study has investigated the role of two polymorphisms in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene (677 C to T and 1298 A to C) resulting in thermolability and reduced MTHFR activity that decreases the pool of methyl-THF and increases the pool of methylene-THF. The study demonstrated that individuals with the MTHFR 677TT, 1298AC and 1298CC genotypes have a decreased risk of developing adult acute lymphocyte (ALL), but not acute myeloid leukemia (AML) suggesting that folate inadequacy may play an important role in development of ALL. Folate deficiency determines DNA strand breaks, leading to genetic instability through uracyl misincorporation and thus to increased cancer risk. It is likely that these mutations increase N5,N10-MTHF at the expense of N5-MTHF available for thymidylate, an essential precursor for DNA synthesis, resulting in decreased DNA uracyl levels and increased serum homocysteine. High plasma levels of homocysteine are a well known risk factor for arterial and

venous thrombosis. In two recent studies individuals homozygous for the mutant alleles of MTHFR with high plasma folate levels had a 2- to 4-fold lower risk of colon cancer than wild-type controls with low plasma folate levels. In order to verify whether MTHFR polymorphisms could have a protective role in susceptibility to developing ALL in the Italian population, we investigated the prevalence of MTHFR C677T in 73 ALL patients at diagnosis (males/females 37/36, median age 28, range 14-70) and 472 healthy controls (males/females 252/220, median age 45 years, range 7-93). The genotyping protocols for the detection of MTHFR C vs T polymorphism were adapted from Frosst. In the patient group 20 cases (27.4%) were 677CC, 39 (53.4%) 677CT and 14 (19.1%) were 677TT while in the control group 201(42.8%) were 677CC, 194(41.1%) 677CT and 77(16.3%) 677TT. The incidence of 677TT among patients with ALL and healthy controls was not statistically significantly different (Fisher's exact test p=0.50) with an OR 1.2 (CI 0.6-2.3). Adjusted ORs using 677CC as reference were respectively CT 2.0 (CI 1.1-3.6) and TT 1.8 (CI 0.9-3.8). Our data show that, at least in the Italian adult ALL population, this mutation does not confer a protective effect. The different geographic distribution of genotypes may in part account for the discrepancy in these results. Moreover folate deficiency in individuals with MTHFR polymorphism may abrogate the protective effect of 677TT genotype. Further studies on different polymorphisms of MTHFR and the evaluation of a large number of patients, including children with ALL, the most common form of leukemia in childhood, should be undertaken. MTHFR polymorphisms may also account for disease susceptibity to antifolate treatment, in particular to methotrexate.

015
MULTIDRUG RESISTANCE-1 AND LUNG RESISTANCE PROTEIN EXPRESSION IN ACUTE NON-LYMPHBLASTIC LEUKEMIA CASES

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Clinical studies about multidrug resistance in acute nonlymphoblastic leukemia (ANLL) have often yielded different results: the SWOG trial did not find any significant role on remission or survival for mdr1, mrp, lrp. In contrast, another recent paper reports that lrp conditions both remission achievement and survival. In the present study we evaluated mRNA expression of mdr1 and lrp by PCR assays in a series of 35 patients with ANLL, 20 men and 15 women, with a median age of 61.5 years. According to FAB criteria, 7 had M1, 18 M2, 2 M3, 7 M4, and 1 M5 disease. The induction treatment for all patients included idarubicin and cytosine-arabinoside, except for M3 (treated according to the AIDA protocol). The aims of the study were: 1) determination of the frequency of mdr1 and lrp expression; 2) finding of a possible relationship with biological characteristics (age, blood counts, marrow blast percentage, FAB subtype, CD34/CD33 expression); 3) finding of a possible role on achievement of complete remission and on duration of overall and disease-free survival. The mdr1 mRNA was expressed in 29% and lrp mRNA in 43% of tested cases. Co-expression of mdr1 and lrp was not significant. As expected, mdr1 expression was significantly associated with CD34+ forms, but not with a particular FAB subtype. The median age of patients included in our study was 61.5 years; this could justify a not significant correlation between age older than 60 and mdr1 that other authors have described. lrp was not correlated with age or immunophenotype, but resulted more frequently detected in M4/M5 forms. Moreover, mdr1 and lrp did not correlate with blood counts or marrow blast percentage. In our study mdr1 and lrp expression did not condition remission achievement, and duration of overall and disease-free survival was not modified by positivity for tested genes. Other tests than molecular PCR reactions (functional?) could be useful to attribute a clinical role to these genes.

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016
COMBINATION OF AMIFOSTINE AND ERYTHROPOIETIN FOR THE TREATMENT OF MYELODYSPLASTIC SYNDROMES: PRELIMINARY RESULTS

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Both amifostine and recombinant erythropoietin (r-EPO) have been demonstrated to be active, as single drugs, in ameliorating cytopenia in a limited proportion of patients with myelodysplastic syndromes (MDS). We are currently evaluating the efficacy of the combination of these two molecules in severely anemic (< 8 g/dL), transfusion–dependent MDS patients. Amifostine (Ethyol, Schering-Plough) is given as a 15' infusion of 200 mg/m^2, three times a week, for three consecutive weeks. r-EPO (Eprex, Janssen-Cilag; Neo-Recormon, Roche; Globuren, Dompe' Biotec) is administered at the fixed dose of 10,000 U s.c. every day, during amifostine treatment, and then at the same dose three times a week. Each course is 5 weeks long. At present, 3 patients have completed ten weeks of treatment and are evaluable for response. The first subject was a 61-year old male with a diagnosis of refractory anemia with excess blasts (RAEB) secondary to radio- and high dose chemotherapy, followed by autologous stem cell transplantation, for multiple myeloma. At the time of amifostine/r-EPO treatment, the patient was in complete remission from myeloma (negative serum and urine immunofixation, no marrow plasma cells) and severely pancytopenic. After one cycle of combined therapy, there was no effect on Hb levels (red-cell transfusion requirement of two units every 2 weeks remained unchanged), while a significant increase of platelet (from 8,000 to 53,000/μL) and WBC count (from 2,100 to 6,000/μL) was observed. However, a rapid leukemic evolution occurred during the second cycle of therapy and the patient died after two months, while having 180,000/μL WBC (90% myeloid blasts) and 315,000/μL platelets. The second patient was a 57-year old male with hypoplastic RAEB and severe pancytopenia, who reduced his red cell transfusional support, under amifostine/r-EPO combined therapy, from two units every 15 days to two units every month. Platelet count also

increased from 15,000 to 55,000/μL in this patient, while WBC count remained unchanged. Both parameters, however, dropped to baseline levels during the interval between the two cycles and after the final interruption of the treatment. The third patient was a 48-year old female with 5q- syndrome. At baseline, she manifested exclusively a transfusion-dependent anemia (two red-cell units every 6 weeks). No transfusional support was necessary during the ten weeks of amifostine/r-EPO combined therapy. Transfusions were again required when therapy was stopped, after two courses. The patient refused to be treated further. No particular toxic effects were recorded during the study in these patients. Our preliminary results suggest that the combination of amifostine and r-EPO may improve cytopenia in some patients with MDS. Continued therapy seems to be necessary to maintain the response. However, modalities of treatment, as applied in the present study, are quite complex and not easily accepted by patients for a long time. A larger experience on homogeneous groups of MDS patients, possibly with different schedules, is needed to assess the therapeutic role of this treatment, in particular in subjects unresponsive or partially responsive to these drugs used as single agents.

017
ASSESSMENT OF MASPIN GENE BY RT-PCR TO INVESTIGATE CIRCULATING BREAST CANCER CELLS DURING CONVENTIONAL CHEMOTHERAPY AND AFTER PERIPHERAL STEM CELL TRANSPLANTATION

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In the past, occult breast cancer cells (BCC) were evaluated by using immunocytochemical, flow cytometry and clonogenic techniques. More recently, the RNA transcript encoding for maspin, a protein likely belonging to the serpin family of protease inhibitors, has been indicated as a specific marker for breast cancer cells. From March 1995 to April 2000, 95 BC patients (73 high risk and 22 metastatic) received conventional chemotherapy plus either single (95) or double (41) or triple (4) peripheral blood stem cell transplantation (PBSCT). Up to now, 71 BC cases had been studied for maspin gene during the various phases of treatment. PBSCT protocols were the following: i) 'Gruppo Oncologico Calabrese' (GOCAM) study for metastatic BC consisting of induction chemotherapy with epirubicin 120 mg/m^2 x 3 cycles following by a double PBSCT; ii) European Union sponsored study for resistant metastatic BC consisting of a mobilization therapy with taxotere following a triple PBCT program; iii) high-dose sequential chemotherapy with PBSCT, as previously published by Milan group; iv) GROCTA study for high-risk RBC consisting of an association of taxol and epirubicin x 3 cycles, cyclophosphamide (6g/m^2) and one single PBSCT. Before induction therapy, 20% of high risk and 42.9% of metastatic BC cases were positive for maspin gene. Altogether, 20%, 38.9%, 9.8% and 13.1% of BC cases were positive at onset, during induction therapy, after PBSCT and during follow-up, respectively. It has also been suggested that the number of chemotherapy cycles affects mobilization of neoplastic cells in peripheral blood. In this study, we evaluated the possible contamination of BCC after each cycle of induction chemotherapy. Fifty-seven BC cases were evaluable after the first course of therapy, 34 for the second and 23 for the third one; whereas maspin were determined in 38 cases after the first PBSCT and 19 after the second one. The rate of contamination was 10.7% after the first course, 17% after the second and 21.7% after the third one (*p*=not significant). Similarly, no substantial difference in terms of contamination was observed between the first and the second PBSCT (7.9% versus 10.5%). In terms of clinical outcome, maspin-positive cases showed the same probability of response as maspin-negative ones. However, BC cases with more than one contaminating sample had a higher probability of progression (82%) compared to those with 1 (55%) or none (45%). Finally, tumor cell contamination failed to demonstrate any significant impact on progression-free or overall survival. These results indicate that a relatively low rate of patients show BCC contamination in their apheretic products after the first course of mobilization chemotherapy, with a trend towards a higher rate of patients with contaminated stem cell harvests after the further courses. However, besides progression rate, cases with no contaminated samples benefited from longer survival.

018
PAMIDRONATE AS SINGLE THERAPY FOR UNTREATED, ASYMPTOMATIC MYELOMA: AN INTERIM REPORT OF A PAIR-MATCHED ANALYSIS WITH HISTORICAL CONTROLS

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Asymptomatic, initial phases of monoclonal gammapathies do not require therapy until overt multiple myeloma (MM) signs or symtoms appear. It has been demonstrated that long-term treatment with pamidronate (PMD), a second generation bisphosphonate, reduces skeletal events in MM, thus improving quality of life of patients. Some evidence also suggests that PMD could have a direct anti-neoplastic activity on myelomatous plasma cells. Aiming to establish whether PMD treatment is able to prevent or at least to delay skeletal-related events or progression of the disease from time of initial diagnosis, we have so far treated with PMD (AREDIA, Novartis, 60 to 90 mg, as 4-hours infusion every four weeks for one year, then every three months, until progression) 35 patients with asymptomatic, stage IA or smoldering myeloma. In particular, criteria for inclusion were: serum M-component > 2 g/dL; bone marrow plasmocytosis > 10%; performance status 0 or 1; no presence of bone lesions, anemia, hypercalcemia, renal failure, bone pain or recurrent infections; no concomitant treatment with steroids. In doubtful cases, the absence of skeletal lesions was confirmed by magnetic resonance (MR) and/or MIBI-scintigraphy studies. Routine hemato-chemical parameters were evaluated at least every three months. Bone X-ray-survey, MR and MIBI-scans were repeated every year or when clinically indicated. No significant reduction of M-compo-

nent (> 10%) was observed in PMD treated patients during the period of the study. These patients were compared with 35 historical controls showing similar characteristics and matched for age, sex, M-component and bone marrow plasmacytosis, who had never received PMD or other bisphosphonates. After a median follow-up of 27 months (range 3-50), there have been 6 progressions to overt MM, requiring chemotherapy: 3 in the PMD group and 3 in the control group (8.5% in both arms, p n.s.). Interestingly, only one of progressions in the PMD group was associated with lytic lesions, while bone lesions were found in all controls who transformed into overt MM. A longer follow-up and a larger number of patients enrolled in this still on-going study are required to determine whether PMD is able to modify the natural history of the disease in this group of patients.

019
BIOLOGICAL EFFECTS OF MEN 11079, A NEW ANTHRACYCLINE ANALOG, ON HUMAN LEUKEMIC CELLS

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The biological activity of MEN 11079, a new anthracycline analog, was investigated in comparison with idarubicin and doxorubicin, two well-characterized drugs, in the human leukemic cell lines K-562 and HEL, and in mononuclear cells from patients with acute myeloid leukemia. Idarubicin and MEN 11079 were more active than doxorubicin in the cytotoxicity test (WST-1 assay) while idarubicin and MEN 11079 ID_{50} values were not significantly different. Though equally active, MEN 11079 seemed to ensure a smaller variability of response than idarubicin in acute myeloid leukemia cells, ID_{50} values ranging between 20-58 ng/mL and 2-350 ng/mL, respectively. Moreover, apoptosis assays (performed by annexin-V assay, propidium iodide and gel electrophoresis of fragmented DNA) and cell cycle studies demonstrated that MEN 11079 was effective at 10-fold lower concentrations compared to idarubicin and doxorubicin. Finally multidrug resistance phenotype, a major obstacle to a successful chemotherapy, was examined studing upregulation of two MDR proteins, P-glycoprotein and MRP-2. Protein levels were analyzed by flow cytometric analysis, and mRNA changes were quantified by semiquantitative RT-PCR. The levels of expression of both MDR proteins increased in response to drug treatment and effects of idarubicin and MEN 11079 were similar.

020
MOLECULAR DETECTION OF MINIMAL RESIDUAL DISEASE IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA

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Acute lymphoblastic leukemia (ALL) is a heterogeneous disease with different molecular patterns; these molecular features may help to provide markers that can be used to monitor disease and so help in developing and improving treatments.[1] The clonally rearranged immunoglobulin heavy chain (IgH) and T-cell receptor (TcR) genes can be used to follow minimal residual disease (MRD) in the majority of B-lineage ALL cases and of T-lineage cases.[2] Two large prospective studies in childhood ALL have shown that MRD analysis can predict outcome, identifying groups with good and poor prognosis.[3,4] In adult ALL there are only a few studies of MRD and its relevance is still unclear.[5,6] We studied a group of adult newly diagnosed ALL patients to test the hypothesis that the level of MRD during follow-up was associated with outcome. Between July 1996 and July 1999, 21 patients (median age 34 years; range 16-57) were classified as common (16), pro B (2), and T-ALL (3), and treated with the 0497 GIMEMA protocol. We excluded cases found to have chromosomal aberrations: 2 with t(9;22), 2 with t(4;11) and 1 with 11q23. We investigated the level of MRD using rearrangements of the TcRγ and TcRδ and Igκ (Kde) genes as molecular markers for the leukemic clone, and heteroduplex PCR analysis to detect the clonal molecular marker.[7] Sensitive detection required determination of the sequence of the marker gene from material obtained at diagnosis, and development of a specific probe that recognizes the leukemic clone, with a minimal target sensitivity of 10^{-4}-10^{-5}. The patients who attained complete morphologic remission were tested for MRD at various time points: post-induction, post-consolidation, every three months for the first year and every six months thereafter. Among ALL-Common patients, we observed 6 with Vd2-Dd3, 3 with VgI-Jg1.3/2.3, 1 with VgIV-Jg1.3/2.3 and 1 with VKII-KDE rearrangements, while in 2 cases we did not find any clonal rearrangement; of T-ALL, 2 cases had VgI-Jg1.3/2.3 rearrangement and 1 had VgIV-Jg1.3/2.3. Seven patients showed evidence of MRD (sensitivity > 10^{-2}) at the end of induction and consolidation therapy: 3 had hematological relapse after 4, 6 and 15 months; 2 relapsed only in the CNS after 6 and 7 months. The other 2 patients (both T-ALL), that are in CR with evidence of MRD, are still in early follow-up. All the 7 patients (common ALL) with no evidence of MRD (sensitivity < 10^{-4}) after induction and after consolidation therapy are still in hematologic remission with a median follow-up of 39 months (range 5 - 46 months) (Figure 1). Our preliminary results seem to confirm that there is an association between the level of MRD at the end of induction and post-consolidation therapy and the probability of relapse. Further data are needed in a large series of homogeneously treated patients to establish the significance of MRD in adult ALL.

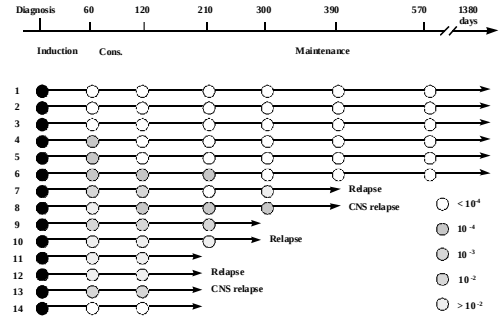

Figure 1: MRD analysis in adult ALL patients

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021
RT-PCR ANALYSIS OF MASPIN GENE EXPRESSION AS A USEFUL TOOL FOR DETECTION OF ISOLATED BREAST CANCER TUMOR CELLS IN BONE MARROW

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Detection of occult metastatic cells in bone marrow of patients with breast cancer might have important prognostic and therapeutic implications as an indicator of disseminated malignant disease. Immunocytology is currently the standard method for detecting isolated tumor cells (ITC). However, RT-PCR amplification of tissue specific mRNA could offer several advantages over the protein based assay, as these techniques are relatively easy and straightforward, very sensitive and suitable for analyzing large numbers of cells. We studied the expression of maspin mRNA in the bone marrow of 41 patients with breast cancer undergoing high-dose chemotherapy in our Institution. Twenty-five patients had high-risk early-stage disease (N+*4) and sixteen had metastases. Bone marrow specimens were collected before starting HDT and following the completion of HDT. To test the specificity of the assay, specimens of bone marrow from 35 healthy donors served as negative controls. Bilateral bone marrow samples from the iliac crests were semipurified by centrifugation on Ficoll-Hypaque density gradient and mononucleated cells were diluted in Trizol to a final concentration of 10^7/mL. RNA was then extracted according to the manufacturer's instructions and 1mg RNA was reverse-transcribed into cDNA. In order to achieve a higher specificity of the assay, previously published experimental conditions were modified by reducing the number of cycles, the duration of amplification and the amount of the primary amplified template subjected to the second PCR. As none of our bone marrow healthy donors had a positive bone marrow the specificity of RT-PCR assay was in our hands, 100% (CI95% 90-100). We consider that the changes we made were quite useful for improving the specificity of the assay, making it able to discriminate between normal controls and BC patients. Sensitivity was assayed by serially diluting MCF-7 in normal PBMC and resulted to be one tumor cell per 10^7 normal cells. Maspin transcript was found in 8 of the 25 high-risk early-stage BC patients before HDT (32%) and 6 of them were in molecular remission after completion of HDT while no patient who was previously negative turned positive. Of the two patients remaining maspin positive one relapsed 7 months later and the other remains disease free after a follow-up of 30 months. Among the 16 patients with metastatic disease, 12 were found to be bone marrow positive before treatment (75%). Fifty percent of previously positive patients became negative after HDT and no negative patient turned positive. In conclusion the maspin RT-PCR assay for BC isolated tumour cells showed high specificity and sensitivity in our hands, with a positive correlation between rate of positive results and stage of disease. Moreover, our data suggest that HDT is capable of inducing BM molecular remission in BC. The clinical significance of BM positivity for ITC should be evaluated in large prospective studies, possibly using a combination of immunocytochemical and molecular methods, including maspin detection.

022
THROMBUS PRECURSOR PROTEIN (TPPTM) LEVELS IN PATIENTS WITH BETA-THALASSEMIA AND THALASSEMIA INTERMEDIA

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Thromboembolic events (TE) are a frequent complication in patients with beta-thalassemia major (TM) and thalassemia intermedia (TI). A recent study reported that the incidence of TE in 735 subjects (683 TM and 53 TI patients) was 3.95 and 9.61% in the TM and TI group, respectively,[1] suggesting that the hypercoagulable state described in TM also exists in patients with TI. TpPTM enzyme immunoassay is a new *in vitro* diagnostic test that has been shown to measure accurately the level of soluble fibrin polymers, the immediate precursors of insoluble fibrin. The aim of our study was to determine soluble fibrin levels in 40 TM (22 females and 18 males, median age 28 years, range 11-43) and 11 TI patients (6 females and 5 males, median age 33 years, range 23-58) and to compare these results with other tests currently used for hypercoagulability screening. We also measured TpPTM levels in a group of 15 healthy donors; the mean value was 0.8±0.5 μg/mL. The results obtained in TM and TI patients are showed in the following table:

	TM (MEAN ± SD)	*TI (MEAN ± SD)*	*p*
TpPTM (mg/mL)	1.0 ± 0.9	5.7 ± 6.2	0.03
ATIII (80-120%)	92 ± 13.5	84.70 ± 13.7	n.s.
PROT.C (80-120%)	58 ± 14	56.2 ± 17.8	n.s.
APC RESIST. (r. .≥2)	3.0 ± 0.8	3.1 ± 1	n.s.

TpPTM levels were significantly (p=0.03) higher in TI than TM patients; the concentration of protein C (PC) was equally decreased in the two groups, while the ATIII levels and APC resistance were normal both in TM and TI patients. In 50 patients, we were able to compare TpPTM levels with ATIII and PC concentration and APC resistance; for this analysis, the threshold of positivity for TpPTM was set at 1.2 µg/mL, corresponding to the median value in the population studied, and we observed that TpPTM levels were significantly higher (p=0.007) in patients with the lowest ATIII concentrations, in agreement with the recent report that elevated plasma levels of thrombin antithrombin III (TAT) complexes are detected in TM patients.[2] In the TI subset 2/11 patients developed deep venous thrombosis and they had TpPTM levels higher than 1.2 µg/mL (6.4 and 6.1). These preliminary results confirm that TI more than TM patients have a subclinical chronic hypercoagulable state; moreover TpPTM enzyme immunoassay could be used in combination with conventional tests for thrombophilic screening for better identification of the patients at high risk of developing thrombosis.

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023
BASOPHILIC BLAST CRISIS OF PH+ CHRONIC MYELOID LEUKEMIA WITH TRANSLOCATION T(7;14)(P15;Q32)

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Ph+ chronic myeloid leukemia (CML) usually progresses to blast crisis (BC) that can have lymphoblastic, myeloblastic or, infrequently, erythroblastic and monoblastic characteristics. CML occasionally progresses to a basophilic and eosinophilic crisis. BC frequently shows additional karyotypic changes such as +8, i(17q) and Ph duplication. We report a case of CML with thrombocytosis that progressed to a basophilic BC with a 46,XX,t(9;22),t(7;14)(p15;q32) in the same clone. A 63-years old female diagnosed as having CML in October 1994, had mild thrombocytosis (520 x 10^9/L) and basophilia (5%) at diagnosis. Karyotype: 46,XX,t(9;22). BCR/ABL positive. Treatment with IFN and hydroxyurea, led to hematopoiesis control until November 1997 when the platelet count increased (up to 1,200 x 10^9/L). At this time the karyotype was 46,XX,t(9;22); basophils 11%, blasts 3%, and the spleen 3 cm palpable. Therapy with hydroxyurea plus thioguanine was effective until February 1999 when the leukocyte count and splenomegaly rapidly increased; in contrast, the platelet counts decreased. After 1 month the blood film showed: Hb 10 g/L; WBC 170 x 10^9/L with basophils 40%, blast 7%; platelets 60 x 10^9/L; the spleen 34 x 15 cm enlarged. Of interest, the basophilic BC arose with coincidental megakaryopoiesis failure and fast, marked spleen enlargement. The bone marrow biopsy showed an enormous increase of granulopoiesis, a decrease of erythropoiesis and almost absent megakaryopoiesis; the blast forms were about 8% and basophils were markedly increased. A mild fibrosis was present. The karyotype was 46,XX,t(9;22),t(7;14)(p15;q32)[20]/46,XX,t(9;22)[3]. Treatment with idarubicin and aracytin led to a marked reduction of leukocytosis and splenomegaly, but the patient died of myocardial failure, 1 month later. To our knowledge, only 2 cases of translocation t(7;14)(p15;q32) in hematologic neoplasias, have been reported: 1 Ph-positive acute lymphocytic leukemia (Maekawa, 1984) and 1 case of childhood AML (Raimondi, 1989). A gene which encodes a class I homoedomain protein, potentially involved in myeloid differentiation, named HOXA9 or HOX1G is located at 7p15. In CML-like cases without t(9;22) or Ph + CML in blast crisis with t(7;11)(p15;p15) HOAX9 is fused with NUP98, a nucleoporin implicated in nucleo-cytoplasmic transport. The T cell receptor γ is also located at 7p15. The T-cell leukemia-lymphoma 1"TCL1", overexpressed in T-cell prolymphocytic leukemia and rearranged in translocation t(7;14)(q35;q32.1), is mapped on chromosme 14 band q32. Moreover in one case of AML with translocation t(5;14)(q33;q32) CEV 14 gene, a transcriptional factor with leucine zipper motif mapped in 14q32, is rearranged to the transmembrane domain of the PDGFRb. Current investigation are on the way to precise the molecular cytogenetics breakpoints and the possible involvements of HOXA9, CEV 14 and TCL1 genes.

024
POSITIVE SELECTION OF PERIPHERAL BLOOD PROGENITOR CELLS: USE OF THE CLINIMACS SYSTEM AS THE ONLY METHOD FOR T-CELL DEPLETION

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Hematopoietic progenitor cells can be mobilized from peripheral blood. These cells express the CD34 antigen and are increasingly being used in allogeneic stem cell transplantation. Selection of CD34+ cells from peripheral blood progenitor cells (PBPC) harvests is generally used to reduce tumor cell contamination in autologous grafts or to have an indirect T-cell depletion in allogeneic grafts. Stable and durable engraftment following myeloablative chemotherapy can be achieved with purified autologous or allogeneic CD34+ cells. Allogeneic PBCP harversts contain high numbers of T-lymphocytes and T-cell depletion is required to reduce the risk of severe acute GVHD in HLA mismatched stem cell transplantation. We have utilized two clinical grade selection devices in the last three years: Isolex 300 (Baxter) and CliniMacs (Miltenyi). PBPC were collected from allogeneic related donors after mobilization with G-CSF 10-15 µg/Kg/die. CD34 positive selection was performed immediately or after overnight storage when two apheresis products were combined. Results of the CD34+ selections are shown in the table. Total mononuclear input cells, percentage of CD34+ cells, percentage and absolute number of CD3+ T cells were similar for the products loaded on both devices. PBPC products selected with Isolex had a higher content of CD34+ cells, but this difference was not statisti-

cally significant. The Clinimacs device gave a comparable number of CD34/Kg, a higher purity, greater efficiency of T-cell depletion, and superior recovery of CD34 and CFU-GM when compared with the Isolex device. The number of residual CD3+ T cells after selection with Clinimacs was constantly below 2e10^4/Kg, and therefore this procedure was safely used as the only method for T-cell depletion in the allogeneic transplant setting.

Device	*N°*	*CD34 % purity*	*CD34/Kg x10^6*	*CD34 yield (% of input)*	*Log T cell depletion*	*CFU-GM yield (% of input)*
ISOLEX	12	83.8±14.9	3.5±2.9	50.0±10.1	2.7±0.3	8.2±8.0
CLINIMACS	15	91.5±6.1	3.1±1.1	70.0±11.4	3.8±0.2	59.0±50.0
		p = 0.12	p = 0.42	p = 0.0001	p=0.0001	p = 0.0004

025
FISH CHARACTERIZATION OF AN ACUTE MYELOID LEUKEMIA -M4EO WITH KARYOTYPIC CHANGES INVOLVINGBOTH CHROMOSOMES16. KARYOTYPE: 46,XX,INV(16)(P13Q22),T(16;17)(P13;Q21)

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Inv(16) is typically associated with acute myelomonocytic leukemia and bone marrow eosinophilia (AML-M4). Secondary chromosomal abnormalities occur in 45% of cases and are mostly represented by trisomy 8 and trisomy 22. Recently, a few cases of inversion-associated translocations have been reported. In these cases the inverted chromosome 16 is also involved in a translocation with different chromosome partners. Using FISH, we characterized a unique case of AML-M4eo showing a typical inversion plus a reciprocal translocation involving the second chromosome 16. Karyotype: inv(16)(p13q22) t(16;17)(p13;q21). The same breakpoint, on 16p13, of the two structural rearrangements raised the question of whether a biallelic involvement of MYH11 or of another gene occurred in this case. The inversion and the translocation were studied by FISH with a panel of locus specific probes for 16p. Inv(16) was investigated using the zit cosmids (27+29 in red and 14+18 in green, distal and proximal to the MYH11 gene/16p13 breakpoint, respectively) in double color experiments, and a mixture of pac clones 20A14 and 28F4 for the 16q22/CBFB gene. The zit cosmids gave only one red spot of hybridization on inv(16) indicating that the region covered by zit18+14 was lost. The inv(16) was confirmed by pacs for the CBFB gene which were split between the short and the long arm of the inverted 16 and by RT-PCR which detected the MYH11-CBFB fusion protein. A panel of 16p probes centromeric to MYH11 and ordered from telomere to centromere (cos 5'MRP-cos 3'MRP-yac876E4-yac879F1-cos331B4-cos301B6) were used to narrow the t(16;17) breakpoint which lies between yac 876E4 and yac 879F1 in a region of 1-2 Mb. Loss of material was not found in the chromosome 16 involved in the translocation. On the basis of these findings the additional rearrangement may involve a new gene at the second 16p13 breakpoint.

026
MULTIDRUG RESISTANCE AND ADULT ACUTE LYMPHOBLASTIC LEUKEMIA: ONSET AND RELAPSE

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Background and Objectives: The role of multidrug resistance (MDR) proteins in adult acute lymphoblastic leukemias is under debate, data are still limited and conflicting. We evaluated the expression of P-glycoprotein (PGP), the multidrug resistance-associated protein (MRP), and the lung resistance related protein (LRP) and the intracellular daunorubicin accumulation (IDA) of blast cells of 80 consecutive adult patients diagnosed at the Division of Hematology of The University Hospital of Udine between 1991 and 1999. Materials and Methods: PGP, LRP and MRP expression was evaluated by flow cytometry using the MRK-16, LRP56 and MRPm6 monoclonal antibodies. The IDA was evaluated by flow cytometry after two hours of incubation with daunorubicin (1,000 ng/mL). PGP, LRP and MRP overexpression (+) was defined for MRK-16, LRP56 and MRPm6 by a mean fluorescence index (MFI) higher than 6, 5 and 3 respectively, i.e. higher than the ones observed in non-MDR cell lines and in normal bone marrow (BM) or peripheral blood (PB) mononuclear cells. A decreased IDA was defined by a normalized fluorescence index lower than 300, that is lower than IDA of non-MDR cell lines or mononuclear cells of normal PB and BM. Results: At onset 37 /80 (46 %) cases overexpressed PGP (PGP+), 8 /51 (16%) LRP , 5 /50 (10%) MRP and 19 /50 (38 %) cases were negative for the overexpression of the three proteins. Simultaneous co-overexpression of two or more proteins was observed in 11 cases: LRP+/PGP+ 7/50 (14%), PGP+/MRP+ 4/50 (8%) . Thirty-seven of the 77 (48 %) had a reduced IDA. Twenty-five patients were also studied at relapse; 18 /25 (72 %) were PGP+, 8/25 (32%) were MRP+, 10/25 (40 %) were LRP+, and 13/25 (52%) simultaneously co-overexpressed more than one protein : 8/25 were PGP+/LRP+, 1/25 was PGP+/MRP+, and 4/25 were PGP+/MRP+/LRP+. Only 3/25 (12%) cases were negative for the over expression of these proteins (p= 0.0392 with respect to onset). Twenty out of 25 (64%) patients had a reduced IDA. Interpretation and Conclusions: These data suggest that in adult acute lymphoblastic leukemias at onset PGP is frequently overexpressed, LRP and MRP are more rare by overexpressed. All these proteins are more frequently overexpressed at relapse (p=0.0433 for PGP, p=0.0399 for LRP, p=0.0252 for MRP) and the blast cells' IDA is more frequently reduced (p=0.0104). This observation suggests that in adult acute lymphoblastic leukemia there is a rapid induction of MDR related proteins, already by first relapse: so the use of non-MDR-related drugs or MDR-modifiers could offer a chance, especially in relapsed patients.

027
EXPRESSION OF MUC1 BY NEOPLASTIC PLASMA CELLS OF MULTIPLE MYELOMA

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MUC1 is a mucin transmembrane glycoprotein normally expressed on the apical surface of simple epithelia. It is over-expressed in adenocarcinomas, such as breast and ovarian cancers, often with loss of polarity and aberrant glycosylation. As a result, cryptic epitopes may be revealed. MUC1 core protein has also been found to be expressed by neoplastic plasma cells of multiple myeloma, but not by normal plasma cells, raising the possibility that MUC1 could be considered as a target antigen for activating immune attack against myeloma. To analyze further the expression of MUC1 in myeloma, we used the MoAbs HMFG1 and SM3, which recognize epitopes in the tandem repeat sequences of the glycoprotein. HMFG1 recognizes MUC1 as expressed by normal mammary epithelial cells, whereas SM3 binds only to the aberrantly glycosylated mucin expressed by breast carcinomas and so recognizes a tumour-associated epitope. HMFG1 reacted with tumor cells from 8/22 patients with myeloma, and with 1/4 patients with plasma cell leukemia. The same pattern of reactivity was seen with SM3. In 3/3 MUC1-negative cases of myeloma, we could amplify the transcript encoding the transmembrane form of MUC1 from highly enriched tumor cells, suggesting that cell surface expression was modulated or downregulated. To investigate potential factors influencing MUC1 expression at the cell surface we analyzed 9 myeloma cell lines. Initially only 5/9 expressed MUC1 protein as recognized by HMFG1, although all lines were positive at the transcript level. Following treatment with neuraminidase to remove the sialic acid residues, 2/4 of the MUC1 protein-negative lines became positive with HMFG1. These findings indicate that expression of MUC1 protein can be modulated by addition of sialic acid and possibly also by downregulation. In order to act as a target for T-cell attack, expression of MUC1-derived peptides in association with MHC I is required. The fact that transcripts are present suggests that target peptides may be produced even when cell surface expression is modulated, and that, if differential expression of peptides can be found in myeloma, induction of T-cell attack should be the goal.

028
GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND TUMOR NECROSIS FACTOR-ALPHA DIFFERENTIATE PRIMITIVE HUMAN CD34+DR- CELLS TO DENDRITIC CELLS BUT THE COMBINATION OF STEM CELL FACTOR AND FLT3-LIGAND IS ESSENTIAL TO SUSTAIN THE LONG-TERM EXPANSION OF EARLY DENDRITIC CELL PRECURSORS

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We studied cytokine-driven differentiation of primitive human CD34+HLA-DR- cells to myeloid dendritic cells (DC). Hemopoietic cells were grown in long-term cultures in the presence of various combination of early-acting cytokines such as stem cell factor (SCF) and FLT3-ligand (FLT3-L) and the differentiating growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-alpha. Two weeks of incubation with GM-CSF and TNF-α generated fully functional DC. However, clonogenic assays demonstrated that colony-forming unit DC (CFU-DC) did not survive beyond 1 week in liquid culture regardless of whether SCF and FLT3-L were added. SCF and FLT3-L alone did not support DC maturation. However, the combination of the two early-acting cytokines allowed a 100-fold expansion of CFU-DC for more than 1 month. Phenotypic analysis demonstrated the differentiation of CD34+DR- cells into CD34-CD33+DR+CD14+ cells which represented intermediate progenitors capable of differentiating into functionally active DC upon further incubation with GM-CSF and TNF-α. As expected, GM-CSF and TNF-α generated DC from committed CD34+DR+ cells. However, only SCF, with or without FLT3-L, induced the expansion of DC precursors for more than 4 weeks as documented by secondary clonogenic assays. Thus, we demonstrated that GM-CSF and TNF-α do not require additional cytokines to generate DC from primitive human CD34+DR- progenitor cells. However, they do force terminal differentiation of DC precursors. Conversely, SCF and FLT3-L do not directly affect DC differentiation but, rather, sustain the long-term expansion of CFU-DC which can be induced to produce DC by GM-CSF and TNF-α.

029
DETECTION OF T(4;14)(P16.3;Q32) CHROMOSOMAL TRANSLOCATION IN MULTIPLE MYELOMA BY RT-PCR ANALYSIS OF IGH-MMSET FUSION TRANSCRIPTS

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We and others have recently identified a novel recurring t(4;14)(p16.3;q32) translocation in multiple myeloma (MM), which leads to an apparent deregulation of the FGFR3 and WHSC1/MMSET genes. As the presence of IGH-MMSET hybrid transcripts has been found in MM cell lines with t(4;14), they may represent a specific tumor-associated marker in MM. In this study, we developed an RT-PCR assay for detecting chimeric transcripts from all of the 4p16.3 breakpoints identified so far, and used it to investigate a representative panel of 53 MM patients and 16 patients with monoclonal gammopathy of uncertain significance (MGUS); in addition, t(4;14) was investigated in all of the MM patients by means of double-color FISH. IGH-MMSET

transcripts were found in 11 (20%) of the 53 MM cases and one (6%) of the 16 cases of MGUS. There was complete concordance between the RT-PCR and FISH analyses of the MM cases. The results of this study indicate that RT-PCR is a sensitive and realiable method of detecting t(4;14) and suggest that it may be useful for monitoring the disease in a significant proportion of patients.

030
IMMUNOHISTOCHEMICAL ANALYSIS OF CYCLIN D1 SHOWS DEREGULATED EXPRESSION IN MULTIPLE MYELOMA WITH THE T(11;14)

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The t(11;14)(q13;q32) chromosomal translocation, the hallmark of mantle cell lymphoma (MCL), is recurrently found in multiple myelomas (MM) by means of conventional cytogenetics. Unlike MCL, recent molecular studies of MM-derived cell lines with t(11;14) have indicated that the breakpoints are highly dispersed over the 11q13 region; however, the fact that cyclin D1 is generally overexpressed in these cell lines suggests that this gene is the target of the translocation. In order to evaluate further the involvement of cyclin D1 in MM, we used immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) to investigate cyclin D1 expression and the presence of chromosome 11 abnormalities in a representative panel of 48 MM patients (40 at diagnosis and 8 at relapse). Cyclin D1 overexpression occurred in 12/48 (25%) of cases; combined IHC and FISH analyses in 39 patients showed cyclin D1 positivity in all of the cases (7/7) bearing the t(11;14), in two of the 13 cases with trisomy 11 and in one of the 19 cases with no apparent abnormalities of chromosome 11. Our data indicate that the t(11;14) translocation in MM leads to cyclin D1 overexpression and that immunohistochemical analysis may represent a reliable means of identifying this lesion in MM.

031
AMIFOSTINE IN THE TREATMENT OF MYELODYSPLASTIC SYNDROMES: CLINICAL AND BIOLOGICAL EFFECTS

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Recently it has been hypothesized that high levels of apoptosis may be responsible for the ineffective hematopoiesis that characterizes myelodysplastic syndromes (MDS); various oncoproteins and cytokines may be involved in the regulation of this phenomenon, through the liberation of intracellular free radicals.[1-3] The antioxidant activity of the aminothiol prodrug amifostine (AMF) justifies its use in MDS, since it determines a reduction of the cellular free radicals, which are mediators of apoptosis, consequently stimulating hematopoiesis.[4-7] We treated with AMF, at the dose of 200 mg/m^2 i.v./3 times a week x 3 consecutive weeks 6 patients with primary MDS (2 RA, 1 RARS, 2 RAEB, 1 RAEB-t), male/female 3/3, median age 63 years (range 54-68), with serious or symptomatic cytopenia, who had not responded to previous therapies. Clonal cytogenetic abnormalities were present in 4 patients (5q- in 2 cases, -Y in 1 case and del(20q) in 1 case). From 2 to 6 cycles of treatment were given at intervals of 2 weeks. The clinical and hematologic responses were evaluated according to the criteria of List *et al.*;[4] the influence of AMF on some biological parameters was also evaluated. In the RARS case and in one RA case a slight reduction of transfusion requirements was observed after the second cycle and there was a significant increase of neutrophils in the RA case with neutropenia. In the RAEB and RAEB-t cases an increase of neutrophils and reticulocytes occurred, with reduction of marrow blasts (from 12% to 5%) in one case. Platelet values did not vary significantly. No adjunctive chromosomal anomalies appeared during treatment; in 2 cases (one with -Y, one with del(20q)) the number of abnormal metaphases decreased. The *in vitro* cultures of the hematopoietic progenitors showed increased BFU-E, CFU-E and CFU-GM in 5 cases. The apoptotic index, evaluated with the TUNEL technique on bone marrow aspirate, diminished significantly from a mean of 24% (range 16-33) to a mean of 5% (range 2-13) (p=0.005), while in the immature bone marrow myeloid cells the expression of bcl-2 oncoprotein, notoriously involved in the regulation of apoptosis increased. The treatment was well tolerated: no significant side effects were observed at the dose used in our study. In conclusion, our findings show a fair hematologic response to AMF in MDS, with *in vitro* confirmation of the stimulation of hematopoiesis and with morphologic demonstration of reduction of apoptosis levels in bone marrow.

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032
CARBOXY-TERMINAL PENTAPEPTIDE OF OSTEOGENIC GROWTH PEPTIDE RETAINS HEMATOLOGIC ACTIVITY *IN VIVO*

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Osteogenic growth peptide (OGP) is a polypeptide showing regulating properties on osteogenic maturation and proliferation. OGP administration increases blood and bone marrow cellularity in mice, and may enhance engraftment of bone marrow transplantats. The carboxy-terminal pentapeptide of OGP retains several properties of the full length polypeptide. We evaluated whether OGP-derived pentapeptide (OGP 10-14) has some activity on peripheral blood cell recovery after cyclophosphamide-induced aplasia and on cell mobilization. *In vivo* results were compared to *ex vivo* colony forming tests performed on bone marrow cells from mice included in the experimental program. Mice were injected with different doses of carboxy-terminal pentapeptide OGP and blood counts were recorded. Peripheral blood stem cell mobilization was evaluated after CTX administration in the presence of OGP 10-14. Hematologic parameters and CD 34+, Sca-1+ cells were sequentially evaluated by cytofluorimetry. CFU counting in experiments performed with *ex vivo* bone marrow cells from control and test mice were performed. OGP 10-14 administration did not induce any toxicity in mice, whereas doses ranging between 0.01 and 10 nmol/mouse were able to enhance band cells and monocyte recovery after cyclophosphamide administration. An increased mobilization of peripheral blood CD 34+ cells after CTX administration was detected. In OGP-treated mice, the absolute number of colonies was increased. These results confirm the role of osteogenic growth peptide on bone marrow cells and could suggest a central role in bone and marrow differentiation/proliferation. A possible role for OGP 10-14 as a mobilizing agent could be hypothesized.

033
FCγRS CHANGES ON PMN OF CANCER PATIENTS UNDERGOING IL-2 IMMUNOTHERAPY

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Interleukin-2 (IL-2) treatment of polymorphonuclear cells (PMN) affects a variety of cellular functions including apoptosis and cytotoxicity. In this study we evaluated the impact of subcutaneous IL-2 administration on FcγRs (CD16, CD32, CD64) expression on PMN isolated from 2 groups of patients, consisting of 6 metastatic renal cell carcinoma (MRCC) and 3 with low-grade non-Hodgkin lymphomas (LGNHL) undergoing a different schedule of IL-2-based immunotherapy. MRCC patients received $6x10^6$ UL/m^2 subcutaneous IL-2 given twice daily for a month. On day 10, subcutaneous IFNα was added to the treatment and given 3 times weekly for 4 weeks. Upon hematological recovery, following autologous stem cell transplantation, LGNHL patients received two weeks of $2x10^6$ IU/m^2 subcutaneous IL-2 given on alternate days followed by $3x10^6$ IU/m^2 infused twice weekly for one year. Basal flow cytometry analysis of FcγRs expression on PMN did not show CD64 expression. By contrast, CD32 was uniformly distributed, whereas CD16 was located on 2 PMN cell subsets defined as $CD16^{low}$ and $CD16^{high}$. CD16+ PMN were electronically sorted, stained with Wright-Giemsa, and analyzed by optical microscopy. According to morphology, neutrophils were identified as the $CD16^{high}$ and eosinophil as the $CD16^{low}$ subsets. Three days after therapy, CD64 was expressed on PMN, promoting intercellular conjugate formation when PMN was coated with a bispecific antibody (Fab anti-CD64 x anti-HER-2/ neu) and incubated in the presence of HER-2/neu-transfected 3T3 cell line. In MRCC patients, CD64 expression was transient, lasting 10-15 days after the beginning of IL-2 treatment. In contrast, in LGNHL patients it remained stably expressed for months. In MRCC patients, no significant amount of serum IL-2 receptor (IL-2R) was detected before IL-2 therapy (range 129-2,129 U/mL). However, after 3 days, the level of IL-2R shed in the serum increased (range 1,900-3,649 U/mL) and reached its maximum peak after day 10 (range 8,848-11,494 U/mL). IL-2R measured in the serum of LGNHL patients during IL-2 treatment was moderately higher than its basal level, suggesting that the downregulation of CD64 observed in MRCC patients may be in part due to the neutralization of IL-2 by the soluble IL-2R released. Although CD32 expression did not show significant changes, IL- 2 treatment increased the number of $CD16^{low}$ eosinophils, leading to a change in CD16 distribution among PMN. Thus, FcγRs changes may be due to *de novo* synthesis of cytokine production induced by the IL-2 therapy. No significant amounts of serum IFNγ, IL-10, and IL-8 were measured. A high serum level of basal IL-6 was found in one MRCC patients. In conclusion, FcγRs may be used for monitoring the immunologic effects of IL-2-based immunotherapy.

034
FLUDARABINE SYNERGIZES WITH ANTI CD20 MONOCLONAL ANTIBODY RITUXIMAB IN COMPLEMENT-MEDIATED CELL LYSIS

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We have previously shown that the anti-CD20 chimeric monoclonal antibody rituximab (Roche) exerts its effects on neoplastic B-lymphoma cell lines in part via complement-dependent cell lysis. In addition membrane expression levels of complement inhibitory proteins CD55 and CD59 play a role in determining susceptibility to lysis. We have identified one t(14;18) positive human B-NHL cell line (Karpas) which is completely resistant to rituximab and complement lysis and we used it for subsequent studies on the possible interaction between this biological therapeutic agent and established antineoplastic drugs. We have exposed Karpas to several chemotherapeutic agents (doxorubicin, idarubicin,

cisplatin, taxol) at different concentrations and time schedules reflecting *in vivo* peak plasma levels and subsequently exposed the cells to rituximab and human complement. The combination of these drugs with rituximab induced only a modest additive cytotoxic effect. In contrast, similar exposure to fludarabine showed a synergistic effect with cell lysis increasing from a mean 14% with ludarabine alone to 69% with both agents. Analysis of the mechanism for this synergistic effect showed that fludarabine downmodulates the membrane expression of CD55 and CD46 complement inhibitors, without altering CD20 levels. Northern analysis demonstrated that the reduction took place at the steady state mRNA level, but no change was detected in the rate of CD55 gene transcription. Furthermore, fludarabine significantly reduced CD55 mRNA stability. This report, while giving rational support for clinical studies with combination of drugs, indicates that previously unexplored mechanisms of action by chemotherapeutic agents may be relevant for their synergistic interaction with biological therapies.

035
SCREENING OF BCR-ABL TRANSCRIPTS IN PHILADELPHIA NEGATIVE ESSENTIAL THROMBOCYTHEMIA

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Essential thrombocythemia (ET) is a chronic myeloproliferative disorder characterized by thrombocytosis, a high incidence of thrombotic complications and a low risk of progression to acute leukemia. One of the diagnostic criteria for ET established by the Polycythemia Vera Study Group (PVSG) is the absence of the Philadelphia chromosome (Ph+) which is in turn the cytogenetic hallmark of chronic myeloid leukemia (CML). Recent studies have reported controversial results on the presence of BCR-ABL rearrangements in ET patients. We evaluated by RT-PCR the presence of the BCR-ABL transcripts in 44 patients diagnosed as having ET following the PSGV criteria. Their median age was 45 years (range: 20-81), the male/female ratio was 0.7 (18/26); eleven patients were studied at the time of the diagnosis (< 6 months), 33 were investigated a median of 48 months (range 6-198) after diagnosis. At diagnosis, all patients were Ph-negative at cytogenetic level; in only one case was a t(5;13) documented. The 33 follow-up patients were treated with hydroxyurea (1 case), busulfan (2 cases), interferon-α (1 case), ASA or ticlopidine (10 cases), other combinations (17 cases; 6/17 including HU, 11/17 including BUS), no treatment (2 cases). All the described BCR-ABL transcripts were tested: the classical P210, resulting from b2a2 or b3a2 rearrangements, the P190 (e1a2 fusion) and the rare P230 (involving BCR exon 19 and ABL exon 2). Primers in the ABL gene were designed in exon 3 (a3), to detect even the rare cases in which ABL exon 2 is lost in the rearrangement (b2a3 and b3a3 junctions). Using RT-PCR, neither the 11 patients studied at diagnosis nor the 33 patients who were investigated at variable times after the onset of the disease resulted BCR-ABL positive. None of the patients progressed to secondary leukemia or showed a transformation to idiopathic myelofibrosis. Our data seem to exclude the possibility of detecting any type of BCR-ABL transcripts in patients with Ph-negative ET diagnosed according to PVSG criteria. Thus, molecular analysis for the detection of BCR-ABL rearrangements, both *classic* (P210) or resulting from alternative junctions (i.e. P190 and P230), should not be considered mandatory in these patients. Nevertheless, we are aware that a small proportion of CML cases (~5%) resemble ET at onset, because of marked thrombocytosis (>1000x10^9/L) and moderate leucocytosis (<20x10^9/L). In these patients, along with the more important cytogenetic analysis for the detection of Ph chromosome and other abnormalities (usually related to a poorer prognosis), molecular studies for BCR-ABL transcripts are strongly indicated.

036
CYTOGENETIC AND FISH STUDIES IN NEWLY DIAGNOSED MULTIPLE MYELOMA

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In malignancies with a low mitotic index such as multiple myeloma, cytogenetic analysis is difficult. Whereas an abnormal karyotype is found in approximately 40% of patients, the use of fluorescence *in situ* hybridization (FISH) and DNA content of plasmacells demonstrated abnormalities in nearly 90% of multiple myeloma (MM) patients. We performed cytogenetic analysis on 21 patients with newly diagnosed MM. The proportion of cases with chromosome alterations was 38%. Among these patients 37.5% were hyperdiploid and 62.5% were pseudodiploid. The most frequent numerical anomalies were gains of chromosomes 3, 9, 15, 18, 21 and losses involved chromosome 13. To improve the detection of aneuploidies, FISH studies with alpha-satellite probes for chromosome 1, 3 and 7 were also performed on the same patients. We chose this panel of probes because these chromosomes are some of the most frequently involved in numerical changes in MM (together with chromosomes 8, 9, 11, 13, 15, and 19). Seventeen, nineteen and seventeen patients were evaluated using alpha-satellite DNA probes for chromosome 1, 3, and 7 respectively. The percentage of aneuploidies assessed by FISH analysis was 71%. Trisomy 3 occurred in 10 of 19 evaluated patients (52.6%) and trisomy 1 in 2 of 17 (11.8%). Aneuploidies of chromosome 7 were found in 6 of 17 cases (35.2%) distributed as follow: 2 patients, trisomy 7 (11.8%); 4 patients, monosomy 7 (23.5%). In conclusion, this report suggests that interphase FISH might significantly improve the detection of aneuploidies in MM. The incidences of numerical changes concerning chromosome 1, 3, and 7 are the same as those seen in previous studies. Chromosomes 3 and 7 are confirmed to be two of the chromosomes most frequently involved in losses and gains. As far as chromosome 1 abnormalities are concerned, it is accepted that structural aberrations are more frequent than numerical changes. This FISH appoach does not permit the detection of translocations and structural aberrations in malignancies. Further studies with other techniques are

needed to clarify the pathogenesis of this disease. However, numerical abnormalities may have a role as prognostic indicators and markers for residual disease analysis studies.

037
AUTOMATED ANALYSIS OF BONE MARROW CELLULAR COMPOSITION USING BLOOD CELL ANALYZERS

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Effective quantitative analysis of cellular composition of bone marrow fluid has never been obtained using automated blood cell counters, owing to difficulties in erythroblast identification, contamination by fat particles, heterogeneity of cell types and maturation levels, low frequency of cell types and very large cells. Two of the latest generation hematology analyzers, the Abbott Cell-Dyn 4000 and the Sysmex XE-2100, can effectively count erythroblasts using laser light flow cytometry and DNA fluorochromes. We compared results of the automated analysis with those of morphologic examination of May-Grünwald-Giemsa-stained films on 400 bone marrow samples obtained by needle aspiration anticoagualted with K3-EDTA. No filtration or any type other manual preparation was required. The correlation between morphology and instrument counts was excellent for the total nucleated cell count and for the neutrophil granulocyte series; the counting of erythroblasts was also well correlated, but showed a small but consistent bias toward lower erythroblast percentages in comparison to the microscope. Squared coefficients of correlation for neutrophil percentages (including immature granulocytes) versus microscopy were $r^2 = 0.778$ for the Cell-Dyn 4000, with a slightly positive mean difference of 3.0%, and and $r^2 = 0.657$ for the XE-2100, with a slightly negative mean difference of -1.2%. Squared coefficients of correlation for erythroblast percentages versus microscopy were $r^2 = 0.539$ for the Cell-Dyn 4000, with a negative mean difference of -11.4%, and and $r^2 = 0.441$ for the XE-2100, with a negative mean difference of -13.4%. The finding of lower cytometric erythroblast counts confirms the observations previously made using flow cytometry and monoclonal antibodies. Both instruments showed good sensitivity in flagging samples with more than 5% of blasts. Moreover, observation of the instrumental cell distribution plots, together with numerical parameters, provided the morphologists with an immediate general perception of the overall quality and characteristics of the samples.

038
EVALUATION OF THE ERYTHROID COMPARTMENT FOLLOWING A NEW PREPARATIVE REGIMEN FOR BONE MARROW TRANSPLANTATION IN CLASS 3 ß-THALASSEMIC PATIENTS

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Transfusion-dependent, irregularly chelated β-thalassemic patients with hepatomegaly and portal fibrosis (risk class 3) before bone marrow transplantation (BMT), have a worse outcome after BMT. Rejection with recurrence of thalassemia and non-rejection mortality are the principal reasons. Twenty-one class 3 beta-thalassemic patients were considered in this study. All patients were prepared for the transplant with hydroxyurea and azathioprine given for 35 days and fludarabine given for 5 days before the BU-CY regimen and BMT. The aim of this study was to evaluate quantitatively the effectiveness of this new extended preparative regimen on the erythroid mass of class 3 beta-thalassemic patients. FACS analysis on hemolyzed bone marrow cells was performed to evaluate the amount of erythroid expansion (CD36, CD71 reactivity) as well as ineffective erythropoiesis (CD45-/AnV+ reactivity), mononuclear cells (CD45, CD14 reactivity), and CD34 positive cells. Values are expressed as median (range) The results show that the extended preparation for the transplant with hydroxyurea, azathioprine and fludarabine before the BU-CY regimen and BMT is particularly effective on the erythroid compartment of class 3 beta-thalassemic patients. *This work was supported by the Berloni Foundation against the Thalassemia.*

	PRE	*POST*	*p values*
WBC (x10^6/mL)	48 (12-123)	8.5 (1-43)	0.0000
CD36 (%)	26 (10-50)	13 (1-47)	0.0007
CD71 (%)	24 (3-57)	6 (0.6-31)	0.0003
CD45-AnV+(%)	20 (1-61)	0.9 (0.08-18)	0.0001
CD34 (%)	1.4 (0.4-3)	0.7 (0-3)	0.024
CD45 (%)	17 (8-39)	12 (0.6-40)	N.S.
CD14 (%)	2 (0.5-7)	1.5 (0-4)	N.S.

039
ERYTHROID EXPANSION AND SEVERITY OF EARLY APOPTOSIS IN β-THALASSEMIA. A QUANTITATIVE EVALUATION

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β thalassemia major is an hereditary disease characterized by ineffective erythropoiesis and severe anemia due to intramedullary programmed cell death (apoptosis) of the erythroid precursors. To characterize and quantify such phenomena better, we studied the immunophenotypic profile of whole bone marrow, the early apoptosis by annexinV reactivity on the erythroid precursors, and soluble transferrin receptor levels (sTfR) in the bone marrow plasma of 78 transfusions-dependent beta-thalassemic patients and 30 healthy controls matched for age and sex. The results are summarized in the Table. Beta thalassemic patients were also arbitrarily stratified into three increasing levels of erythroid hyperplasia. Statistically significant differences in the reactivity of the considered variables as well as in some clinical feature were observed between each erythroid hyperplasia level and the others. CD36 and the CD71 reactivity on all bone marrow cells and annexin V reactivity on the erythroid precursors, seem to be good indicators of erythroid expansion in beta-thalassemic patients. Moreover, quantitative evaluation of both erythroid expansion and the severity of early apoptosis could have an impact on the problem of the occasional per-

sistence of the beta-thalassemic clone after bone marrow transplantation. *This work was supported by the Berloni Foundation against Thalassemia.*

040
AUTOMATED COUNTING OF NUCLEATED RED BLOOD CELLS IN MYELOPROLIFERATIVE AND OTHER HEMOPOIETIC DISORDERS

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The last advance in flow-cytometric assessment of erythropoiesis is represented by automated recognition of nucleated red blood cells (NRBC) in peripheral blood. Circulating NRBC are found in severe hematopoietic disorders such as myeloproliferative syndromes and acute leukemias. Moreover the identification of circulating NRBC provides important clinical clues in: 1) para-physiologic states (premature babies); 2) intense erythropoietic stimulation (immune hemolytic anemia, hemolytic crisis in congenital anemias, microangiopathic anemia, severe thalassemia or sickle cell anemia, recovery from aplasia or bone marrow transplantation); 3) metastatic infiltration of bone marrow by tumor cells; 4) hypoxic states (severe hemorrhage, cardiac and pulmonary disorders, fetal asphyxia). Automated blood cell counters cannot easily distinguish NRBC from white blood cells (WBC). NRBC are variably and unpredictably included within lymphocytes or the noise component, according to their heterogeneous size and sensitivity to lysis. NRBCs are a cause of pseudolymphocytosis and can mimic lymphoproliferative disorders in instrumental reports. The majority of blood cell counters flag circulating NRBC through increased noise or disappearance of the separation between lymphocytes and noise. The specificity of the NRBC flag was reported to be above 90%; sensitivity, on the other hand, has shown a much wider range of variability (20-90%). Fully automated methods based on nuclear fluorescence are now available in multiparametric automated blood cell analyzers. We report here the results of the first European evaluation of NRBC counting with the Sysmex XE-2100. Intact WBC and NRBC are stained with a fluorescent dye. In the two-dimensional cytogram the intensity of lateral fluorescent light is represented on the x-axis, while the intensity of the forward scattered light is represented on the y-axis. NRBC are identified as a well separated cell cluster on the left side of the WBC population. We assessed imprecision, inaccuracy, clinical sensitivity and specificity of the Sysmex XE-2100 NRBC method by using ICSH and NCCLS statistical guidelines on a population of 300 samples, including: 80 healthy subjects, 61 newborn and premature babies, 33 lymphoproliferative disorders, 14 myeloproliferative syndromes, 21 acute leukemias, 9 thalassemia, 35 leukopenia after chemiotherapy and/or bone marrow transplantation, 42 miscellaneous. The level of imprecision was very low, due to the very high reproducibility (CV 12.8%) even in samples with very low NRBC count. Correlation between the XE-2100 and the microscope reference method showed a high level of accuracy (R^2 = 0.985; mean difference -1.55). The ability of the XE-2100 to identify specimens with circulating NRBC is indicated by the sensitivity of 94.8%. Specificity was also excellent (92.2%). Our results point out that the Sysmex XE-2100 method for NRBCs in peripheral blood is linear, precise and reproducible; it shows an excellent correlation with the microscope and is ready for clinical use.

041
SEVERITY OF EARLY APOPTOSIS AND ERYTHROID EXPANSION IN BETA THALASSEMIA. QUANTITATIVE EVALUATION

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ß-thalassemia major is an hereditary disease characterized by ineffective erythropoiesis and severe anemia due to the intramedullary programmed cell death (apoptosis) of the erythroid precursors. To better characterize and quantify such phenomena, we studied the immunophenotipic profile of the whole bone marrow, the early apoptosis by AnnexinV reactivity on the erythroid precursors, the soluble transferrin receptor levels (sTfR) in the bone marrow plasma of 78 transfusions dependent Beta thalassemic patients and 30 healthy controls matched for age and sex. The results are summarized in the table. Beta thalassemic patients were also arbitrarily stratified into three increasing levels of erythroid hyperplasia. Statistically significant differences in the reactivity of the considered variables as well as in some clinical feature were observed between each erythroid hyperplasia level and the others. The CD36 and the CD71 reactivity on the whole bone marrow cells and the AnV reactivity on the erythroid precursors, seem to be good indicators of the erythroid expansion in b thalassemic patients. Moreover the quantitative evaluation of both the erythroid expansion and the severity of the early apoptosis could have an impact on the problem of the occasionally persistence of the b thalassemic clone after bone marrow transplantation. *This work was supported by Berloni Foundation against Thalassemia.*

	CD36+	*CD71+*	*CD45-/AnV+*	*CD34+*
	CD71+	0.94*		
CD45-/AnV+	0.93*	0.89*		
CD34+	0.63*	0.64*	0.57*	
sTfR	0.49#	0.54#	0.41#	0.16

**p<0.0000 #p<0.005*

042
REARRANGEMENT OF ABR, AN ACTIVE BCR-RELATED GENE ON CHROMOSOME 17P, WITH ABL IN A PATIENT WITH CHRONIC MYELOID LEUKEMIA AND THE CLASSICAL B3A2 TRANSCRIPT

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While we were testing, by nested RT-PCR, a series of P210-positive chronic myeloid leukemia (CML) patients at diagnosis for the presence of alternative splicing sites of BCR, gene localized downstream of M-bcr, we found a case in which ABL exon 2 (a2) was also rearranged with the ABR gene. ABR is an active BCR-related gene located on chromosome 17p; ABR displays great homology with BCR (68% identity), interacts with members of the Rho family in cellular signaling and is particularly expressed in the brain. In our case the junction was between ABL exon 2 and a region of ABR corresponding to BCR exon 16, therefore outside M-bcr. Conventional cytogenetic studies performed on bone marrow and FISH analysis on interphase nuclei with a BCR-ABL translocation DNA probe showed a classical t(9;22). Metaphase FISH was done with a 17 painting probe, an ABL cosmid probe and a centromere probe for chromosome 9; this analysis demonstrated that chromosome 17 does not rearrange with any other chromosome. We therefore tested the patient's sample with a set of primers ABR-specific and located 3' than the region homologous with M-bcr, to avoid the possible amplification of the classical BCR-ABL rearrangement. RT-PCR highlighted a specific band, detectable only at the second step, indicating a small amount of ABR-ABL transcript. It is plausible that there is a small population of cells of unknown Ph status (i.e. Ph+ or Ph-) that bear an atypical fusion between ABL and the BCR-related gene, ABR. Further studies are required to investigate the prevalence of ABR-ABL expression in patients with BCR-ABL rearrangements and its biological and clinical significance; however, this finding shows the tendency of ABL and BCR (or related genes) to join.

043

INHIBITION OF THE INTERLEUKIN-6 PATHWAY POTENTIATES APOPTOSIS INDUCTION BY DEXAMETHASONE AND ZOLEDRONATE IN HUMAN MYELOMA CELLS

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Interleukin-6 (IL-6) is a major growth promoting and survival factor for human myeloma cells. Selective inhibition of IL-6 activity is therefore a reasonable approach to increasing the efficacy of presently available anti-myeloma agents. Sant7 is an IL-6 receptor superantagonist which prevents the assembly of functional IL-6 receptors and blocks an IL-6-mediated signaling pathway. *In vitro* cytotoxicity assays were performed by the trypan blue exclusion test and hemocytometric cell counts. Induction of apoptosis was analyzed with propidium iodide, annexin-V staining and the Mebstain method. We investigated the effects of Sant7 on growth and apoptosis of IL-6-sensitive human myeloma cell lines and of freshly isolated cells from myeloma patients. Moreover we evaluated whether Sant7 could increase the anti-myeloma activity of dexamethasone and of the novel bisphosphonate, zoledronate, given alone or in combination. We found that Sant7 has a clear anti-myeloma activity and also enhances the growth inhibition and apoptosis induced by dexamethasone and/or zoledronate on established myeloma cell lines and primary myeloma cells. Combined treatment with all three agents results in high levels of cell death. We conclude that selective interference with the IL-6 pathway provides a promising model for the experimental treatment of human myeloma.

044

THE HUMAN BLADDER CARCINOMA CELL LINE 5637 RELEASES GM-CSF WHICH INDUCES HUMAN NEUTROPHILS, MONOCYTES AND LYMPHOCYTES TO PRODUCE HB-EGF, A KNOWN GROWTH AND ANGIOGENIC FACTOR FOR A NUMBER OF EPITHELIAL CANCERS

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Heparin-binding EGF-like growth factor (HB-EGF) is a widely expressed member of the EGF superfamily which induces mitogenic and/or chemotactic activities towards different types of cells, such as smooth muscle, endothelial and normal/neoplastic epithelial cells, fibroblasts and astrocytes, through binding to EGF receptor tyrosine kinases 1 or 4 (HER-1, -4). As a membrane-bound molecule, HB-EGF exerts growth activity and adhesion capabilities and possesses the unique property of being the receptor for diphtheria toxin (DT). HB-EGF supports angiogenic activities partly through induction of secondary factors, including VEGF, and partly through its effects on smooth muscle and endothelial cells. We have previously shown that recombinant GM-CSF can specifically regulate the production of HB-EGF in normal and neoplastic myeloid cells *in vitro*. Using molecular (RT-PCR cloning, Northern blot, flow cytometry, ELISA) and functional (mitogenic activity on BALB/c 3T3 cells, sensitivity to the pro-apoptotic effect of DT) approaches we studied whether cancer cells producing GM-CSF were able to induce myeloid and lymphoid cells, such as neutrophils (PMN), monocytes (Mo) and lymphocytes (PBL), to produce HB-EGF, a potential growth and angiogenic factor for numerous types of carcinoma. To this purpose, we used the human bladder carcinoma cell line 5637 known to produce high amounts of GM-CSF, as a number of carcinomas do *in vivo*. We found that the conditioned medium of the cell line 5637 was a powerful inducer of HB-EGF in PMN, Mo and PBL, in a fashion similar to that observed in our previous experiments using recombinant GM-CSF *in vitro*. Inhibition tests with neutralizing anti-GM-CSF and anti-GM-CSF receptor mAbs specifically inhibited the production of HB-EGF by PMN, Mo and PBL. Because the 5637 cells bear HER-1, the receptor for HB-EGF, this cell line was able to release GM-CSF to induce potentially infiltrating reactive PMN, Mo and PBL to produce HB-EGF which would in turn support the proliferation of carcinoma cells themselves binding to HER-1 tyrosine kinase. Though HB-EGF is an autocrine growth factor for some types of epithelial cancers *in vivo*, our data provide evidence that inflammation- and immunity-related cells can be driven by the neoplastic cells which they infiltrate *in vivo* to produce factors, namely HB-EGF, favoring neoplastic growth and angionesis.

045
CD30-MEDIATED PATHWAYS MODULATE CXCR4/SDF-1 ACTIVITY IN THE CD4+/CD30+ CELL LINE L540

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The so far elucidated role of CD30 (a tumor necrosis factor-family receptor upregulated by interleukin-4(IL-4) and preferentially expressed/released by persistently activated Th2/0 lymphocytes) in immune priming mechanisms as well as in the pathogenesis of HIV infection provides evidence that CD30-induced activities integrate chemokine-driven modulation of cellular functions. We evaluated the possibility that signals transduced through CD30 may regulate some chemokines and/or chemokine receptors. To this purpose, we used the CD4+/CD30+ cell line L540, which is an established model for studying CD30-mediated activation. L540 cells constitutively expressed the SDF-1 receptor CXCR4 on their membrane (MFI at flow cytometry: range 101-120; mAb from Pharmingen) and released low amounts of MIP-1a (range 20-41 pg/mL) and RANTES (0.3-80 pg/mL) (Amersham ELISA kit) in standard culture conditions, while PMA stimulation induced high amounts of both chemokines. Agonistic anti-CD30 mAbs (M44 and M67, Immunex) induced nuclear mobilization of the p50/p65 NFkB complex in supershift assays. At 48 hours, there was downregulation of membrane CD30 (p=0.008), that correlated with increased sCD30 concentration in culture supernatants (mean±SEM: basal 19.3±5.5 vs stimulated 99.8±12 U/mL, p=0.0006; DAKO ELISA kit). By contrast, membrane CXCR4 was upregulated, followed by a genuine, clear-cut enhancement of the chemotactic activity exerted by SDF-1 on L540 cells. This CD30-mediated effect was coupled to an evident decrease in the rate of cell proliferation and associated with no or little effect on the production of RANTES (<=3 pg/mL) and with an apparent inhibitory effect on the release of MIP-1a (mean±SEM: basal 33.9±9vs stimulated 10.8±2.3 pg/mL, p=0.026), though RPA showed a faint, early induction of MIP-1a mRNA. Thus, CD30 cross-linking can lead to a modulation of CXCR4 and MIP-1a expression in L540 cells with functional effects, suggesting CD30-related cellular pathways could be involved in T-cell priming and activation mechanisms.

046
BONE SURGERY MAY ACT AS A STIMULUS FOR MOBILIZATION OF MESENCHYMAL PROGENITOR CELLS IN PERIPHERAL BLOOD

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Bone marrow derived mesenchymal stem cells (MSCs) can serve as a powerful tool for tissue regeneration since they can be selectively induced to differentiate into any of a variety of tissues including bone, cartilage, tendon and adipose tissue. In normal subjects, MPCs can be detected in bone marrow (BM) but not in peripheral blood (PB), however we cannot exclude the hypothesis that in particular conditions such as tissue damage, MPCs circulate in PB. Based on the knowledge that mesenchymal progenitors can differentiate into osteoblasts, we designed this study to verify whether bone tissue damage is a stimulus to MPC mobilization. We analyzed 19 patients who underwent bone surgery for maxillofacial pathologies. Serial samples of PB were analyzed at different times after surgery starting from day 1 to day 14 and then weekly until day 28. Ten mL of PB were separated by gradient centrifugation and the mononuclear cells were cultured in complete alpha medium in humidified atmosphere at 37°C for 2-5 weeks. In 5 out of 19 cases we observed stromal cell growth. The number of fibroblast colony-forming cells (CFU-F) progressively increased from day 2 (1.5 per 10^6 MNCs) to day 10 (7 per 10^6 MNCs) and became undetectable in 4 cases after 4 weeks. In only one case did we detect a small number of CFU-F (0.3 per 10^6 MNCs) until the 5th week. Stromal cell growth was never observed in samples from normal donors (n=22). Immunohistochemistry analysis was performed to confirm the stromal origin of these cells and we found that they did not express CD45, CD34, CD14, CD41, or vWF. In order to evaluate the functional properties of these stromal cells, we tested for their capacity to support allogeneic hematopoietic progenitors under long-term culture conditions, using normal bone marrow stromal layers as controls. We found that peripheral blood derived stromal cells showed a maintained capacity to support LTC-IC. We conclude that bone tissue damage probably acts as potent stimulus for MSCs mobilization but the factors responsible for this and the hypothetical contribution of these cells to bone repair require further investigation.

047
RITUXIMAB AS TREATMENT OF AUTOIMMUNE DISEASES

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Several diseases or syndromes are associated with polyclonal or monoclonal immunoglobulins that have abnormal physical properties and in many cases react or cross react with self antigens carried by erythrocytes, platelets and other normal cells. Since immunoglobulins are produced by plasma-cells that originate from B-lymphocytes and CD20 is a B-lymphocyte lineage marker, the use of an anti-CD20 monoclonal antibody can help to control the secretion of the abnormal immunoglobulins. We report the preliminary results of an ongoing clinical trial which tests the therapeutic effect of the human-mouse chimeric monoclonal antibody rituximab (Mabthera, Roche) in antibody-related immune diseases. Patients included in the study must be 18 or more years old, with an active disease resistant to full dose conventional treatments and should have interrupted other immunosoppressive or cytotoxic drugs. Treatment with steroids, if strictly necessary, is allowed during rituximab administration, but only patients who reach a substantial dose reduction or steroid suspension are considered responders. Table 1 summarizes patients' clinical characteristics before treatment. Rituximab was given iv at a fixed dose of 500 mg, corresponding to 240 to 330 mg/m^2, weekly, for a total of four doses. In three cases of type 2 mixed cryoglob-

ulinemia, rituximab administration was followed by the disappearance of purpura and arthralgia. Response duration was 3 months in one case and is not yet evaluable in the other two cases. In one patient with cold agglutinin hemolytic anemia, a complete hematologic remission was achieved, lasting for more than 6 months without any other treatment. One case of warm antibody autoimmune hemolytic anemia and one case of chronic Werlhof's disease failed to respond. In one case of myasthenia gravis, that developed after an allogeneic bone marrow transplantation, rituximab allowed the dose of prednisone to be reduced from > 0.5 to 0.1 mg/Kg/day and that of pyridostigmine from 4 to 2 mg/Kg/day. This was a very significant therapeutic achievement, because in this case the myasthenia gravis had, for many years, required intolerable amount of corticosteroids, with many severe metabolic and infectious complications. In this case rituximab not only allowed a very significant reduction of prednisone, but substantially improved the neurologic syndrome, with a Karnofsky's performance score that increased progressively from 50 to 90, after 12 months. At the same time, the titer of anti-acetylcholine receptor antibodies decreased from 50 to 7 nmol/L. In all cases CD20+ lymphocytes disappeared from peripheral blood for a minimun of 3 months; in contrast there was no substantial change in the immunoglobulin level. Two thrombotic episodes were observed, one affecting the left retinal artery and the other affecting the popliteal vein. It was not clear whether these were related to rituximab administration. In conclusion, these preliminary data support larger studies of rituximab treatment in polyclonal and monoclonal immune diseases.

Table 1. Patients' clinical characteristics before treatment with Rituximab.

Pts.	*Sex/age*	*Disease*	*Previous treatment*	*Diagnosis to Rtx interval (mos.)*
DM	M/58	Type II MC	IFN, EDX, PDN, DNZ, PL	46
CR	F/59	Type II MC	PDN	102
SG	F/67	Type II MC	PDN, PL	8
CS	M/72	Cold agglutinin anemia	PDN, AZA	6
MP	M/53	Warm antibody AHA	PDN, AZA,	7
VF	F/64	ITP	PDN, IG	6
MD	M/33	Myasthenia gravis	CSA, PDN, IG	120

Rtx: rituximab; MC: mixed cryoglobulinemia; AHA: autoimmune hemolytic anemia; ITP: immune trombocytopenic porpora; IFN: interferon; PDN: prednisone; DNZ: danazol; PL: plasmapheresis; AZA: azathioprine; IG: immunoglobulin.

048
PROTEASOME INHIBITORS: EFFECTS ON NORMAL AND MALIGNANT HEMATOPOIETIC PROGENITOR CELLS

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The ubiquitin-dependent multicatalytic protease complex or proteasome regulates the degradation of many cytoplasmic and nuclear proteins involved in cell cycle control, apoptosis and tumor growth. Inhibition of the proteasome complex can lead to cell cycle arrest, activation of caspases and ultimately cell death. Thus, proteasome inhibitors (PI) may be used to modulate the apoptotic process in actively proliferating leukemic cells with little effect on quiescent or terminally differentiated normal cells. In the present study we tested the effect of a cell permeable proteasome inhibitor with chymotrypsin-like activity on a number of myeloid leukemia cell lines and on fresh chronic myelogenous leukemia (CML) samples. The proteasome inhibition results in substantial cytotoxicity in the myeloid cell lines with IC_{50} ranging from 5 (HL60) to 2500 nM (KG1a). Pre-treatment with proteasome inhibitors enhances cytotoxicity induced by taxol and cisplatinum. The dose of the proteasome inhibitor which induces 50% inhibition in the growth of CFU-GM is 15 nM for CD34 enriched cells from patients affected by CML and 50 nM for normal subjects, thus indicating a preferential effect of the drug on neoplastic cells. Furthermore, proteasome inhibition induces apoptotic cell death, as revealed by ultrastructural changes, nuclear DNA fragmentation, cleavage of poly (ADP-ribose) polymerase (PARP) and of beta-catenin. Apoptosis is antagonized by ectopic expression of Bcl-2, but not by inactivating mutations of p53. This event is associated with a slight accumulation of Bcl-2, a decrease of Bax, but no changes in Bcl-Xl protein expression at any time point. In the Ph+ cell lines, the levels of the BCR-ABL protein are downregulated only after 48 hours of treatment with 10 nM PI. Our data, taken together, indicate that proteasome inhibitors, alone or in association with other cytotoxic agents, have anti-tumor activity against myeloid malignancies and have limited effects on normal hematopoietic progenitor cells.

049
THALIDOMIDE IN IDIOPATHIC OR MYELOPROLIFERATIVE DISEASE ASSOCIATED MYELOFIBROSIS

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In the last few years trials have been reported regarding the use of thalidomide in various hematologic malignancies. Most recent reports concern the use of the drug in multiple myeloma, and produce encouraging results. Additional reports have shown that thalidomide could be effective in other hematologic disorders, particularly in myeloproliferative disorders (MPD). Marked neovascularization of marrow stroma in MPD has been observed and this reaction is postulated to be secondary to release of bFGF, PDGF, and TGF. Thalidomide appears to inhibit bFGF-stimulated angiogenesis and TNFα production. Some trials on the use of thalidomide in MPD are ongoing, though only sporadic cases of myelofibrosis have been published as far as we know. At our institution three patients with idiopathic myelofibrosis (iMF) and two with other myeloproliferative disorders with associated MF (aMF) were given thalidomide, starting with a dose of 200 mg/die, increasing by 100 mg every two weeks, according to tolerance and response. Two iMF patients were therapy naive, whereas the other 3 patients (1 iMF and 2 aMF) had failed to respond to previous therapy

(hydroxyurea, interferon). Median age was 52 years (average 36-64). All patients had hepatosplenomegaly and three were transfusion dependent (all iMF patients). One patient had an abnormal karyotype (47,XY,+8). Two iMF patients with Hb levels < 8 g/dL before therapy became transfusion independent and experienced significant reduction in splenomegaly and improvement in platelet counts. Both have been receiving therapy for 4.5 months and their actual dose is 600 mg and 400 mg, with mild side effects including somnolence and constipation. The third iMF patient developed hemolytic anemia requiring splenectomy before any response to thalidomide could become evident. He now has a good performance status, receiving thalidomide at 400mg/die with good tolerance. Neither aMF patient showed hematologic improvement at the dose of 400mg/die, but the median time on thalidomide is only two months. In conclusion, in the dose range of 200-400mg/die thalidomide was relatively well tolerated in our patients, with grade 0-1 WHO toxicity. Side effects included somnolence, constipation and dry mouth. Our data suggest the possibility that thalidomide could have substantial efficacy in previously untreated idiopathic myelofibrosis (probably in the early proliferative phase). Treatment failure has been observed in MPD-associated myelofibrosis. Further large studies are needed to confirm these finding and to evaluate the role of thalidomide used alone and in combination therapy (i.e αIFN) in Philadelphia negative myeloproliferative disorders.

050
LIPOSOMAL DAUNORUBICIN IN THE TREATMENT OF LYMPHOMA

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It has been shown that liposomal daunorubicin (daunoxome) has improved clinical effects and reduced toxicity compared to conventional daunorubicin because of its selective and higher accumulation in neoplastic tissues. This pharmacokinetic property is due to imperfect angiogenesis and increased capillary permeability of some tumors. Moreover, daunoxome appears able to bypass multidrug resistance partially. We treated 10 newly diagnosed patients with aggressive non-Hodgkin's Lymphoma (older than 60 years: median 70; range 61-80) with P-VABEC regimen substituting daunoxome (40 mg/m^2) for daunorubicin. Non-hematologic toxicity was mild; in particular no patient showed nausea or vomiting despite no antiemetic therapy being administered; only one had diarrhea. The overall response rate was 60% (6 out of 10 patients): 5 patients achieved a complete response, 1 patient reached a partial response, 2 had no response, 1 died of ictus and 1 did not tolerate daunoxome infusion (lumbar pain). After a median follow-up of six months off therapy (range 5-7), only one patient has relapsed. This study confirms the efficacy of daunoxome in the treatment of lymphoproliferative-disorders, and shows that this drug, because of reduced side-effects, offers the patients a better quality of life.

051
A NOVEL BCR-ABL TRANSCRIPT E8-A2 ASSOCIATED WITH PHILADELPHIA CHROMOSOME POSITIVE CHRONIC MYELOID LEUKEMIA

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Some of chronic myeloid leukemia (CML) Ph+ patients may express rare BCR-ABL transcripts. In a subset of patients, we[1, 2] and others[3] previously described a new variant of BCR-ABL transcripts named e8-a2 or e8-int-a2,[1, 2] which gives rise to a p200 kDa BCR-ABL protein, in which the breakpoint is positioned on the 3' part on the BCR gene. These novel types of chimeric BCR-ABL mRNA transcript were detected in patients with Philadelphia chromosome positive (Ph+) chronic myeloid leukemia by reverse-transcription polymerase chain reaction (RT-PCR).[4-8] Conditions for RT and PCR for BCR-ABL have already been described.[7, 9] In our case an atypical amplification product was detected using primer R112 (BCR exon e1) and AZ (for the e1a2 junction), which was considerably larger than expected: 1,145 vs. 331 base pairs (bp).[9] BCR-ABL cDNA PCR products were directly sequenced and analyzed by FASTA3 analysis. In both strands, the fragment investigated showed high homology between the 5' part of exon 8 of the BCR gene (e8) and exon a2 of the ABL gene. The consequent BCR-ABL transcript was translated into a BCR-ABL protein slightly larger than p185 BCR-ABL and smaller than p210 BCR-ABL and p230 BCR-ABL: this protein of 1,804 amino acid residues and with a molecular mass of 197.5 kilodaltons (kDa) was called p200 BCR-ABL. Furthermore, we observed that sequence motifs similar to consensus binding sites of the lymphoid-associated translisin protein are present on both participating strands at 22q11 and 9q34 recombination sites, respectively. Clinically there was a good response to alfa-interferon therapy with complete hematologic response and major karyotypic conversion. A similar case was described by Byrne *et al.*[3] Our case[1] is the first description of occurrences of either an intra-exonic break in the generation of a BCR-ABL fusion transcript or of insertion of intronic sequence to generate an in-frame BCR-ABL transcript. The preservation of the translational frame for bcr-abl supports the concept that this gene product is important for the development and maintenance of CML cells.[10] Our data show that breakpoints outside M-bcr have to be considered in Ph+ CML and that variant BCR-ABL fusion transcripts and proteins can be expressed in Ph+ CML patients. *Acknowledgments. This work was supported by Italian Association of Cancer Research (A.I.R.C.) "Tumor associated antigen", by Italian C.N.R. no. 98.00526.CT04, by M.U.R.S.T. COFIN 1999 "Ph+ Leukemia", by M.U.R.S.T (S. Tura 40% 1998 and 1999), by University of Bologna Fund (M. Cavo 40%) target projects and by "30 Ore per la Vita" A.I.L. grants.*

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052
REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION FOR THE DETECTION OF MINIMAL RESIDUAL DISEASE IN MULTIPLE MYELOMA USING JUNCTIONAL REGION SPECIFIC TAQMAN PROBE AND PATIENT SPECIFIC PRIMER SET

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Analysis of minimal residual disease (MRD) can predict outcome in hematologic malignancies.[1] Our previously reported study in multiple myeloma (MM) patients obtaining PCR negativity has shown that MRD analysis using immunoglobulin (Ig) gene rearrangement (IgH) as PCR targets can identify a group of patients with prolonged survival and low rate of relapse.[2] This MRD-based risk group assignment was based on qualitative assessment but the kinetics of tumor reduction could be more predictive. Consequently, the level of MRD needs to be defined precisely in follow-up samples.[3] However, current PCR methods do not allow easy and accurate quantification.[4] We have tested *real-time* quantitative PCR (Q-PCR) using TaqMan technology and compared its sensitivity with our conventional MRD-PCR method (re-amplification of PCR amplified Ig gene rearrangement using clone-specific patients specific primers). In Q-PCR the generated specific PCR product is measured at each cycle (real-time) by cleavage of a fluorogenic intrinsic TaqMan probe. The junctional regions of rearranged Ig gene define the specificity and sensitivity of PCR-based MRD detection in MM and are generally used to design either a patient-specific primer set or probes. We have chosen a similar approach, with the design of patient-specific primers and a *common* TaqMan probe at the position of the junctional regions. We developed primers/probe combinations for Q-PCR analysis of IgH rearrangements in three randomly chosen MM patients. In one patient, several bone marrow follow-up samples were analyzed for the presence of MRD.[5] The sensitivity of the Q-PCR technique appeared to be comparable to method using reamplification of patients specific primers. Although it is still a relatively expensive method, Q-PCR allows sensitive, reproducible and quantitative MRD detection with a high throughput of samples making semi-automation possible. We consider this novel technique as an important step forward towards routinely performed diagnostic MRD studies.

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053
ALLOGENEIC BONE MARROW TRANSPLANTATION USING G-CSF PRIMED BONE MARROW

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In allogeneic transplantation, G-CSF priming of bone marrow cells before harvest may shorten time to engraftment and may lower the risk of GvHD; this latter effect could be expected to occur by G-CSF-induced cytokine modulation (Th2 polarization). We describe our experience in six cases of allogeneic bone marrow (BM) transplantation in which we used, as inoculum, bone marrow cells harvested after G-CSF priming of the donor. Glycosylated G-CSF (Myelostim – Italfarmaco) at the dosage of 5 mg/Kg/day was administered to 6 donor for 3 days, s.c, and bone marrow harvests were taken on the 4th day. Underlying diseases were: 3 CML, 1 AML, 2 ALL; in all cases donor\recipient pairs were HLA identical siblings, but in five cases there was ABO group mismatch and therefore bone marrow cells were further manipulated with a cell separator. Busulphan/cyclophosphamide were used as conditioning regimen and a short course of cyclosporin A + methotrexate as GvHD prophylaxis. For each case we evaluated graft characteristics and post-transplant hematopoietic recovery. G-CSF stimulated BM transplants were compared to a group of 15 allogeneic peripheral blood progenitor cell (PBPC) tranplants done in our Institute. G-CSF primed bone marrow harvests showed, compared to PBPC collections, a lower number of CD34+ cells (2.95 vs 8.3 x 10^6/Kg; $p = 0.018$) However these two groups of transplants did not differ in term of platelet recovery (Plt>50,000/mm^3); $p = 0.47$, and in duration of severe bone marrow aplasia (days of ANC<100/mm^3) $p = 0.11$ although we found a difference in terms of neutrophil recovery (ANC>500/cmm at median day +15.5 for G-CSF primed BM group vs days +12 for the PBSC group $p = 0.048$). In our preliminary experience G-CSF primed allogeneic bone marrow seems to lead to fast hematopoietic recovery, although longer follow-up is needed to determine the chronic GvHD rate.

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NOVEL TYPES OF BCR-ABL TRANSCRIPT WITH BREAKPOINTS IN BCR EXON 8 FOUND IN PHILADELPHIA POSITIVE PATIENTS WITH TYPICAL CHRONIC MYELOID LEUKEMIA RETAIN THE SEQUENCE ENCODING FOR THE DBL- AND CDC24 –HOMOLOGY DOMAINS BUT NOT THE PLECHSTRIN HOMOLOGY ONE

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A small proportion[1,2] of Ph+ chronic myeloid leukemia (CML) patients fail to express a b2/a2 or b3/a2 transcript. We recently described a novel type of the chimeric bcr-abl mRNA transcript in a patient with a Philadelphia chromosome positive CML. We show that these novel bcr-abl transcripts retain the DBL homology (DH) domain and the recently recognized CDC24 homology domain, but not the pleckstrin homology (PH) domain of the bcr gene. We recently described a novel type of the chimeric bcr-abl mRNA transcript in a patient with a Philadelphia chromosome positive chronic myeloid leukemia: sequence analysis of the fusion region showed a join between part of exon e8 of the bcr gene and an intronic sequence of abl intron 1b spliced on exon a2 of the abl gene, giving rise to an in-frame e8-int-a2 bcr-abl transcript, translated into a 197.5 kDa BCR-ABL protein of 1,804 amino acid residues, which we named P200 BCR-ABL.[1,2] A similar bcr-abl transcript was also described by other.[3] In this work, employing protein comparison analysis (pFAM) we show that these novel bcr-abl transcripts retain the DBL homology (DH) domain and the recently recognized CDC24 homology domain, but not the pleckstrin homology (PH) domain of the bcr gene. This observation, along with the myeloid immunophenotype of the tumor and, at least in one case, the patient's correspondingly good response to alfa-interferon therapy,[4,5] suggests that P200 BCR-ABL is more similar to P210 BCR-ABL,[5] in which the DH, CDC24 and PH domains are all maintained, than to P185,[6-7] in which these domains are all lost.

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MOLECULAR FOLLOW-UP OF PATIENTS WITH MULTIPLE MYELOMA WHO WERE REINFUSED WITH HIGHLY PURIFIED CD34+ CELLS TO SUPPORT SINGLE OR TANDEM HIGH-DOSE CHEMOTHERAPY

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Eighty-two patients with advanced multiple myeloma (MM) were enrolled in 2 sequential clinical studies of 1 or 2

courses of myeloablative therapy with stem cell support. Conditioning regimens consisted of high-dose melphalan (MEL) with or without total body irradiation (TX1 = 35) and MEL as the first preparative regimen, followed within 6 months by busulfan and melphalan (TX2 = 47). Overall, the complete remission (CR) rate of evaluated patients was 13.8% and 41% for single and double autotransplant, respectively ($p = 0.04$). Moreover, 3 patients undergoing TX2 achieved molecular remission[1-4] and 2 remain PCR-negative 36 and 24 months after autografting. Thus, whereas multiple cycles of high-dose therapy may be beneficial for patients with myeloma, the clinical impact of tumor cell purging remains highly questionable. Eighty-two patients with advanced multiple myeloma (MM) were enrolled in 2 sequential clinical studies of 1 or 2 courses of myeloablative therapy with stem cell support. Conditioning regimens consisted of high-dose melphalan (MEL) with or without total body irradiation (TX1 = 35) and MEL as the first preparative regimen, followed within 6 months by busulfan and melphalan (TX2 = 47). On the basis of adequate stem cell harvest, 31 patients (TX1 = 13; TX2 = 18) were transplanted with highly purified CD34+ cells. Positively selected stem cells did not adversely affect hematopoietic reconstitution compared with unmanipulated peripheral blood stem cell. Overall, the complete remission (CR) rate of evaluated patients was 13.8% and 41% for single and double autotransplant, respectively ($p = 0.04$). Moreover, 3 patients undergoing TX2 achieved molecular remission and 2 remain PCR-negative 36 and 24 months after autografting. The median event-free survival (EFS) for TX1 and TX2 was 17 and 35 months, respectively ($p = 0.03$). Actuarial 3-year overall survival for patients treated with 1 or 2 transplants is 76% and 92%, respectively (p = NS). On multivariate analysis, better EFS was associated with low β2 microglobulin (β2-M) level at diagnosis and TX2, whereas overall survival was correlated with β2-M. Positive selection of CD34+ cells did not influence the achievement of clinical or molecular CR,[5,6] or remission duration or survival of MM patients. Thus, whereas multiple cycles of high-dose therapy may be beneficial for patients with myeloma, the clinical impact of tumor cell purging remains highly questionable.

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EXPRESSION OF THE RARE E1/A3 AND B2/A3 TYPES OF BCR/ABL TRANSCRIPT LACKING ABL EXON 2 IN CHRONIC MYELOID LEUKEMIA PATIENTS

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The role of the different BCR/ABL fusion proteins in determining the phenotype of the Philadelphia chromosome-positive (Ph-positive) leukemias is still unclear.[1] Most chronic myeloid leukemia (CML) patients express BCR/ABL transcripts with a b3/a2 or b2/a2 fusion and a translated P210 protein. Here we report the finding of a CML patient showing concomitant expression of both the rare e1/a3 and b2/a3 types of BCR/ABL transcripts, both lacking the ABL exon 2 sequences. A 68-year old female patient was diagnosed as being in chronic-phase CML in June 1997. At that time, her peripheral blood count showed 38.4 x 10^9/L WBC (88% neutrophils, 1% basophils, 1% promyelocytes, 3% lymphocytes, 7% monocytes), 629 x 10^9/L platelets. The hemoglobin level was 12.9 g/dL, and an alkaline neutrophil phosphatase score of 10 (normal range, 10 to 100). The spleen was not palpable. Bone biopsy showed increased cellularity of the bone marrow, marked eosinophilia, and normal megakaryocytes. Cytogenetic analysis of 40 bone marrow metaphases by Giemsa banding showed a karyotype with 46XX, t(9;22)(q34;q11). The presence of BCR/ABL transcripts was studied at diagnosis by reverse-transcription polymerase chain reaction (RT-PCR).[2] Briefly, an amplification product was detected both using primers R112 and AZ (usually positive for e1/a2 transcript in some Ph+ ALL) and using primers EA12 and AZ (usually positive for b2/a2 or b3/a2 transcript): in both reactions the fragments were considerably smaller than expected (approximately 160-170 bp less).[3-6] They were both detectable after a single step of amplification. Specific half-nested RT-PCR with primers EA500 (ABL exon 3), EA122 and with R112 confirmed the amplification of abnormal BCR/ABL transcripts of 141 bp and 123 bp, respectively. Both fragments were directly sequenced and submitted to comparison by FASTA3: the two different transcripts, respectively with a b2a3 and e1a3 types of junction, lacked ABL exon 2 sequences. Semi-quantitative analysis of b2a3 and e1a3 transcripts was performed as described elsewhere[1] and revealed 380,000 and 280,000 amounts of bcr/abl transcript per µg of RNA, respectively. The transcripts with the e1a3 junction probably arose through a mechanism of alternative splicing of the longer form, as a single Ph-chromosome

was detectable in all the mitoses obtained from bone marrow cells of the patient. The finding that hybrid BCR/ABL genes lacking the ABL exon 2 sequences were naturally occurring in a small number of patients with Ph-positive leukemias and generally associated with an ALL phenotype or a rapidly progressive CML phenotype could be in favor of this hypothesis. Here we show that both main BCR/ABL fusion transcripts lacking ABL exon 2 sequences can be simultaneously expressed by the same Ph-positive clone in a patient showing a classical form of CML and this adds further complexity to an enigma that puzzles[7-8] clinical and molecular hematologists: the relationship existing between the BCR/ABL hybrid gene structure and the leukemia phenotype.

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CLINICAL VALUE OF QUANTITATIVE LONG-TERM ASSESSMENT OF BCR-ABL CHIMERIC TRANSCRIPT IN CHRONIC MYELOGENOUS LEUKEMIA PATIENTS AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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For purposes of therapeutic decision making, we used quantitative PCR[1] for molecular follow-up of 55 patients with cronic myeloid leukemia (CML) in complete remission (CR) after allogeneic bone marrow transplantation (BMT) from HLA compatible donors.[2-4] A total of 402 bone marrow samples from 40 patients transplanted in chronic phase (group 1) and 15 in accelerated/blastic phase (group 2) were analyzed by qualitative[5,6] and quantitative[1-7] PCR. Regarding clinical outcome, 34/40 (85%) group 1 vs. 8/15 (54%) group 2 patients are alive. Only 1/40 (2.5%) group 1 patients relapsed, as against 6/15 (40%) in group 2 ($p = 0.0002$). At qualitative PCR, 8/40 (19%) group 1 vs. 9/15 (60%) group 2 patients were positive, with a significantly greater total number of positive samples in group 2 (33/129, 27% vs. 16/273, 5%; p<0.001). The probability of qualitative PCR positivity >1 year after BMT was significantly lower in group 1 patients (4/40 pts, 10% vs. 9/15 pts, 60%; $p = 0.01$). At quantitative PCR, 4/8 (50%) group 1 patients were positive only once (< 400 transcripts/µgRNA). In group 2, 9/15 (60%) patients had 3 or more positive samples (always with >4,000 copies/µgRNA); therapeutic interventions (cyclosporin A discontinuation, temporary alpha-interferon or donor lymphocyte infusion) restored molecular remission in 4/9 (44%) cases. This study indicates that quantitative PCR could provide practical indications[8] capable of directing therapeutic interventions for transplanted CML patients, especially those transplanted in accelerated/blastic phase, for whom intensive monitoring is required.

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VOLUNTEER UNRELATED DONOR TRANSPLANTS FOR CHRONIC MYELOID LEUKEMIA IN CHRONIC PHASE ARE ASSOCIATED WITH A LOW INCIDENCE OF GVHD AND RELAPSE AFTER A SEROTHERAPY-CONTAINING CONDITIONING REGIMEN

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From September 1995 to December 1999, 18 consecutive adult patients with chronic myeloid leukemia (CML) in chronic phase underwent volunteer unrelated donor transplants. HLA typing required molecular identity at the HLA A, B, DRB1, DQB loci. One patient, however, had one class I mismatch. The mean interval between diagnosis and transplant was 32 ± 26 months. Conditioning was based on single fraction total body irradiation (8 or 10 Gy), 120 mg/Kg Edx and rabbit antithymocytic globulin (ATG) (Fresenius, Bad Homburg, D) 3 mg/Kg/die from days -6 to -2 (total dose 15 mg/Kg). GVHD prevention consisted of Cyclosporin A and short methotrexate. The patient population had a median age of 35 years (range 22-50); median donor age was 35 years (range 19-52). All patients engrafted, with a median time to 0.5 x 10^9/L PMN of 21 days and to 50 x 10^9/L platelets of 27 days. All patients were evaluable for acute GVHD; it occurred in 8 (I° grade 3, II° grade 2, IV° grade 3). Chronic GVHD (survival >100 days) has occurred in 2/15 patients at risk, in both cases limited. Fourteen patients are alive, with a median follow up of 21 months (range 3-52). No hematologic relapses have occurred: one patient had a molecular relapse and received interferon-α, after which he became negative; two other patients had transient molecular positivity which disappeared without intervention. Causes of death were acute GVHD/infections (3) and multiorgan failure (1). Actuarial disease-free survival at one year is 77% (95% CI, 59 - 97), with no additional events after that point to 4 years. These data show that a low incidence of severe GVHD, both acute and chronic, is achievable in most patients who received low dose ATG pre-transplants, without an increase in relapse. These results are similar, if not better, than those obtained in patients receiving transplants from HLA matched siblings.

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COMPLETE REMISSION OF SEVERE APLASTIC ANAEMIA WITH AUTOLOGOUS HAEMATOLOGIC RECONSTITUTION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: A REPORT OF 2 CASES

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Severe aplastic anemia (SAA) is a potentially fatal disorder that can be successsfully cured by allogeneic bone marow transplantation (BMT). However, an immunosuppressive regimen, usually including antilymphocyte globulin (ALG), is able to induce partial or total remission, by restoring autologous hematopoiesis, in nearly 80% of cases. We report the clinical and hematologic data of 2 patients who showed autologous recovery of hematopoiesis after allogeneic BMT, and are still alive and free from transfusions. The 1st patient, a 29-year old female, came to our observation on February 1992. A diagnosis of SAA had been made by another Institution, and she had been treated with high doses of steroids without response. She was submitted by us to BMT from her HLA and ABO identical brother in May 1992. She received cyclophosphamide (CY) (200 mg/kg over 4 days) as preparative regimen, and cyclosporine (CsA) and methotrexate (MTX) for graft-versus-host disease (GVHD) prophylaxis. After 28 days, because of graft failure, a 2nd BMT (CY + antilymphocyte globulin (ALG) + thoracoabdominal irradiation as preparative regimen) was performed, followed by a slow hematologic recovery (after G-CSF : 10 μg/Kg/die), which was detectable only from day + 41. On day + 70 , while on CsA and low dose steroids, the patient showed mixed chimerism (with only 7/15 metaphases of donor origin) and on day + 132 a complete autologous recovery was documented. CsA was discontinued on day + 540, and she is still in complete remission, without any therapy. The 2nd patient, a 41-year old female, was transplanted in January 1995, from her HLA and ABO identical brother, because of SAA, diagnosed 13 months before, at another Institution, and unsuccessfully treated with corticosteroids. The preparative regimen consisted of CY and ALG, and she received CsA and MTX for GVHD prophylaxis. After an early engraftment, first detected on day + 15, a subsequent drop of peripheral blood counts was observed from day + 22. G-CSF was promptly added (in association with corticosteroids and CsA), followed by a gradual hematopoietic recovery, which proved to be autologous. The last transfusion was performed on day + 62, and she reached normal peripheral blood values, while on CsA, after 15 months.

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DIFFERENTIAL REGULATION OF NUCLEAR FACTOR-ERYTHROID 2 EXPRESSION BY INTERLEUKIN-4 AND TRANSFORMING GROWTH FACTOR BETA-1 IN TWO MEGAKARYOBLASTIC CELL LINES (JURL MK-1 AND HEL) AND IN PRIMARY HUMAN MEGAKARYOCYTIC CELLS

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Nuclear factor-erythroid 2 (NF-E2) belongs to the basic-leucine zipper family of transcription factors and two isoforms of the human NF-E2 gene have been isolated (a and f NF-E2). The factor is involved in the regulation of globin gene transcription and is also essential for terminal megakaryocyte maturation and platelet production. However, the regulation of NF-E2 expression in megakaryocytic cells is currently obscure, as indeed are its critical targeted genes. In the present study, we investigated whether negative regulators of megakaryocytopoiesis, such as interleukin-4 (IL-4) and transforming growth factor-beta1 (TGF-β1), act through modulation of NF-E2 in two megakaryoblastic cell lines (JURL-MK1 and HEL) and in primary human megakaryocytic cells derived from normal bone marrow CD34+ cells. CD34+cells were induced to differentiate along the megakaryocytic lineage in liquid culture for 14-16 days by continuous addition of 100 ng/mL thrombopoietin. NF-E2 expression was monitored at both mRNA and protein levels by RT-PCR and immunofluorescence, respectively. Furthermore, the protein-DNA interaction was also evaluated by gel-shift assay in the two cell lines. IL-4 and TGF-β1 inhibited the growth of MK-1 and HEL cells in a dose-dependent way. IL-4 downmodulates the expression of the NF-E2 transcription factor in the two megakaryoblastic cell lines either at the protein or the mRNA level (both a and f isoforms) and in normal CD34-derived megakaryocytic cells. Downmodulation of the gene expression was associated with reduced binding of the protein to the complementary DNA sequence in cell lines. By contrast, TGF-β1 did not affect protein expression and mRNA NF-E2 abundance was partially affected since the f isoform was not influenced by the inhibitory incubation either in cell lines or in normal CD34-derived megakaryocytic cells. In conclusion, our results suggest that negative regulators of megakaryocytopoiesis may induce downmodulation of NF-E2 gene activity (as shown by interleukin-4) or may not involve NF-E2 transcription factor (e.g. transforming growth factor-beta1) in its intracellular pathway.

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NEW AND UNFREQUENT CBFβ-MYH11 FUSION TRANSCRIPTS: CLINICAL AND MOLECULAR FINDINGS IN ACUTE MYELOID LEUKEMIA PATIENTS

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Ten different CBFβ-MYH11 fusion transcripts have been reported. Two female patients with inv(16) acute myeloblastic leukemia were positive for type D and E CBFβ-MYH11 transcripts, respectively. We investigated the relationship of these rare transcripts with clinical presentation and therapeutic outcome. The pericentric inversion of chromosome 16, inv (16), and the related translocation t(16;16), associated with AML-M4 with abnormal eosinophils (M4Eo), fuse the CBFβ (core binding factor beta subunit) (16q22) to the MYH11 gene (16p13). Ten different CBFβ-MYH11 fusion transcripts have been reported. More than 85% of the positive patients have type A, and transcripts D and E account for many of the rest.[1] Two female patients with inv(16) AML were positive for type D and E transcripts. At diagnosis, both our patients showed typical AML-M4 with eosinophilic abnormalities, and no atypical clinical or laboratory features were recognizable. We investigated the relationship of these type D and E transcripts with the clinical presentation and therapeutic outcome. CBFβ and MHY11 primers specific amplifications and specific enzyme restriction digestions were used, as reported by us.[2,3] In patient #1, after amplification, a 1,157 base pairs (bp) product was obtained. Following digestion with two restriction enzymes, PstI and AccI, two fragments of 630 bp and 527 bp (PstI) and of 769 bp and 388 bp (AccI) were obtained, respectively. Both these analyses were compatible with a type D CBFβ (exon 5)-MYH11 (exon 8).[4] Similarly, in patient #2, a 1,364 bp product was found, and after restriction digestions with PstI and AccI two bands of 769 bp and 595 bp and four bands of 630, 386, 285 and 63 bp, respectively were obtained: in this case a type E CBFβ (exon5)-MYH11(exon7) was identified. Sequence analysis confirmed the breakpoints. The breakpoints of CBFβ gene occurs in intron 5 (nucleotide 495) in nearly 99% of cases, comprising our two variant ones; in rare cases the breakpoints are located in intron 4 (nucleotide 399). Within the MYH11 gene, the breakpoint occurs at least in eight different points; seven different exons (from exons 7 to 13) are variably included in CBFβ-MYH11 fusion transcripts. Fusion breakpoints mostly occur at exon boundaries but rare cases of intraexonic breaks have also been recently reported.[5] Inv(16) positive AML is associated with a good prognosis, particularly after induction and consolidation chemotherapy including intermediate/high dosage Aracytin.[3,6,7] In both our patients, the response to chemotherapy was excellent with complete clinical and cytogenetic remission (overall survival: 19 and 30 months).[8] Concerning the clinical value of molecular remission, at present no conclusion can be reached for type A CBFβ-MYH11, since patients with long lasting clinical remission may display either negative or positive PCR results in their molecular follow-up.[9,10] Nevertheless due to the limited number of patients with rare CBFβ-MYH11 fusion transcript analyzed, to our knowledge, no information about the value of detection of CBFβ-MYH11 transcripts during follow-up can be obtained from literature. In this context, the present study is the first report of PCR negativisation at remission in inv(16) AML patients without type A transcript. This finding, coupled with the typical clinical and morphologic features found at presentation, suggest that the clinical outcome of patients with type D and E transcripts may be rather similar to those with type A.

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062
MOLECULAR MONITORING OF MINIMAL RESIDUAL DISEASE IN PATIENTS IN LONG-TERM COMPLETE REMISSION FOLLOWING ALLOGENEIC STEM CELL TRANSPLANTATION FOR MULTIPLE MYELOMA

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In the present study we used a polymerase chain reaction (PCR)-based strategy to retrospectively detect the presence of minimal residual disease (MRD) in serial post-transplant bone marrow samples obtained from 13 multiple myeloma (MM) patients in remission after allogeneic transplantation of hemopoietic stem cells (allo SCT). For this purpose, patient-specific primers were generated from complementarity determining regions 2 and 3 of the rearranged IgH gene. The level of sensitivity of the PCR-based assay ranged from 1 in 100,000 to 1 in 1,000,000 normal marrow cells. All patients were PCR-positive at the time of allo SCT. Following transplantation, 9 patients out of 12 who attained stringently defined complete remission (CR) became PCR-negative at a median of 6 months (range, 3 to 120 months). In contrast, in the remaining 4 patients a clonal product was demonstrated for 12 to 72 months after transplantation. All PCR-negative patients were serially monitored for MRD for a median of 36 months, and they always tested PCR-negative. In particular, in 7 of the patients there were no residual myeloma cells detected by PCR in any sample that was analyzed up to 36, 36, 36, 48, 72, 72 and 120 months after transplantation. All these patients are presently alive, relapse-free, 36 to 188 months (median, 82 months) after allo SCT. It is concluded that allo SCT has the potential ability to induce sustained serologic and molecular CR in selected patients with MM. Larger molecular monitoring studies are required to assess the prognostic relevance of MRD detection in MM.

063
HETEROGENEITY OF THE AMOUNT OF BCR-ABL TRANSCRIPT BY REAL-TIME QUANTITATIVE PCR IN P230 CHRONIC MYELOID LEUKEMIA PATIENTS

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A small proportion of Philadelphia positive chronic myeloid leukemia (CML) patient display a BCR-ABL transcript resulting from a fusion between BCR exon 19 and ABL exon 2, encoding the large P230 hybrid protein. This rearrangement is usually associated with a mild form of CML, defined as *chronic neutrophilic leukemia* (CNL). Nonetheless, recent data seem to suggest heterogeneity in the clinical course of P230-positive patients, since some of them have aggressive disease with rapid progression to the blastic phase and/or resistance to interferon (IFN) therapy, while other cases show an indolent disease. In most of these P230 cases the presence of e14a2 (P210) transcripts is completely undetectable, but we found a case expressing this transcript as a result of alternative splicing of the BCR gene. We analyzed 5 P230-positive CML patients with a new quantitative PCR technique (Real-Time Taqman) which employs the fluorescent reaction resulting from the hybridization of a specific probe with the target sequence and we found a great variability in the copy amount of the P230 rearrangement, ranging from more than 400,000 copies in a patient who rapidly evolved to an accelerated phase to 20,000 copies in a patient who remained in a prolonged chronic phase. Our results show that a high copy number relates to more aggressive disease, even through the reason for this correlation remains uncertain.

064
A MULTIPLEX RT-PCR STRATEGY FOR RAPID MOLECULAR DIAGNOSIS OF THE MOST COMMON FUSION GENES IN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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More than 50% of patients with acute lymphoblastic leukemia (ALL) presently show detectable genetic alterations. These alterations contribute to a better classification of the disease and, in several instances, provide independent prognostic factors of clinical outcome. These considerations strengthen the need for a rapid and careful identification of

all the most common genetic lesions. To this purpose Pallisgard *et al.* (Blood 1998; 92:574) recently proposed a multiplex RT-PCR analysis for quick detection of the most common fusion genes occurring in patients with acute leukemias. In this study, in 46 ALL cases (median age 29 years, range 16 to 58 years) we used a modified version of this multiplex RT-PCR method, adapted to identify the alterations frequently occurring in ALL patients, and compared the observed results to those achieved by cytogenetic, Southern-blot, and standard RT-PCR analyses. As a first step of our RT-PCR multiplex assay two reaction tubes were setup to amplify the BCR/ABL p190 (e2a2) and p210 (b2a2 and b3a2) isoforms, ALL1/AF4, ALL1/ENL (vial No.1), and E2A/PBX1, TEL/AML1, SIL/TAL, HLF/E2A (vial No.2). Oligoprimers to amplify the E2A gene were also added to each tube as an internal control. In addition, to avoid false positive results, a negative control, consisting of all reagents without RNA, was performed in each experiment. If an amplification product was obtained in one tube, then a series of RT-PCR reactions were performed in order to confirm and identify precisely the specific fusion gene. Amplified products were observed in 25/46 cases (54%). These included BCR/ABL p190 in 6/46 patients (13%), BCR/ABL p210 in 5/46 (11%), ALL1/AF4 in 5/46 (11%), ALL1/ELL in 1/46 (2%), TEL/AML1 in 3/46 (6%), E2A/PBX1 in 5/46 (11%). With respect to comparison with cytogenetic data t(9;22), t(4;11), t(12;21), t(1;19) balanced translocations, when present, were always confirmed by multiplex PCR. In addition, BCR/ABL, ALL1/AF4, ALL1/ENL, TEL/AML1 and E2A/PBX1 fusion transcripts were found in 4, 2, 1, 2 and 3 patients, respectively, without evidence of the corresponding karyotypic alteration. In conclusion, our data demonstrate that this multiplex RT-PCR strategy is a powerful assay for rapid screening of the most common genetic alterations associated with ALLs. These observations could prove extremely relevant in order to provide rapid molecular diagnosis for risk-adapted treatment of ALL patients.

065
ANTI CD20 MONOCLONAL ANTIBODY TREATMENT IN HAIRY CELL LEUKEMIA PATIENTS. REPORT OF A CASE

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CD20 is a transmembrane protein expressed on virtually all B-cells including those in B-cell lymphomas and leukemias. It does not modulate, nor shed or internalize and is not expressed on hematopoietic stem cells. It therefore represents an ideal target for antibody immunotherapy. Its function is not well defined, although it may serve as a calcium channel involved in B-cell activation, differentiation and proliferation. *In vivo* administration of anti CD20 monoclonal antibody (MoAb) induces tumor cell lysis through several mechanisms including activation of C1q component of complement, antibody dependent cellular cytolysis (ADCC) and induction of apoptosis. Encouraging results have been obtained with anti CD20 therapy in patients with different lymphoid malignancies including those with follicular lymphomas in which neoplastic B-cells display large amounts of CD20 on the cell surface. We report here on a patient with typical hairy cell leukemia in leukemic phase progressed after 2-CdA treatment. Pre-clinical *in vitro* studies showed that after 3 hours incubation of hairy cells (HC) with anti CD20 MoAb and human serum, 75% of the cells underwent lysis as evaluated by orange acridine test in flow cytometry. The patient was then treated with anti CD20 MoAb (Mabthera, Roche) at a dose of 375 mg/m^2 continuous infusion for 24 hours. WBC count, cytological and immunophenotypic studies were performed on peripheral blood before treatment and 6, 12, 24 and 72 hours after starting CD20 administration. Soon after 6 hours from the onset of CD20 treatment, there was a dramatic reduction, both in proportion and absolute number, of HC count from 7.1 x 10^9/L to 1.9 x 10^9/L together with an evident increase in the proportion of neutrophils from 11% to 68%. These results were stable for only 24 hours after which HC and neutrophils returned to the previous values. In conclusion, on the basis of these results, we demonstrated a very dramatic sensitivity of HC to the lytic effect of anti CD20 MoAb which unfortunately lasted only 24 hours. The very fast recovery of circulating neoplastic cells was probably due to the rapid *consumption* of the MoAb because of the very high number of HC expressing a large amount of CD20 receptor. Secondly, the possibility of a temporary displacement of the HC coupled to the anti CD20 MoAb cannot be excluded.

066
FLUORESCENCE *IN SITU* HYBRIDIZATION FOR THE REFINEMENT OF VARIANT AND COMPLEX TRANSLOCATIONS IN MYELOPROLIFERATIVE SYNDROMES, IN MYELODYSPLASIA AND IN ACUTE MYELOID LEUKEMIA

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Some patients affected by chronic myeloid leukemia (CML) and acute promyelocytic leukemia (APL) may harbor t(9;22) and t(15;17) or variant translocations undetectable by conventional cytogenetic (CC) analyses. Moreover other patients with myeloproliferative syndromes (MPS) or myelodysplastic syndromes (MDS) may show a complex cytogenetic picture by CC. In these patients we decided to apply FISH for better refinement of the chromosome pattern. In CML and in APL cases we have used both the probes revealing the standard gene rearrangements, i.e. the minor BCR/ABL probe and the RARα/PML probe, and probes painting all chromosomes 9,22 and 15, 17. In MPS and in MDS patients the probes used were those for the chromosomes that, by CC, were presumably involved in the complex karyotype. All probes were commercially obtained and applied according to manufacturers guidelines (Appligene Oncor). In a CML patient with a masked Ph a BCR/ABL rearrangement was identified by FISH, which showed a fused signal within the long arm of chromosome 22. In another CML case CC detected the Ph chromosome but numbers 9 appeared normal. FISH confirmed the presence of the t(9;22). In an APL case without the t(15;17) on CC this translocation due to an insertion was documented by FISH. In two APL cases the use of FISH allowed better definition of the variant rearrangement, namely a t(15;17;20) and a t(1;15;17).

In another two APL cases an isochromosome for the long arms of number 17 was seen on CC. In these two patients FISH identified a fused signal on both arms of the isochromosome in one case, while on only one isochromosome arm in the other. In two patients with MPS and in two with MDS the complex karyotypes partially defined by CC were fully clarified by FISH with multiple differently labeled probes. In conclusion FISH is of paramount importance for the diagnosis of CML and APL, unmasking the specific rearrangement even in cases in which CC has not identified a translocation. FISH is complementary to CC and mandatory for a better definition of variant translocations and complex karyotypes.

067
NUMERICAL ALTERATIONS OF CHROMOSOME 17 AND P53 PROTEIN EXPRESSION IN NON-HODGKIN'S LYMPHOMAS IN LEUKEMIC PHASE

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The p53 tumor suppressor gene, which is inactivated by deletion and/or mutation in most types of solid tumors, is localized on chromosome 17. p53 mutations lead to a marked increase in protein half-life with high intracellular concentration of the p53 protein. A strong correlation between numerical chromosome 17 alterations and p53 gene mutation has been reported in a number of solid tumors. Since the detection of the p53 protein by immunologic techniques is strongly associated with the gene mutation, we investigated the number of copies of chromosome 17 and p53 protein expression in 29 cases of non-Hodgkin's lymphoma (NHL) in leukemic phase at diagnosis (n=19) or in progression (n=10). An enzymatic *in situ* hybridization with the centromere specific DNA probe was used to identify the number of copies of chromosome 17. To identify p53 protein accumulation we used the DO-7 (Dako) monoclonal antibody immunocytochemical technique. The overall frequency of p53 protein expression was 41% (12/29 cases) with a percentage of p53 positive cells (24%±29) significantly lower than the percentage of peripheral blood leukemic cells (71.5%±8.6). According to the phase of the disease, p53 accumulation was more frequent in progression (50%) than at diagnosis (29%). A numerical alteration of chromosome 17 (3 and/or 4 copies) was observed in 4 cases (14%) with a percentage of polysomic cells of 23%±13. No monosomic cases were observed. According to p53 immunostaining, two polysomic cases were p53 positive. Furthermore, we observed the coexistence of polysomic and normal cells, as well as p53 positive and negative cells, within the same leukemic population. Despite the high frequency of p53 expression observed, no correlation was found with the numerical alterations of the chromosome 17. The coexistence of normal and polysomic, as well as p53 positive and negative cells, highlights the heterogeneity of the leukemic population. As observed in chronic lymphocytic leukemia, p53 deregulation in NHL in leukemic phase appears to be involved in the progression of the disease. *(S. Masi was supported by a fellowship from FIRC).*

068
MOLECULAR CHARACTERIZATION IN 9 PATIENTS AFFECTED BY PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal stem cell disorder characterized by the expansion of a hematopoietic cell clone unable to produce the glycosyl-phosphatidyl inositol (GPI) anchor. The clinical symptoms are intravascular hemolysis and hemoglobinuria. In some cases a relationship between aplastic anemia and PNH has been observed. In 15 % of patients a complete clinical remission has been described. The gene involved in PNH (PIG-A gene) is localized on chromosome X and is splitted in 6 exons. A variety of mutations have so far been reported. The aim of this study was to investigate the molecular defect in 8 patients with hemolytic PNH (4 males and 4 females, age 24-48 years). Moreover, a 51-year old female who had spontaneously recovered from severe hemolytic PNH at the age of 32 years was studied. The diagnosis of PNH was made by Ham's and sucrose hemolysis tests and by red cell CD55 and CD59 antigen determination. The study of the entire codifying region and flanking intronic sequences of PIG-A gene was done by SSCP analysis and sequencing. The DNA sequence revealed the presence of one known and 8 new mutations. Results are reported in the Table together with some hematologic data at the time of the study. Mutation C55T has been already described in 5 PNH patients, but its nature was controversial. C55T was the only mutation found in patient SL, who spontaneously recovered from PNH. It was also detected in patient DTI, in association with mutation T728C. To exclude the somatic origin of this variant, mucous membrane cells were analyzed and found to be positive. C55T was also present in 1/100 normal alleles investigated, thus confirming the polymorphic nature of the variant. Mutations so far identified could be useful as molecular markers for monitoring the course of the disease during therapy.

Table 1.

Pts.	*Hb g/dL*	*Tx*	*% CD55/59–*	*Mutation*	*Effect*
ZT	7.3	occasional	36/35	del 258-264	fs; stop codon 93 aa
AL	8.9	no	17/18	del C423	fs; stop codon 170 aa
SD	9.6	occasional	39/33	del G 342	fs; stop codon 170 aa
PL	7.0	2U/mo	47/59	dupl 604-610	fs; stop codon 203 aa
CM	7.5	no	99/99	del T689	fs; stop codon 245 aa
CP	7.3	occasional	21/36	del T774	fs; stop codon 259 aa
DC	10.5	no	29/28	del 1251-1254	fs; stop codon 422 aa
DTI	6.6	occasional	71/84	C55T; T728C	Arg19-Trp; Leu 243-Pro
SL	13.1	no	0/0	nt 55 C-T	Arg 19-Trp

069
REACTIVATION OF HEPATITIS VIRUSES UNDER STANDARD OR HIGH DOSE CHEMOTHERAPY

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Background: Liver damage linked to hepatitis viruses B (HBV) and C (HCV) frequently occurs during chemotherapy. Its frequency is a function of the diffusion of previous, often unrecognized chronic infection with HBV or HCV. HBV is present in about 1% and HCV in up to 5% of adult italians. A massive increase of viral replication occurs under the immunosuppression of chemotherapy, and then upon immune reconstitution a large viral load is presented to the immune system. Severe *hepatic* reactivations then occur, jeopardizing treatment schedules and producing a high mortality rate. *Patients*: We prospectively assessed the prevalence of HBV and HCV infection in a cohort of 77 patients (males/females 32/45, mean age 42 years, range 19-87). Fifty-one (66%) had a lymphoproliferative disease, 14 (18%) a myeloproliferative disease and 12 (16%) breast cancer. Sixty-six received standard regimens, and 9 high-dose chemotherapy. Follow-up was performed by quarterly testing for liver enzymes and viral markers, including HBV-DNA and HCV-RNA by PCR. *Interventions*: HBsAg positive patients received lamivudine (100-150 mg/day) either as preemptive prophylaxis or as treatment for HBV reactivation. Anti-HCV positive patients received α-interferon (IFN, 3 MU daily) if their alanine aminotransferase (ALT) levels exceeded twice normal, and if there was no marked cytopenia. *Results*: Upon enrollment, 55.8% of patients had no serologic evidence of HBV or HCV, 31.2% had features of past HBV infection (anti-HBc and/or anti-HBs positive, HBsAg negative), 5.2% were HBsAg positive, 5.2% were anti-HCV positive without HBV markers and 2.6% anti-HCV positive with HBV markers. Of the 4 HBsAg positive subjects, one was HBeAg positive and 3 HBeAg negative; all however were HBV-DNA positive by PCR. Liver damage, became evident in 2 cases who did not receive lamivudine prophylaxis. In both, an ALT peak above 20 times normal appeared, heralded by a 2-3 log rise in HBV-DNA concentrations. Lamivudine therapy induced a prompt fall, within 15 days, of serum HBV-DNA and of ALT. In both patients it was possible to reintroduce chemotherapy under lamivudine coverage. The remaining two patients underwent pre-emptive therapy and become HBV-DNA negative without any ALT increase during or after chemotherapy. Treatment with lamivudine was continued for 3 months after completion of chemotherapy. None of the 26 patients with markers of past HBV infection (including 2 anti-HCV positive) became HBsAg or HBV-DNA positive during the observation period, nor had a rise in ALT. All 7 anti-HCV positive subjects were HCV-RNA positive by PCR. Remarkably, 1/69 anti-HCV negative subjects was HCV-RNA positive. Only one of the HCV-RNA positive cases had ALT beyond twice normal and received IFN. His HCV-RNA level fell by 2 logs, and became undetectable by the end of treatment. *Conclusions*: Reactivation of hepatitis viruses is a clinically significant event only for HBV infection, while HCV does not seem to cause major cytolytic damage even under immunosuppression. Previous HBV infection, at variance with the experience of other groups, did not represent a high risk for HBV reactivation. Pre-emptive treatment with lamivudine prevents HBV reactivation, and is nowadays a must for all HbsAg-positive patients undergoing chemotherapy.

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070
MOLECULAR REMISSION IN A CASE OF ADULT ANLL P210 BCR/ABL POSITIVE

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Adult ANLL BCR/ABL P210 positive has an estimated prevalence of about 3% . There is consensus that the prognosis of this variant is very poor and sometimes it is difficult to distinguish between a chronic myeloid leukemia blast crisis and a *de novo* ANLL with t(9;22) and P210 BCR/ABL hybrid.[1] We now report the case of a 63-year old woman, admitted in February because of leukocytosis (WBC 200x 10^6/L), mild thrombocytopenia (PLT 83x10^6/L) and anemia (Hgb 8.68 g/L). Peripheral blood and bone marrow specimens, immunophenotype and citochemistry were consistent with an acute myeloid leukemia (FAB M4). Nested RT-PCR[2] showed the BCR/ABL (P210 b3a2) rearrangement and the FISH evaluation of the hybrid was positive in 10% of the examinated nuclei. The patient was treated according to the following schedule : liposomial daunorubicin (daunoXome) 80 mg/ m^2 (by 6 hours i.v. infusion, on days 1,2,3,4) and ARA-C 100 mg/m^2 i.v. (for 7 consecutive days given by continuous infusion). G-CSF was administered s.c. starting from day +10. Adverse events consisted of hematologic (WHO grade 4) and gastrointestinal (WHO grade 3) toxicity. Post-induction bone marrow evaluation showed complete haematological remission, confirmed by the disappearance of the BCR/ABL transcript. The patient was then consolidated with a second course of chemotherapy and subsequent maintenance therapy with α-IFN. The patient is still in molecular remission after 3 months of follow up.

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071
CHARACTERIZATION OF LEUKEMIC-ASSOCIATED PHENOTYPES FOR THE DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE LEUKEMIAS

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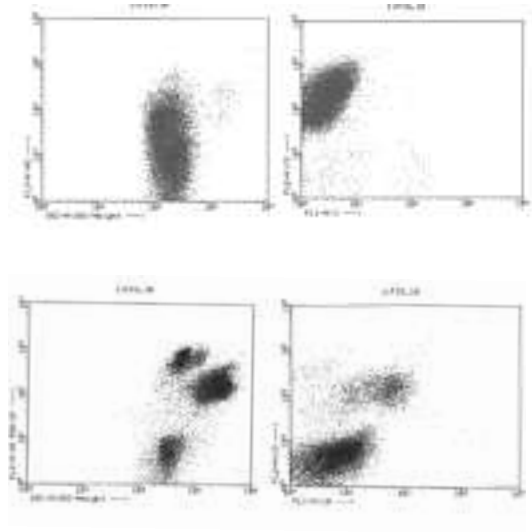
Immunologic studies of leukemic blasts at diagnosis have become critical also in identifying biphenotypic leukemias, acute myeloid leukemia (AML) expressing lymphoid markers and acute lymphocytic leukemia (ALL) expressing myeloid markers.At present, while the prognostic value of individual antigen expressions is still controversial, the existence of leukemic-associated phenotypes has been suggested to be a valuable tool for the detection of minimal residual disease (MRD) in acute leukemia patients. The aim of the present study was to analyze the proportion of acute leukemia patients (both with lymphoid and myeloid leukemias) in which the immunologic detection of leukemic-associated phenotypes was suitable for the distinction of leukemic and normal cells. For this purpose we prospectively investigated the immunophenotype of blast cells from 91 patients at diagnosis with a large panel of monoclonal antibodies in triple staining combinations analyzed at flow cytometry, with a method employing CD45-gating of AML and ALL blasts, and CD19/CD45-gating of CD45-/+ B-ALL blasts (Figure 1A), in order to detect lineage infidelity, antigenic overexpression, and asynchronous antigenic expression, as well as aberrant light-scatter patterns. In the analysis of the 61 AML cases, leukemic cells with immunophenotypes comparable to their normal counterparts were detected in 33 patients (54%). Twenty-eight of the 61 AML cases analyzed (46%) showed the existence of at least one aberrant phenotype: expression of non-myeloid antigens such as CD7 (9 patients with normal karyotype, 1 with inv19, 1 with –5, +mar), CD7+CD19+ (1 pt), CD7+cy79a+ CD56+ (1 pt), CD7+CD22+ CD56+(1 pt), CD7+CD2 +cyCD3+ (in a young girl with t(2;14)), CD19 + (4 pts), CD19+ cy79a + CD56+ (1 pt with t(8;21)), CD19+ CD56+ (1 pt with t(8;21),del Y), CD19+ cyCD22+ (1pt), CD56+ (3 pts, one with t(8;21)); asynchronous antigen expression such as CD117+CD15+ (7 pts); absence of expression of myeloid antigens such as lack of CD33 (1pt). Myeloid markers were present in 11 cases of 21 B- ALL : cyMPO+ (1 pt), cyMPO+ CD13+ CD33+ (1 pt), cyMPO+ CD33+ (1 pt), CD13+ CD33+ (5 pts, one with t(9;22)), CD33+ (2 pts), CD33+CD15+ (1 pt with an abnormal SSClog/CD45 distribution of leukemic cells. Figure 1B). Two cases had a mature B-ALL immunophenotype, of which one had overexpression of CD20 and t(8;14), del 6. In the analysis of T-ALL (5 patients) aberrant markers were present in 2 cases: CD117+CD13+CD33+ and CD33+cy79a+. Finally 1 undifferentiated, 2 biphenotypic and 1 not-differentiated ALL leukemias were studied. These results suggest that immunologic methods for the detection of MRD based on the existence of aberrant phenotypes could still be used in a large number of leukemic patients.



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PERSISTENCE OF PML-RAR ALPHA FUSION TRANSCRIPT IN PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA CLINICAL REMISSION

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The t(15;17) translocation and its molecular equivalent PML-RARα fusion gene are genetic markers of acute promyelocytic leukemia (APL). PML-RARα RT-PCR is commonly used to monitor APL patients, since it has been demonstrated that the persistence of the fusion transcript is strongly associated with APL relapse within 2-3 months. We used RT-PCR to monitor 2 APL patients (1 male and 1 female, aged 68 and 64 years, respectively) who had clinical and hematologic relapse after remission lasting 2 years obtained with AIDA protocol. Salvage treatment consisted of all-trans retinoic acid (45 mg/m^2/die for 30 days) plus idarubicin (12 mg/m^2/die for 4 days). Both patients achieved a second hematologic complete response (CR)è. During the chemotherapy-induced aplastic phase, both experienced severe pneumonia.The man also had an *Enterococcus faecium* sepsis and subsequently *Candida parapsilosis* sepsis. Therefore, the 2 patients were not submitted to any consolidation course. The RT-PCR performed after the re-induction treatment demonstrated the persistence of the PML-RAR α fusion transcript. The two patients were monitored by RT-PCR every three months and this analysis always confirmed the persistence of the transcript. After a follow-up of 2 and 3 years from the achievement of second remission, both patients are still in CR with persistent positivity of RT-PCR. These two cases are in contrast with previous experiences, that report a strict correlation between positive PCR and APL relapse, and suggest the possibility that the mere presence of PML-RARα fusion transcript may not imply inevitable hematologic relapse within a few months after its

appearance. The biological conditions which determine this discordant molecular behavior remain unclear. Additional studies are needed to clarify the significance of the molecular follow-up in APL patients.

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P-GLYCOPROTEIN, MULTIDRUG RESISTANCE RELATED PROTEIN AND LUNG RESISTANCE ASSOCIATED PROTEIN EXPRESSION IN ACUTE NON LYMPHOCYTIC LEUKEMIAS: AN ONSET VERSUS RELAPSES ANALYSIS

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Background and Objectives. In Acute Leukemia data about the expression of multidrug resistance related proteins (MRP-I) at relapse is still limited and sometimes conflicting. We evaluated P-glycoprotein (PGP), lung resistance associated protein (LRP) and MRP-1 expression in 40 patients paired for an onset versus relapse event. The objective was to evaluate whether a standard chemotherapy induction regimen containing at least one (range 1 to 4, median 2) anticancer drug involved in the multidrug resitance (MDR) phenomena, could significantly affect the blast cells' MDR profile. Design and Methods. Peripheral or bone marrow samples were collected from all 40 patient at the time of diagnosis and at 1st relapse which occurred 3 to 72 months after the diagnosis. Cells, cryopreserved until 24 hours before the study, were prepared for the MDR analysis at a single Institution by testing vitality and normal peripheral leukocyte contamination. All samples contained more than 85% of available leukemic cells. PGP, MRP-1 and LRP expression was evaluated by means of specific anti PGP (MRK-16), anti MRP-1 (MRPm6) and anti LRP (LRP-56) MoAb and an indirect flow cytometric method. Results were expressed by the mean fluorescence index (MFI) calculated as the ratio between the mean fluorescence of sample and the one of the respective negative control. Results. Four out of 19 patients classified as PGP-non-overexpressing (PGP expression not exceeding that of non-MDR cell lines) at onset increased their protein expression until they became PGP-overexpressing at relapse. No patient was classified as overexpressing at onset while 7 were classified as overexpressing at relapse. Seven out of 30 non-LRP-overexpressing at onset became overexpressing at relapse. In the global analysis of the 40 paired onset versus relapse cases, no significant differences were found in protein expressions. The mean PGP LRP and MRP-1 expressions were 7.0, 3.7, 1.6 at onset and 6.6, 5.2, 2.2 at relapse respectively ($p > 0.33$). When the percentage of increase or decrease of the protein expression was calculated for each patient by dividing the protein expression at relapse for the one at diagnosis, no relationship was found between this index and disease free survival or number of MDR involved drugs used during the induction therapy. Interpretation and conclusion. Our data on paired onset versus relapse patients suggest that no relevant changes are likely to develop at first relapse in patients treated with high or standard dose arabinosyl cytidine and one to 4 drugs involved in the MDR phenomena. The higher PGP, LRP and MRP-1 expressions reported by several authors in patients evaluated at relapse with respect to series of patients studied at onset may be related to the higher % of MDR overexpressing cases relapsing after chemotherapy rather than to a therapy-induced development of PGP, LRP or MRP-1 overexpression.

074
AMIFOSTINE DOES NOT PROTECT MULTIDRUG RESISTANCE TUMOR CELL LINES AGAINST THE TOXIC EFFECTS OF ANTHRACYCLINES OR MITOXANTRONE

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Background and Objectives. The major obstacles to effective chemotherapy are drug toxicity to normal tissues and drug resistance. In cell lines drug resistance may be overcome by increasing the drug dose and in a clinical setting the benefit of such a strategy is reflected by the improvement in the outcome of leukemic or lymphomas patients treated with high-dose chemotherapy followed by rescue with autologous bone marrow or peripheral stem cell transplantation. The concept of selective cytoprotection of normal tissues from the potential cytotoxic effects of anticancer drugs my be an alternative approach for the delivery of high-dose chemotherapy to tumor cells. Amifostine (Ethiol), a cytoprotectant, protects hematopoietic progenitors and other normal tissues from the toxicity of ionizing radiations and several antineoplastics, including anthracyclines. The aim of this work was to investigate the amifostine and WR-1065 (the active metabolite) activity on cell lines showing multidrug resistance (MDR) associated with P-glycoprotein (PGP), multidrug resistance protein (MRP) or lung-related resistance protein (LRP) overexpression. This target has a clinical implication since it has already been observed that a wide range of human cancers including acute leukemias often overexpress one or more of these proteins. *Design and Methods*. Five pairs of human cell lines including a parental drug sensitive line (CCRF CEM, HL60, LOVO 109, GLC4, SW1573) and the respective drug selected MDR resistant subline (the PGP overexpressing cell lines CEM VLB, HL60 DNR, LOVO DX; the MRP overexpressing cell line GLC4 ADR; the LRP overexpressing cell line SW1573/2R120) were incubated with increasing doses of daunorubicin, doxorubicin, idarubicin or mitoxantrone with or without amifostine or WR-1065. After 7 days of incubation, cell growth was determined using the MTT microcultured colorimetric assay. *Results*. The toxicity of 15 minutes preincubation with amifostine or WR-1065 was tested in all cell lines. The inhibition dose 50 (ID_{50}) ranged from 19 to 80 mg/mL for amifostine and from 14 to 75 mg/mL for WR-1065. In a 7-day incubation with increasing doses of anticancer agents the presence of amifostine or WR-1065 (15 minutes preincubation with concentrations of 1, 5 or 10 mg/mL) did not modify the drug cytotoxic profile in any of the MDR negative or positive cell lines. *Interpretation and Conclusion*. These results show that amifostine and WR-1065 did not protect MDR cell lines against anthracyclines or mitoxantrone. The obser-

vation that even the active metabolite WR-1065 gave no protection against drug toxicity strengthened the data and provides further validation for the *in vivo* testing of safety and efficacy of this class of protecting agents in leukemias.

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ANTHRACYCLINES FOR LEUKEMIC PATIENTS WITH MULTIDRUG RESISTANCE: THE INDUCTION OF APOPTOSIS IS SIGNIFICANTLY HIGHER WHEN P-GLYCOPROTEIN OVEREXPRESSING CELLS ARE EXPOSED TO LIPOSOME-ENCAPSULATED DAUNORUBICIN THAN TO FREE DAUNORUBICIN

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Background and Objectives. The liposome-encapsulated daunorubicin (DNR), daunoxome (DNX), has been developed in an attempt to increase the delivery of the anthracycline to tumors while sparing toxicity to normal tissues. In cell lines, the small liposomes that characterize DNX, causes different DNR cellular kinetics in MDR cell lines overexpressing the P-glycoprotein (PGP); this difference seems to be the key to the higher DNX toxicity as compared to that of the free anthracycline. Also leukemic blast cells exposed to DNX show different DNR cellular kinetics with respect to those exposed to the free anthracycline. Moreover, also in blast cells, DNR cellular kinetics appears to be affected by the blasts' PGP expression. The aim of this work was to check whether the liposomal DNR transport affects leukemic cell killing. *Design and Methods.* Bone marrow or peripheral leukemic blast cells obtained from 40 patients with acute lymphocytic (n = 9) or non-lymphocytic (n = 31) leukemia were checked for PGP expression and cultured in the presence of 300 ng/mL DNR or DNX. After 24 hours of exposure, cellular DNR content (cell normalized mean fluorescence index NMFI) and the percentage of apoptotic cells (annexin V positive/propidium iodide negative cells) were evaluated by flow cytometry. *Results.* Out of the 40 leukemic patients, 16 had comparable PGP expression (PGP-non-overexpressing) while 26 had higher PGP expression (PGP-overexpressing) to that non-MDR cell lines used as controls. In PGP-non-overexpressing patients the DNR cellular content and the drug killing after 24 hour drug exposure was comparable for DNR or DNX (mean NMFI 134.84±42.9 versus 146.3±44.4; % of annexin V positive/ PI negative cells 28.0±15.9 versus 29.7±16.4). In PGP overexpressing patients the mean DNR cellular content was 67.4±25.3 after free DNR exposure and 78.3±30.0 after DNX (p = 0.0004). The mean percentage of the cell killing was 11.62±5.70 against 17.52±10.2 for DNX .While this difference seemed quite small it was statistically significant (p=0.017). *Interpretation and conclusions.* According to preliminary tests in cell lines, DNX seems to be as toxic as free DNR in PGP-non-overexpressing leukemic cells and more toxic than the free drug in PGP-overexpressing leukemic cells. We conclude that DNX is worth testing for the treatment of acute non-lymphocytic leukemias, especially if secondary or developed, in elderly patients in whom, even at onset, PGP overexpression is a common event.